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BOOK OF ABSTRACTS

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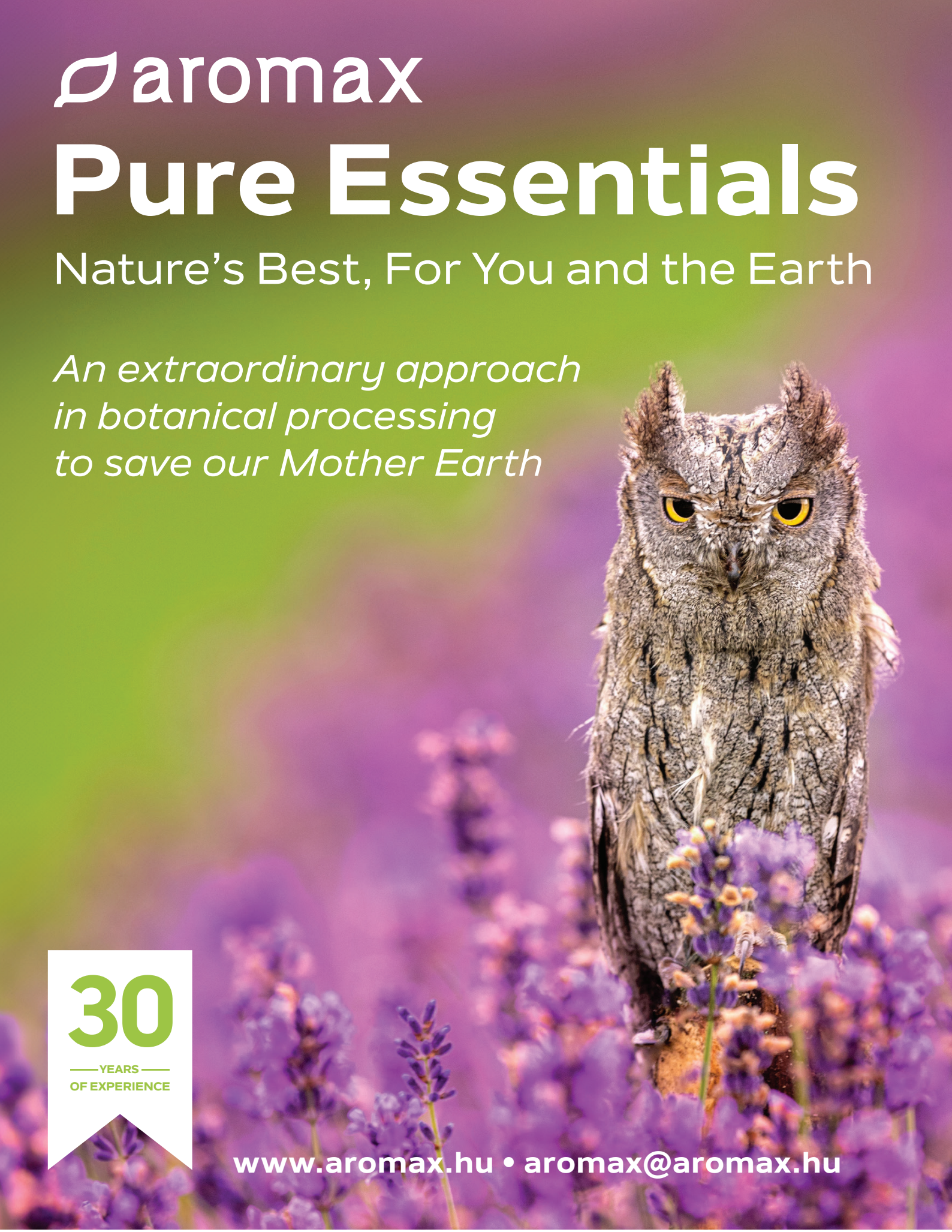

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AWARD LECTURES

AWARD LECTURE

Biologically active volatile components from liverworts and their application to cosmetics foods and medicines

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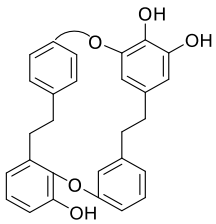
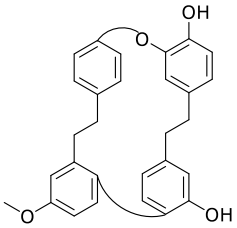
Liverworts are found everywhere in the world, except in the sea and they are placed taxonomically between the algae and the pteridophytes. There are about more than 6000 liverwort species in the world. Almost all liverworts possess beautiful cellular oil bodies.

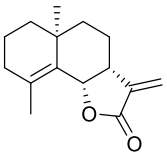
Many species of liverworts possess characteristic fragrant odors and an intense pungent, sweet, or bitter taste. Generally, liverworts are not damaged by bacteria, fungi, insect larvae and adults, snails, slugs and other small mammals. Although liverworts possess such bioactive products, their isolation and structural elucidation were neglected for almost a century. Since 1972, we collected more than 800 species of liverworts around the world and chemically analyzed with respect to their chemistry, pharmacology, and application as sources of cosmetics and human diets, and as medicinal or agricultural agents. The biological activities of liverworts are due to the terpenoids and aromatic compounds which are present in the oil bodies in each species. Several hundred new compounds have been isolated from the



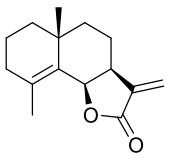
(**1**) marchantin A



(**2**) riccardin A



(**3**) (+)-frullanolide



(**4**) (-)-frullanolide

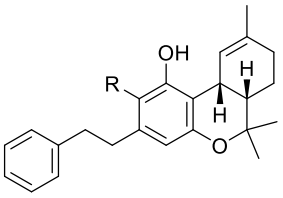
essential oils and solvent extracts of liverworts and more than 60 new carbon skeletal terpenoids and aromatic compounds such as bis-bibenzyls, marchantin A (**1**) and riccardin A (**2**), which are very rare natural products, were found. Most of the liverworts studied elaborate characteristic scent, pungent, and bitter tasting compounds, many of which show antimicrobial, antifungal, antiviral, allergenic contact dermatitis, cytotoxic, insecticidal, anti-HIV, superoxide anion radical release, plant growth regulatory, neurotrophic, NO production inhibitory, muscle relaxing, antiobesity, piscicidal, nematocidal activity and many others. The most characteristic chemical phenomenon of the liverworts is that most of the sesqui- and diterpenoids are enantiomers to those found in higher plants. It is very noteworthy that different liverwort species of the same genus like *Frullania tamarisci* and *F. dilatata* (Frullaniaceae) each produces sesquiterpene lactone enantiomers, (+)-frullanolide (**3)** and (-)-frullanolide (**4**).

When the large thalloid liverwort, *Conocephalum conicum* was completed sealed into plastic sac which was covered by a glass plate for 1, 6 and 9 months, its morphology and each chemical profile were dramatically changed. The major monoterpene, (+)-bornyl acetate (**5**) included in the original thallus, disappeared and menthyl cinnamate (**6**) was newly created. The chemical profiles of the cultured *C. conicum* are very similar to that of the Japanese most expensive mushroom *Tricholoma matsutake* which is used as consommé soup in Japan. Thus the production of the volatiles of *T. matsutake* can be produced limitless from the liverwort in laboratory and industry scales.

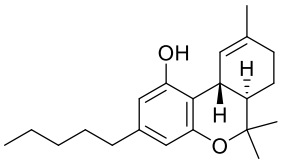
When the thalloid liverwort *Marchantia paleacea* subsp. *diptera* was cultured with the same condition as mentioned above, (*S*)-(-)-perillaldehyde (**7**) which is the most important aroma for *Perilla frutescens* (Lamiaceae) and used as the Japanese cuisine and herbal medicines, and not included in the original liverwort, was elaborated in 50% yield, along with 1-perillyl alcohol (**8**) and shisool (**9**). Thus (*S*)-(-)-perillaldehyde (**7**) can be created limitless for a year in this simple manner.

Almost all of *Radula* liverwort species mainly produce bibenzyls and prenyl bibenzyls. It is noteworthy that *R. perrottetii* and *R. marginata* biosynthesize perrottetinene (PET) (**10**) and perrottetinenic acid (**11**), the structures of which are very similar to that of well-known psychoactive compound, tetrahydrocannabinoid (THC) (**11**) obtained from *Cannabis sativa*. PET (10) showed the same psycho activity as that of THC and more potent antiinflammatory activity than THC (**12**).

In this paper, bio- and chemical diversity of liverworts and their bio- and pharmacological activities, including characteristic odor and taste, as well as the possibility of liverworts as cosmetics, foods and medicals are surveyed.



(10) perrottetinene R=H
(11) perrottetinenic acid



(12) tetrahydrocannabinoid (THC)

Table 1 Scent and pungent compounds from liverworts

Species name	**Compound**	**Odor property**
Asterella species	Skatole (**13**)	Stool, feces
Chandonanthus hirtellus	Dictyotene (**14**), ectocarpene (**15**)	Ocean smell
Cheilolejeunea imbricata	(R)-Dodec-2-en-1,5-olide (**16**), (R)-tetradec-2-en-1,5-olide (**16**)	Milky
Chiloscyphus pallidus	(E) and (Z)-Dec-2-enal (**17, 18**),	Stink bug
Leptolejeunea leratii	4-Methoxystyrene (**19**)	Myrrh-like
Conocephalum conicum (cultured)	1-Octen-3-ol (**20**), 1-octen-3-yl acetate (**21**), methyl cinnamate (**22**)	Strong mushroomy Medicinal
Corsinia coriandrina	(E)-Coriandrin (**23**), (Z)-coriandrin (**24**), (E)-O-methyltridentatol (**25**), (Z)-O-methyltridentatol (**26**)	Faint sulfur
Cyathodium foetidissimum	Skatole (**13**), 4-methoxystyrene (**27**), 3,4-dimethoxystyrene (**28**), bicyclogermacrene (**29**), iso-lepidozene (**30**), lunularin (**31**), 2-aminoacetophenone (**32**)	Feces Myrrh like
Fossombronia angulosa	Dictyotene (**14**), multifidene (**33**), dictyopterene (**34**)	Ocean smell
Frullania tamarisci subsp. tamarisci,	Tamariscol (**35**)	Carnation
Lophocolea bidentata	(–)-2-Methylisoborneol (**36**)	Earthy
Mannia fragrans	Grimaldone (**37**)	Strong mossy
Marchantia paleacea subsp. diptera (cultured)	(–)-(S)-Perillaldehyde (**7**) 1-perillyl alcohol (**8**) shisool **(9**)	Perilla frutescens-like
Plagiochila sciophila	Bicyclohumulenone (**38**)	Strong mossy
Porella vernicosa complex	Polygodial (**39**)	Strong pungent
Wiesnerella denudata	1-Octen-3-ol (**20**), 1-octen-3-yl acetate (**21**), nerol (**40**), neryl acetate (**42**), linalyl acetate (**43**)	Sweet citrus
		Pungent
Chiloscyphus polyanthos Diplophyllum albicans	ent-Diplophyllolide (**44**),	Weak hot
Fossombronia alaskana Lobatiriccardia yakushimensis, Pellia endiviifolia	Sacculatal (**45**) 1β-hydroxysacculatal (**46**),	Persistent hot
Plagiochila fruticosa P. yokogurensis	Plagiochiline A (**47**), plagiochiline I (**48**)	Persistent hot
Porella vernicosa complex	(–)-Polygodial (**49**)	Persistent hot
Wiesnerella denudata	Tulipinolide (**50**)	Weak hot

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AWARD LECTURE

Application of essential oils in animal health from the one health perspective

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Abstract

The interest and demand on plant based products - and essential oils in particular - in animal nutrition, welfare and health care has risen tremendously over the last two decades. This could be attributed on one side to the fact that pet and horse owners inquire more often after soft natural remedies and care products to treat their companion animals. On the other side, the increasing use of ‚botanical' feed additives and a changing treatment of farm animals to reduce the application of antibiotics and synthetic drugs was driven by several European Regulations, especially in organic production. Animal healthcare is, in addition, more and more focussed on the One Health approach, that aims to sustainably balance and optimize the health of people, animals and ecosystems.

The hype in using essential oils under the category flavouring additives has been, however, cut down in the meantime by the resp. European Authorities due to strong quantitative restrictions. The general claims of essential oils as antiinflammatory, antimicrobial and antioxidant substances are no longer sufficient for registration. The application as e.g. animal welfare or veterinary medicinal products requires many more scientific data on absorption, mechanism of action, impact on the microbiome, effective concentrations, (environmental) toxicology a.s.o. An increasing number of papers have been published in recent years to contribute to the understanding of the value that essential oils can provide to animal health and welfare. The recently started EU-COST-Project MedPlants4Vet aims at collecting all these data to establish a consistent database, to identify and fill gaps in knowledge and to further stimulate the European Authorities in issueing practicable guidelines for the authorization of botanicals incl. essential oils with respect to One Health.

PLENARY LECTURES

PL-1

Global trade of essential oils

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Essential oils are complex mixture of volatile chemicals mainly found in plants. Animals, liverworts, mosses, microorganisms and marine organisms also bear natural volatiles. Essential oils can be extracted from their matrix by distillation (e.g. water distillation, water and steam distillation, steam distillation) or expression in the case of citrus fruits. Supercritical fluid extraction and solvent extraction are used to extract aromatic extracts which are not technically considered essential oils.

Essential oils are ingredients of flavour and fragrance materials for use mainly in food and beverage, perfumery, cosmetics and toiletries, wellness and aromatherapy, spa and relaxation, healthcare, pharmaceutical and chemical industries. They also possess various biological activities and in recent years their use in feeds as antibiotic substitute and growth promoter is on the rise.

Global market of essential oils was valued at USD 21.8 billion in 2022 with a projected rise by 7.9% to 40.1 billion in 2030. Orange oil tops the list of most globally traded essential oils followed by lemon, lime, peppermint, corn mint, citronella, spearmint, geranium, clove leaf, eucalyptus, jasmine, tea tree, rosemary, lavender oils, etc. Europe dominates the market with Brazil, China, USA, India, Indonesia, Mexico, Morocco, Egypt, Guatemala, etc. as major producing countries.

PL-2

Advanced sustainable analytical methods for the analysis of essential oils

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Keywords: Alternative sustainable gas carrier, sub-critical Fluid Chromatography, bio-solvents

1. Introduction

The purpose of this research is to explore the performance of alternative carrier gasses such as nitrogen and hydrogen instead of the most common helium for routine analysis of the volatile fraction of essential oils in gas chromatography (GC) and the use sub-critical CO2 with bio-solvents as modifier for the analysis of the non-volatile fraction.

2. Material and Methods

The volatile fraction of essential oils was investigated by means of GC-FID and GC-MS analysis while the non-volatile fraction by SFC-PDA.

The optimal linear velocities of helium, hydrogen, and nitrogen carrier gasses were calculated by Golay curve approach. To compare the performance during routine analyses, *Citrus* essential oils were analyzed in GC-FID and GC-MS methods using nitrogen and hydrogen carrier gases, respectively.

The analyses of the non-volatile fraction were carried out by testing several columns. Mobile phase was composed of CO_2 and bio-EtOH. Analyses were carried out under gradient conditions. The oven temperature and BPR were set at 44°C and 120 bar, respectively

3. Results

The developed GC-FID method allowed a satisfactory separation of all components in about 47 min, in accordance with analysis times (ca. 45 min) obtained using helium as carrier gas. The GC-MS optimized method using H_2 as carrier gas allowed the separation and identification of 55 volatile compounds in comparable, He-based analysis times. Also, most compounds showed a spectral similarity of more than 90%. Absolute correspondence was also registered between experimental and reference LRI. A more environmentally friendly analytical strategy aimed at the separation and quantification of coumarins, furocoumarins and polymethoxyflavones in cold-pressed *Citrus* essential oils. This method can be applied for the rapid analysis of cold-pressed *Citrus* EOs using the SFC-UV system, without the need for expensive instrumentation to determine target compounds. To satisfy the requirements of the most important organizations focused on cosmetics, however, a new sensitive SFC-UV method for the analysis of oxygen heterocyclic compounds at trace levels in finished products is currently being studied

4. Conclusions

Carrier gas switching to H_2 for analysis carried out by GC-MS did not necessitate to adjust or to modify mass spectral database containing MS spectra and LRI values. In fact, absolute correspondence between experimental and reference data were obtained.

Furthermore, regarding the GC-FID analysis the use of N_2 as carrier gas allowing to obtain comparable helium-based GC-FID analysis time.

For the separation of oxygen heterocyclic compounds in cold-pressed *Citrus* EOs is a challenge due to the wide variety of compounds and due to small structural differences between the compounds. The results obtained show that SFC-UV is a perfectly suited method to investigate the essential oil composition, because of the great number of compounds separated in a reduced analysis time (around 10 min), and with a very short time for re-equilibration of the system at the end of the gradient analysis

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

PL-3

Healthy smells and beyond: Aromatherapy today

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Keywords: olfaction, mood, depression, (psycho)pharmacological mechanism

Abstract

By definition, the purpose of aromatherapy is to cure, alleviate and prevent illnesses using essential oils of high quality (e.g., according to the European Pharmacopeia). Its application as a complementary treatment method has increased during the last decades. Clinical studies as well as investigations on the elucidation of the pharmacological mechanisms of essential oils in the context of aromatherapy have caught the interest of researchers. This review gives an overview of recent developments and challenges in scientific and applied aromatherapy, with a focus on mental disorders.

1. Introduction

Essential oils have been used by mankind for ages due to their numerous biological activities. Today, their antimicrobial impact is valuable in the cosmetic and food industries for preservation, as well as in medicine and pharmacy as a promising complementary antbiotic in times of increasing bacterial resistance. Further, their insect-repellent activity enables their use as natural repellents, and their expectorant effect is implemented in pulmological therapies, just to name a few.

In aromatherapy, essential oils are mainly applied via inhalation or on the skin (massages, baths, etc.). This does not only involve their pharmacological activity after absorption into the bloodstream, but also the psychopharmacological impact due to the strong odor of these volatile mixtures of terpenoids and phenylpropanoids. Since the number of people suffering from depression, sleeping disorders, and anxiety noticeably rose since 2019 [1], the use of essential oils to improve wellbeing and alleviate mental and psychosomatic disorders increased within the last decades. Further, the professional application of aromatherapy has experienced increasing approval among medical and pharmaceutical experts. [2,3] This is even more interesting, as the pharmacological mechanisms, due to the complexity of the oils, can hardly be elucidated. In contrast to conventional drugs (“one disease – one target – one drug”-approach), the mixture of hundreds of compounds can affect different target-structures and additionally may emit synergistic, additive or antagonistic effects between each other. Therefore, the scientists postulate the main components of an essential oil as leading substances and responsible for its activity. Further, the odor of the oils impedes the elucidation of the mechanism of action via its impact on the olfactory system, affecting emotions.

2. Material and Methods

Information was taken from reviews, and research articles, and websites selected due to their actuality and, according to the author, high impact on this topic.

3. Discussion and Conclusions

Due to a high overlap and strong connection between regions of the olfactory and the emotional brain, aromatherapy has a high impact on alleviating mild to moderate mental disorders. Clinical research over the last three decades has compellingly proven the effectiveness of this therapy. However, clinical studies on the elucidation of pharmacological mechanisms are difficult due to the complexity of the oils. Therefore, various *in silico* models to calculate quantitative/qualitative structure-activity-relationships (QSAR) are used to confirm the outcomes of *in vivo* animal model experiments. Still, further research will be necessary to get a closer understanding in the mechanisms of action of essential oils used in aromatherapy.

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Conflict of Interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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PL-4

Pampering or stress?- Optimisation of volatile formation in plants

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Keywords: stress, elicitation, volatiles, biosynthesis, environment

Abstract

During crop cultivation, growers generally are struggling to assure optimal conditions for the plants as stress usually reduces growth and biomass. At the same time, data have been accumulating about the influence of unfavorable conditions in the quality of the crop. There are also some scientific explanations known for this reactions like the opportunistic emission of volatile isoprenoids, the carbon allocation and the surplus of reductive power theories. Numerous studies investigated the effects of drought, heavy metals, salts, bacterial or other infections, etc. on the volatile accumulation and composition. It seems, that the reactions of different plant species, moreover, varieties may show different behavior. In recent years, data are also accumulating proving, that certain conditions may initiate enhanced gene expression and accelerating the synthesis of special compounds. Nevertheless, it remains a basic question and important task to create the balance between accumulation of volatiles and that of dry matter in order to assure a proper yield of essential oil. Biotic and abiotic elicitation may be a tool in improving the production of secondary metabolites. However, while it is a relatively accepted alternative in vitro, optimisation among in vivo (field) conditions may be a long way, as the result depends on the growth dynamics of the plans and weather conditions, among others.

1. Introduction

The quality of aromatic plants and therefore the quality of the essential oil (EO) produced from them is influenced by numerous biotic and abiotic factors (Fig a). The genetic background is a potential for the plant's all possible traits, however, manifestation of this potential depends on the actual developmental status and growing conditions. Besides, we could improve or even deteriorate the quality by postharvest handling, among them by the extraction method of volatiles. Environment is a very complex system itself in this complex situation. When cultivating any crop, growers generally are struggling to assure the optimal conditions according to the need of the plants to achieve high yields. Nevertheless, in the last period, numerous studies have been investigating the optimum circumstances from the aspect of the quality of the crop. Is the optimum for the biomass and for the accumulation of active ingredients necessarily the same? And what is actually the optimum? Where are the borderlines between optimum, tolerable conditions and stress effects? To what an extent the stress reactions are depending on the genotype, on interacting environmental factors or on the duration of the effect? These are basic questions, for which unfortunately, the majority of published data do not give a satisfactory answer.

2. Discussion

Stress in plant life is defined as any external factors or deviations from optimal conditions that adversely affect plant homeostasis. Many papers may be cited where the volatile production was really enhanced under stress conditions, but unfortunately, there is also a big number of data where this kind of response could not be proved. There are different theories explaining the connection between environmental stresses and the production of secondary metabolites (SMs) in plants. In this context, now we are focusing on the accumulation of volatile compounds.

One of these theories is the hypothesis of opportunistic emission of volatile isoprenoids due to stress conditions. Owen and Peñuelas [1] concluded, that changes in the synthesis of higher isoprenoids (gibberellic acids, abscisic acid, sterols or carotenoids) may be responsible indirectly for the concentration of other, "non-essential" isoprenoids, like volatiles. The production of these latter ones might depend on the available remaining IPP and DMAPP precursors. This theory presumes a competition between different isoprenoid compounds under unfavourable environmental conditions.

The other hypothesis behind the elevated SM content is the surplus of reductive power theory [2]. According to this, -especially in case of drought and heat stress-, the closure of stomata inhibits the uptake of CO_2, in consequence of which the consumption of reduction equivalents for CO_2 fixation is stopping, generating an oxidative stress with oversupply of reduction equivalents. Due to this, the metabolism is shifted towards processes

using the reduction equivalents, among others to the synthesis of isoprenoids. While this is an interesting and possible explanation, the transport of these reduction equivalents between the cell organelles has not been clarified until now. While excess NADH is primarily developing in mitochondria, the cellular compartmentalization in plant metabolism is highly complex [3], thus, intracellular transport mechanisms must be very important and influenced by the stress, too.

Additionally, the carbon allocation theory should be mentioned. To some extent similarly to the previously mentioned explanation, the closure of stomata results in decreased CO2 uptake and fixation which, in consequence leads to reduced biomass. By carbon allocation however, the plant tries to compensate the situation and increase the synthesis of SMs which frequently may contribute to the protection of the organism. However, if we want to conclude an accelerated biosynthesis of the stressed plants, we should be sure that the reference value, the biomass is the same. If biomass is reduced, the unchanged intensity of volatile synthesis would result in higher ratios of them even without a real stimulation of the biosynthetic processes [4]. In the literature, several publications can be found for both situation: increase of volatiles with parallel increase of the yield, or elevation of volatiles but with unchanged or even decreased yields [5].

In the majority of references, changing amount of volatiles due to unfavorable environmental conditions is concluded as result of changed biosynthesis. Nevertheless, very few articles are dealing with eventual losses of the volatiles, however, emission and/or evaporation of accumulated volatiles could contribute to the actual concentrations. In this case, the rate and intensity of these losses largely depends not only on the weather (temperature, wind, humidity) but on the tissue and species specific accumulation structures in the plant.

In the last years, more and more studies are focusing also on genetic aspects with the goal to reveal more details about the detected changes in volatile accumulation. These studies help us to clarify, if the stimulation of biosynthesis of SMs and (among others) of volatile compounds is only a passive shift due to the mentioned over-reduced status, decreased precursor flow, etc. or it is an active change initiated at the level of upregulation of certain genes involved in terpenoid metabolism. Data about enhanced gene expression are accumulating, e.g. [6,7]

In the practice, the main question arises: shall we keep the plants under optimal conditions or apply some unfavorable effects to improve the quality. Usually, we have to establish a balance between biomass (yield) and volatile concentration (quality) in the plant material. For production of EOs, both of these components may be of high importance as the amount of this product is formed by multiplication: kg biomass x volatile %. Interestingly, various situations have been described. For example Petropoulos et al. [8] found, that drought stress reduced the fress mass of the plants by appr. 35% but the concentration of volatiles increased to such an extent that the overall EO yield pro m2 was basically the same both in stressed and control plants. Nevertheless, there are also cases where the slight increase in volatile accumulation is not able to compensate the reduction of biomass like in thyme [4]. In our former study on lemon balm, thyme, peppermint, marjoram, a parallel decrease of both the biomass and volatile content of the drug has been established [9]. As result of that, the yield of EO was severely dropped, although the rate of decrease was somewhat different for the investigated species, Therefore the connection between the changes in biomass and volatile production must be clarified in each situation very carefully in order to start with optimization (Fig b).

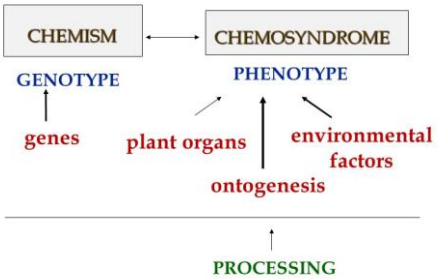

Fig (a) Factors influencing the quality of the production of secondary metabolites

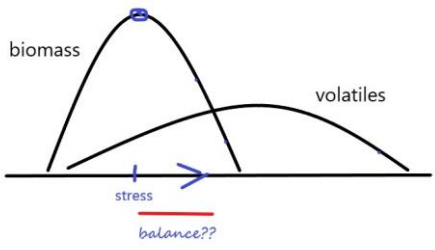

Fig (b) Biomass and volatile accumulation curves in consequence during changing environmental conditions

Unfortunately, the majority of publications is dealing only with a single genotype of a single species, however, some references indicate, that stress responses may depend also on intraspecific variety, their reactions are not necesserily uniform, like in lemon balm [10]. We need more data about that in the future.

Beside the mentioned total volatile production, in most cases compositional spectrum and ratio of components is of primarily importance, too. In the studies on stress related reactions of EO producing species, the findings

represent a mixed picture. Only to mention some examples: Petropoulos et al. [8] described in parsley significant quantitative changes of several components due to dry conditions. In a larger study we found, that in case of peppermint the composition changed only slightly, while in marjoram and thyme the ratio of terpinene type compounds was elevated in drought stressed plants [9].

Elicitation is a relatively new aspect for enhancement of SMs, thus also isoprenoid and other volatile compounds as it may induce plant immune responses and trigger defense reactions [11]. Both biotic elicitors (with microorganism origin) and abiotic ones can be applied. These latter factors include light, salinity, drought, high emperatue, heavy metals and hormones as jasmonic acid, salicylic acid, gibberellic acid treatmens. This method seems to be an accepted tool in several in vitro cultures [12] although less frequently for terpenoids, than other SMs. In vivo application of chemical elicitation is however, much less frequently discussed and the papers are extremely heterogenous concerning species, growing conditions and treatment methods. Some dillemmas based on our own experiments in connecion with this issue will be also presented in the lecture.

3. Conclusions

As volatiles may contribute to defense reactions, stress conditions definetly have significant effects on their synthesis in plants. In order to utilize this phenomenon and produce high quality herbs with appropriate yield at the same time, all conditions and treatments must be adjusted to the target species/variety. There are still many questions to answer for an in vivo technology optimisation.

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PL-5

Antimicrobial activity of essential oils and essential oil components on food-borne pathogens – recent applications and future perspectives

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Keywords: essential oils, food preservatives, food-borne pathogens

Essential oils (EOs) and their components have excellent antimicrobial activity which empowers their use as food preservatives against food-borne pathogens. Most of the EOs has the GRAS status by the FDA and components are on the list of flavoring substances by the EFSA. Some limitation aspects have to be considered before using essential oils or components as food preservatives, namely their strong aroma, low water solubility, possible hypersensitization effect and difficulty of the standardization of essential oils. To overcome these problems several solutions were presented. Essential oils as food preservatives can be used directly by adding to the food mainly in the form of nano emulsions or nano capsules, or incorporated into edible films on the surface of foods. In some types of active packaging EOs or components have no direct contact to the food; they are present in vapor form and can be adsorbed by the packed food. Efficient dose of EOs could lead to unpleasant changes in the sensorial characteristics of foods therefore EOs and components are often combined with other natural or synthetic preservatives utilizing the synergistic effect among these substances. Other application of EOs against food-borne pathogens is their use as sanitizing or disinfection agents. In this aspect EOs has several advantages compared to commercial disinfectants: they are able to eliminate multi-species biofilms from industrial surfaces, can be used in vapor phase and need no rinsing. Considering all pros and cons essential oils and their components can have a future as food antimicrobials.

PL-6

Formulation of essential oils: Rational design and nanotechnology

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Keywords: rational design, nanotechnology, essential oils, Pickering emulsions, lipid nanoparticles, targeted delivery

1. Introduction

There is a great deal of respect for essential oils (EOs) because they are known to have medicinal effects, such as antimicrobial, anti-inflammatory, and anticancer. The problem, however, arises from their volatility, hydrophobicity, and instability, which present significant challenges in the clinical use of EOs. This presentation looks at the rational design of EO formulations via nanotechnology, focusing on controlled release for cancer treatment through the Enhanced Permeation and Retention (EPR) principle and topical therapy targeting biofilm treatment and nail fungal infections.

2. Rational Design of Essential Oils for controlled delivery in cancer treatment

The architecture of tumors' blood vessels and poor lymphatic drainage cause the accumulation of nanoparticles in tumor tissues. Encapsulation of EOs within nanoparticles ensures stability while being delivered and controlled release at the tumor site. For the rational design of these nanoformulations, appropriate materials have to be chosen for the nanocarriers, such as liposomes, polymeric nanoparticles, or lipid-based systems that can enhance the solubility and bioavailability of essential oils. This targeted delivery is crucial for maximizing the therapeutic index and minimizing the adverse effects associated with systemic EO administration.

3. Nanoformulations of Essential Oils in Biofilm Treatment and Nail Fungal Infections

The local application of essential oils is a promising option against traditional therapies for biofilm-based infections and nail fungus because they have strong antimicrobial and antifungal properties. Biofilms are communities of bacteria structured with self-produced extracellular matrix that pose a significant challenge due to their resistance to antibiotics or immune responses. EOs like tea tree oil and eucalyptus oil have been found useful in treating them by destroying the biofilm matrix, thus making bacteria more susceptible to antimicrobials.

From a nanotechnology perspective, encapsulating EOs into nanoparticles or nano gels can improve their penetration and retention at the infection site. For example, compared to free EOs, nanoemulsions containing EOs showed better efficacy against biofilms due to improved stability and sustained release characteristics. Moreover, such nanoformulations can be engineered so that when triggered by specific stimuli like pH or enzymatic activity, they release the active EO ingredient and deliver it exactly to the infection area.

Another condition where essential oils may work well is nail fungal infections, referred to as onychomycosis. Poor nail penetration and prolonged treatment durations sometimes limit traditional treatments. Nanotechnology can overcome these barriers by enhancing the delivery of EOs through the dense keratin structure of the nail. Formulating EOs into nanostructured lipid carriers (NLCs) or Pickering nanoemulsions can significantly improve their permeation and retention within the nail bed, offering a more effective and convenient treatment option.

4. Conclusions

The conjunction of nanotechnology with the rational design of essential oil formulations is a powerful approach to overcoming the limitations brought by EOs properties and enhancing their therapeutic potential. The enhanced permeation and retention effect principle can be useful in treating cancer systemically using nanoparticles loaded with EO, which helps improve efficacy while minimizing side effects. In terms of local application, nanotechnology provides better stability, penetration, and action of EOs against biofilm-related diseases and nail fungal infections. Further improvements in this area will result in innovative, efficient, and safe EO-linked therapies for various medical conditions.

KEYNOTE LECTURE

KEYNOTE LECTURE

The value of having a multidisciplinary approach to essential oil research – reflecting on two decades of studying the medicinal-aromatic flora of South Africa

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Introduction: Southern Africa is considered one of the richest centres of plant diversity in the world, with an estimated 24,300 plant taxa. South Africa, renowned for its botanical diversity and ranked among the top ten countries in the world for plant species abundance, contributes to about 10% of the world flora. The country further harbours three distinct biodiversity hotspots namely, the Cape Floristic Region (Western Cape), the Succulent Karoo ecoregion (Northern Cape and Namibia) and the Maputaland-Pondoland-Albany corridor (Eastern Cape), making it one of the 25 known biodiversity hotspots globally. Despite South Africa having this unique botanical heritage, very few medicinal and aromatic plants are harvested from the wild and actively traded at informal markets, while only a fraction of these proceed to large scale commercialisation. This is partly due to the lack of basic research, which is crucial for the optimised commercialisation of herbal products. Furthermore, insufficient knowledge of the raw material, in relation to chemical variation and proper biomarker identification contributes to the void of South African-derived commercial products on the international markets.

Over the years there has been increasing interest in medicinal and aromatic plant research, with a strong emphasis on essential oils, whose applications span the pharmaceutical, cosmetic, food and beverage, as well as the fragrance and aromatherapy industries. Based on historical and modern day uses, essential oils rank as the most extensively used plant metabolites for their anti-infective properties. Numerous essential oils and their major constituents are known to exhibit promising antimicrobial activities and can therefore be a good source of biologically active molecules for therapeutic applications. Our phytomedicine research group has over the years embarked on research involving South African indigenous aromatic plants and some commercially important essential oils, focusing on establishing quality control protocols and biological activities with the aim of providing crucial information to encourage their commercial development.

Several examples emanating from our research other past 2 decades will be highlighted under the following over-arching categories and focal points of research.

Bioprospecting: Bioprospecting of medicinal and aromatic plants in South Africa offers significant opportunities due to the country's rich biodiversity, which includes a wide variety of unique flora with potential therapeutic and commercial value. We have worked with several multinational companies to explore several plants from South Africa for possible use in the flavour and fragrance industries. However, as much as what this initiative may hold unique opportunities one needs to remain mindful to the challenges such as ensuring sustainable harvesting, protecting intellectual property rights, and navigating complex regulatory frameworks which must be addressed to fully realize these benefits. Moreover, engaging local communities and respecting traditional knowledge are crucial for ethical and equitable bioprospecting practices.

Chemical profiling and quality control: Chemical fingerprinting is a crucial component in characterizing plant material and requires a dedicated approach to develop analytical methods for the profiling of complex plant matrices including essential oils. Our research group developed comprehensive essential oil profiles for selected indigenous aromatic plants based on gas chromatography coupled to mass spectrometry (GC-MS) and the more sensitive GCxGC-MS. A few examples will be presented to illustrate the comprehensive profiling of commercially important *Warburgia salutaris, Leonotis leonurus* and *Eriocephalus.* Furthermore the use of more environmentally friendly methods which are rapid and more cost-effective (e.g vibrational spectroscopy) will be presented as a

valuable approach in the routine quality assessment of essential oils.

Chemometrics and multivariate data analysis: Extensive chemotypic variation in aromatic plants makes standardization of essential oils very difficult. It is therefore important to document variation for quality control purposes and to ensure that chemotypes with favourable chemical profiles are identified for commercialization. Furthermore, chemotaxonomy studies have assisted in differentiating some taxonomic allies where interchangeable use due to morphological similarities has been reported. In this regard, our group has used untargeted metabolomics approaches to analyse GC-MS data of large sample sizes in SIMCA-P14® software, to document chemotypic variation within several plant species including *Croton* species, *Leonotis leonurus* and *Tagetes minuta*. Chemotaxonomic differentiation of several species within a genus was possible and *Salvia* species and *Lippia* species, will be discussed as examples.

Biological activity: The bulk of the biological activity work on essential oils has focused primarily on investigating the anti-infective properties of essential oils. Antimicrobial tests have been performed on a range of pathogens associated with skin disorders, bromodosis, food pathogens and other popularly occurring pathogens. The interaction of essential oils from different species, or with essential oil compounds was investigated to document synergism when these are used in combination for subsequent formulation into polyherbal combinations. A few examples will be discussed to also show the application of software such as SynergyFinder® to assist in optimising various combinations to obtain a synergistic outcome. The effect of stereochemistry on the biological activity of essential oils and essential oil compounds is also illustrated.

Biochemometrics: Untargeted metabolomics approaches have become popular in the identification of active constituents within a complex chemical matrix. Our group has applied biochemometrics to analyse large sample sets based on integrating GC-MS data with biological activity data and the subsequent identification of active essential oil constituents in these complex data matrices. Examples are discussed where orthogonal projections to latent structures discriminant analysis (OPLS-DA) enabled the identification of putative biomarkers with antimicrobial, antituberculosis and anxiolytic effects.

The several examples presented above displays how rewarding a comprehensive approach to essential oil research may be. Furthermore, aromatic plants and essentials oils have proven to be an excellent subject matter to build human capital and develop academic capacity in our country where only 6% of the population holds a degree as their highest level of education.

ORAL ABSTRACTS

OL-01

Use and abuse of retention indices in essential oils analysis

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Keywords: Gas chromatography, Kováts index, van den Dool & Kratz indices, linear retention index, identification

Abstract

The identification of essential oil (EO) constituents is a challenging task. Automatic searching in mass spectra libraries is not sufficient, as compounds in EO, usually, present isomeric structural relationships. Even before the coupling of gas chromatographs to mass spectrometry (MS) lead to commercial equipment, identification of EO components was possible by co-injection of authentic standards, whenever available, and/or by the calculation of their retention indices (RI). The RI concept was developed by Kóvats in 1958 to be used in isothermal analyses, as was the common approach at that time. A few years later, van den Dool and Kratz published a RI modification for programmed-temperature analyses. RI do provide a useful tentative indication of the possible molecule(s), especially if combined with MS data. After more than 60 years the system proved its efficacy. However, a worrying trend is the increasing number of articles in which the original concepts or RI are misused, leading to misidentification and, what is worse, propagating wrong data which, in turn, serve as a (wrong) basis for new misidentification, perpetuating the propagation of error. Herein we discuss some causes of this misuse and propose a roadmap for the reliable use of retention indices.

1. Introduction

For those who are younger than 50, it is hard to imagine how essential oil (EO) constituents were isolated, quantified and identified. To have a good picture, see the book of Ernest Günther [1]. All changed when gas chromatography (GC) was developed in the 1950s. As all chromatography, GC is a separation technique, and identification requires additional techniques. Kováts indices [2] and van den Dool and Kratz linear retention indices [3], made identification possible when no internal standards were available, which is quite common in natural products chemistry. The use of retention indices successfully contributed to expand the applications of GC especially in the analysis of EO. A few years after the appearance of GC, its hyphenation to MS was developed. Now, the fast development of commercial equipment and, latterly the arrival of computers, libraries of spectra and automatic searching software have made GC–MS one of the most (if not *the* most) important and widespread tool for the analysis of volatiles in general, and essential oils in particular [4]. However, with automation, and less training and skilled operators required, reduced critical evaluation of generated data, leading to misuse, misidentification of compounds and, in some cases, abuse in the use of retention indices is apparent. Consequently, more publications containing identification errors appear. Some of these commonly found misidentifications are discussed and some simple procedures proposed to, at least, reduce their occurrence. As a case study, the confusion regarding the identification of cymene isomers is presented and discussed.

2. Material and Methods

Standards of *o*-cymene, *m*-cymene, *p*-cymene, *n*-decane, *n*-undecane, *n*-dodecane and *n*-tridecane were purchased from Sigma-Aldrich Co. (Milwaukee, WI). Dichloromethane "Absolv" grade was used as solvent and purchased from Tedia (Brazil). Two stock solutions were prepared: one with *n*-decane and *n*-undecane and another with *n*-dodecane and *n*-tridecane. Each cymene standard was added separately to two vials, one with the C_{10}-C_{11} and other with the C_{12}-C_{13} stock solutions, so that six test solutions were obtained. The GC–MS analyses were performed using an Agilent 7890A GC coupled to an Agilent 5975C mass detector in electron ionization mode, at 70 eV. A DB-5ms fused silica capillary column (5%-phenyl-95%-methylsilicone, 30 m × 0.25 mm × 0.25 µm, Agilent) and an HP-INNOWax (polyethylene glycol, 25 m × 0.25 mm × 0.20 µm, Agilent) were used. The injector was maintained at 250 ºC. A volume of 1.0 µL of each test sample was injected in split mode (1:50). The oven temperature was kept constant at 130 ºC for isothermal analyses and was varied from 60 to 240 ºC at a rate of 3 ºC/min for temperature programmed analyses. The carrier gas used was helium (1.0 mL/min). The transfer line

was kept at 260 ºC, the ionization source at 220 ºC and analyzer at 150 ºC. The mass scan range was from 40 to 350 u at a rate of 3.15 scans/s. All data were processed using ChemStation software (Agilent Technologies). Mass spectra were interpreted using commercial spectrum libraries, which also incorporate retention index values, from various sources.

3. Results

Since neither MS nor RI are adequate for isomer differentiation, identification is best based on injection of authentic standards, which is quite a reasonable requirement in this case, since all cymenes are available commercially. Under the experimental conditions and using capillary columns comprising 5%-phenyl-95%-methylsilicone (DB-5, HP-5 or similar), the first compound to elute is *m*-cymene, closely followed by *p*-cymene with *o*-cymene coming as a last, well resolved peak. Because the difference in the RI of *m*-cymene and *p*-cymene is only two or three units, these isomers can be reliably identified only by using standards, since noticeable deviations from the tabulated values of the RI can be observed when different non-polar phases are used (and even when using the same type of phase but from different manufacturers).

4. Discussion and Conclusions

Perhaps the easiest way to solve the identification problem for cymenes would be to change the stationary phase from a non-polar to a polar one. Unfortunately, the problem remains. In polyethylene glycol phases, *meta* and *para* isomers elute very close too. This also highlights that newer stationary phases, such as ionic liquids, simply do not have tabulated listings of RI data, so this excludes these phases from using RI databases to support identification, most likely delegating MS to be the only identification available with the attendant risks this implies. The cymene isomers case is illustrative, but several other types of error, invariably related to poor judgement, including poor peer-review processing, result in much wrong data published. The misuse of GC–MS data and, particularly, of retention indices in the analysis of EO is clearly related to a poor or lack of understanding on the concept of RI and its limitations leading to, not rarely, abuse on the conclusions authors could get from the analytical information. To minimize errors and use RI properly, simple procedures can be applied. A roadmap for using retention indices is proposed [5].

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OL-02

Predicted response factors as an efficient and validated analytical method to screen for volatiles in *Cannabis sativa* inflorescences

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Keywords: *Cannabis sativa*, volatile compounds, predicted response factors, GC-FID, methodology

Abstract

Objective: The objective of this study is to validate a method resorting to the application of predicted response factors (PRFs) in the context of a robust routine quantitative screening of all volatile constituents of *Cannabis sativa* L. inflorescences for quality control purposes. **Methods:** Ground cannabis inflorescences were extracted with pentane and spiked with a methyl octanoate internal standard. Samples were analyzed by GC-FID, and compounds quantitated using PRFs. The method was validated by examining linearity, accuracy, repeatability, intermediate precision and limit of quantitation, as well as comparing hydrodistillation with solvent-extraction results. **Results:** The method afforded linear response over realistic concentrations for volatile constituents. Compilation of interlaboratory assays indicates mean accuracy of 102% of the assigned value, with an expanded uncertainty of ± 42%. Repeatability was ≤±8% between 0.03 and 4 mg/g. Intermediate precision was ±27% for individual analytes and ± 20% for "total terpenes" (sum of analytes), and robust across the concentration working range. Obtained profiles and yields calculated via solvent extracts were comparable to distilled essential oils. **Conclusions:** The combination of a quick solvent extraction and PRFs using GC-FID allowed for the high throughput untargeted screening of cannabis volatile constituents, without being restricted to compounds available as commercial standards. This approach is compatible with routine quality control of aromatic plant material such as cannabis inflorescences.

1. Introduction

Since *Cannabis sativa* L. legalization for recreational use in Canada in 2018, great attention has been devoted to the hypothesized "entourage effect" [1], a debated theory [2] suggesting that the psychoactive effects of cannabinoids are modulated by the plant's terpenoid volatile constituents (VCs) – with recent results beginning to provide insight in that regard [3]. Therefore, conducting VCs assays on all cannabis batches is now standard practice in Canada. Most testing facilities use targeted quantitation, which, however, fails to properly account for all VCs in cannabis owing to the lack of available standards for many analytes, and requires cumbersome calibration. Our objective was to implement and validate an efficient and untargeted GC-FID screening and quantification method of cannabis VCs in routine. To this effect, we examined the application of the PRFs approach proposed by the *International Organization of the Flavor Industry* (IOFI) [4] and validated it for the challenging case of a complex natural extract under screening conditions.

2. Material and Methods

Sample preparation: ≈1.0000 g dried cannabis inflorescences, finely ground with electrical blade grinders, were mixed with ≈10 mL GC grade pentane. Samples were spiked with a methyl octanoate (MO) solution at a known concentration then vigorously agitated for 15 min. A filtered liquid aliquot was used for GC analysis. A "deterpenated" cannabis matrix was prepared by lyophilizing a ground sample for several days while heating the sample at 40 °C, until minimal amounts of VCs could be observed by GC in a pentane extract. For seven cannabis samples already tested by solvent extraction, ≈ 20 g ground inflorescences were distilled with 300 mL water for 4 h using a Clevenger apparatus. The % v/m yield was converted to % m/m assuming a specific gravity of ≈ 0.9.

GC-FID analysis: Analyses were carried out on Agilent 7890A GC featuring a split/splitless injector and two FID detectors. The GC apparatus was equipped with two capillary columns (DB-5 and DB-Wax) of the same dimensions (10 m × 0.10 mm × 0.10 µm film thickness), fitted onto the same injection port. The temperature program was from 35 °C (1 minute), increased by 9 °C/minute to 260 °C (10 minutes). The injection port was heated to 250 °C. The injection volume was set to 1 µL, with the split set to 50:1 (0.03 µL & 300:1 for EOs). The carrier gas was hydrogen, used with constant flow mode at a flow rate of 0.7 mL/minute. The FID detectors were heated to 250 °C, with hydrogen flow set to 40 mL/min, airflow to 450 mL/min, and makeup gas (nitrogen) flow to 45 mL/min. Compounds were identified from their retention indices on DB-5 and DB-Wax columns, as well as by occasional concurrent GC-MS verification, and quantitated against MO using PRFs. [4]

Validation parameters: The protocol validity was assessed by examining linearity of MO response across working range; accuracy by comparison of measured vs. assigned analytes concentrations in reference samples obtained

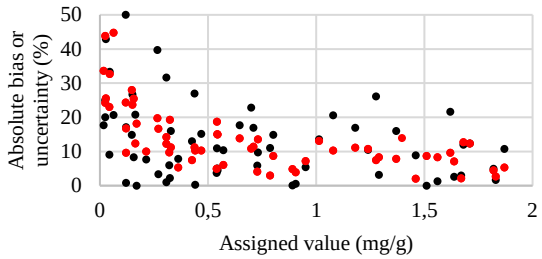

Figure 1: Scatterplot of absolute laboratory bias (•) and interlaboratory assigned value uncertainties (•) for 61 individual VC determinations at various concentrations.

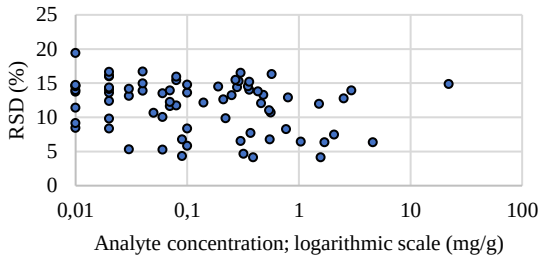

Figure 2: RSD of repeated quantitation of cannabis inflorescences VCs against their mean concentration in the plant material, showing no trend in results repeatability.

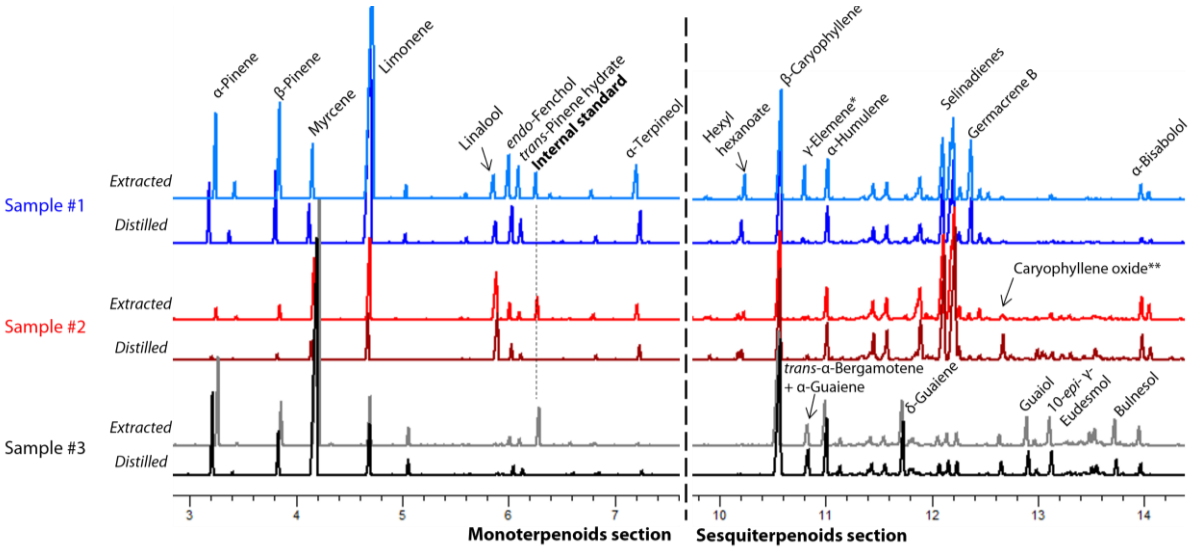

Figure 3: Qualitative comparisons of GC-FID (DB-5 column) profile sections for solvent-extracted VCs and hydrodistilled EOs for three chemically diverse cannabis inflorescence accessions. Slight shifts in retention times are due to the utilization of two different GC instruments (and columns) for the investigation; chromatograms were manually aligned. *See discussion. **Caryophyllene oxide is more prominent in the distilled oil following partial oxidation of the EO.

through interlaboratory assays (NSI Lab Solutions, NC, USA and ASTM International, PA, USA; 7 rounds between 2020 and 2024); instrumental repeatability by repeated analyte contents determination in a deterpenated cannabis matrix spiked with VC standards; intermediate precision by repeated analysis of two representative cannabis varieties, using six independent samples per variety, each sample being homogenized and tested in triplicate; and limit of quantitation (LOQ) defined as a signal/noise ratio >10. The yield of essential oils distilled were compared to the calculated total VC content using the PRFs, and a visual comparison of the VC profile obtained by distillation and solvent extraction was conducted for three samples exhibiting different compositions.

3. Results

The injection method afforded linear response (r^2=0.9988) over a concentration of methyl octanoate corresponding to the equivalent of 0.02 mg/g to 26 mg/g in the dried inflorescences. To determine method accuracy, results obtained from the quantitation methodology described herein were compared to the consensus (mean) values obtained interlaboratory assays. A total of 61 individual compound concentrations, within a range of 0 to 2 mg/g inflorescences, were compared to the concentrations measured by the relative response factor approach, with a Pearson coefficient of r=0.9871 ($p < 0.01$). The average relative bias between the laboratory measured values and the mean study values was +2%. The expanded uncertainty (with $\alpha = 0.05$) was determined with the Nordtest approach [5] from interlaboratory assays to be ± 42%, with bias being more important in the lower concentration range. The total uncertainty component attributable to the laboratory bias was not significantly different from the uncertainty of the assigned values via a t-test ($p = 0.82$; figure 1). Repeatability was assessed by triplicate injections of spikes of 18 different VCs at 5 concentration levels in vacuum-deterpenated inflorescences, and examining the relative standard deviation (RSD) of the measured response. At 0.01 mg/g, the mean RSD (expanded to 95% confidence) for all VCs was 25%, and was below 8% for concentrations between 0.03 and 4 mg/g. Intermediate

precision was evaluated by quantitating non-negligible, non-coeluting VCs in two cannabis varieties tested each as six independent samples, individually tested in triplicate. A RSD was calculated for each triplicate, and the mean RSD for each compound was computed across the 12 samples. For 50 compounds, the mean RSD (expanded to 95% confidence) was 27%, and it was 20% for the sum of all VCs. The RSD was uncorrelated ($r = 0.0173$) with the concentration of individual analytes (figure 2). Limit of quantitation (LOQ) was set to 0.01 mg/g by spiking various terpenes at 0.01 mg/g in vacuum-dried inflorescences and validating that all signal/noise ratios were $\geq$ 11.9. Total VCs content quantified in solvent extracts by the PRFs approach were on average 108% ± 10% (95% confidence) of the essential oil yield obtained by distillation across a range of 1.6 to 4.2% m/m. The profiles obtained via both methods showed similar qualitative patterns (figure 3).

4. Discussion and Conclusions

Surprisingly, few similar approaches to quantifying VCs directly in solvent-extracted aromatic plant material occur amongst papers citing PRFs. Beyond studies where our laboratory was involved in data acquisition [6–9], the closest instance was a study used PRFs to quantitate VCs in tea leaves in combination with solvent-assisted flavour evaporation [10]. Most other studies applying PRFs deal directly with essential oils or liquid flagrance, and not with raw plant material, highlighting the relevance of providing validation data for this application.

In addition to the other validation parameters described above, specificity of the analysis is ensured by the combination of retention indices from two complementary GC phases, as is commonly used in the field of essential oils profiling. The expanded method uncertainty was assessed using the compilation of interlaboratory assays in the absence of any realistic certified reference material of cannabis inflorescences for VCs. Attempts to establish an in-house reference material proved fruitless, as terpene contents inevitably decreased upon keeping. Whereas the expanded uncertainty of ±42% may seem high, one must consider that the uncertainty associated to interlaboratory-assigned values is equivalent to the laboratory bias, implying that individual VC determinations are associated with a large uncertainty across laboratories. Repeatability (±8%) and intermediate precision (± 27%) were satisfactory in view of the interlaboratory assays biases. As for total content of VCs, they were slightly higher (108%) when using the pentane extraction and PRFs compared with hydrodistillation. The moderate difference might in part be explained by the more important recovery of some VCs using solvent, and the fact that germacrene B and its thermal rearrangement product γ-elemene, which are often quantitatively important analytes in cannabis, are less prominent in EOs (e.g., sample #1, figure 5), possibly degraded. Otherwise, the qualitative comparability of the resulting chromatographic profiles is excellent, indicating equivalence between both experimental approaches. Overall, the ease of application of solvent extraction combined to PRFs for the purpose of routine quality control far outweighs the slight total VCs content difference.

In conclusion, the combination of a quick solvent extraction and PRFs using GC-FID allowed for the high throughput untargeted screening of cannabis VCs, without being restricted to compounds available as commercial standards. This approach is compatible with routine applications and allows testing of a large diversity of cannabis varieties. This protocol could be extended to other plants to offer an efficient tool to investigate chemotypes, ecological patterns and aromatic plants breeding programs.

Conflict of Interest

The authors declare that the method described here is offered as a paying service in their laboratory.

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OL-03

Preparative gas chromatography for the isolation of volatile terpenes for diabetes treatment

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Keywords: preparative gas chromatography, β-caryophyllene, diabetes, biological assays

1. Introduction

Despite the significant progress made using oral anti-diabetic agents, current treatments for diabetes are far from perfect, requiring to find novel viable alternatives. In this regard, recent studies have shown that some spices and herbs are active in reducing high blood glucose, thanks to the biological activities of the volatile fraction. In this study, *Piper nigrum* essential oil was characterized by means of GC-MS analysis, and fractionated through preparative gas chromatography prior to biological assays.

2. Material and Methods

The volatile fraction of *Piper nigrum* essential oil was investigated by means of GC-MS analysis. Target fractions were isolated from the essential oils by means of the preparative gas chromatographic system prior to biological assays.

3. Results

In a first step, the essential oil was analysed through GC-MS analysis to define the overall chemical composition. After that, a suitable preparative gas chromatographic system was exploited to isolate target fractions from the essential oil. In order to collect the highest amount of target fractions per analysis, wide bore capillary columns were exploited, allowing a consistent reduction in terms of total time analysis. In the first steps, the entire monoterpene and sesquiterpene fractions were collected, separately, prior to biological assays. Secondarily, specific subfractions, showing the highest biological activity, were evaluated for their anti-diabetic action.

4. Conclusions

This study demonstrated the usefulness of preparative gas chromatography as analytical tool prior to biological assays. Thanks to the selection of specific fractions of interest, it was possible to evaluate the specific contribution of the terpenes investigated to the resulting antidiabetic activity.

ACKNOWLEDGEMENTS

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

OL-04

Verification of Agarwood essential oil authenticity with handheld near infrared spectrometer

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Keywords: Agarwood oil, Aquilaria Crassna, Near infrared spectroscopy, Handheld NIRS, Chemometrics

Abstract

Agarwood essential oil is one of the most expensive plant extract materials. Agarwood oil is produced from several Aquilaria and Gyrinops tree species through fungal infection on injured trees. The quality of oil varies depending on the type of injury, species, environmental conditions, and extraction process. Due to its rarity, the attar is more often adulterated with low-quality oil from other species of the same genus, closely related essential oils from non-agarwood trees, and various synthetic chemicals that may harm health. A simple agarwood oil testing procedure is needed to build consumers' confidence and save CITES-protected endangered species. Regrettably, the market lacks a simple, quick, reliable, and cost-effective analytical technique for instant authenticity verification at trading or customs checkpoints. We have demonstrated that handheld near-infrared spectroscopy (NIRS) can confidently handle such a complex analytical issue. In this experiment, NIRvascan Smart NIRS Transmissive Model T1 and Model T11 (900 nm to 2150nm) are used on twenty agarwood oil specimens claimed to be distilled from Aquilaria Crassna species from different marketplaces and distillers. The chemometric analysis shows that samples measured at distillation factories have almost the same NIR signal characteristics, whereas the samples collected from trading sources significantly differ. It can be concluded that handheld NIRS devices could be used to identify adulteration in agarwood oil or other similarly important non-agarwood essential oils based on the inherent NIRS characteristics of each oil produced from a single species.

1. Introduction

Agarwood, also known as oud, is a highly prized aromatic resinous wood that forms within some species of Aquilaria and Gyrinops trees when they become infected with a particular type of mold (Phaeoacremonium parasitica) [1]. This infection triggers a response in the tree, causing it to produce a dark, fragrant resin in the heartwood. Perfumes, incense, and traditional medicines value this resin-embedded wood for its distinctive and complex fragrance [2]. Some regions where wild agarwood trees are found in South and Southeast Asia include Indonesia, Malaysia, Thailand, Vietnam, Cambodia, Laos, India, and Sri Lanka [1]. However, wild agarwood-producing trees are rare due to overharvesting and habitat loss. Agarwood oil, distilled from different species and regions, has its own specific scent profile. As there is a difference in users' preferences for scent profiles, a particular scent is more valuable to one customer than another species' scent. Rogue traders exploit customers' preferences by blending low-quality oil from one species with a similar one, or by adding synthetic or similar scents from low-cost essential oil adulterants. Consumers' trust in traded agarwood oil, the business's overall sustainability, the livelihood of numerous farmers, and the preservation of endangered wild species all depend on a straightforward agarwood oil testing procedure.

Although GC-MS analysis is highly effective for chemical characterization, it is not accessible to most enterprises and small producers and does not allow on-site measurements. The implications of modern handheld molecular spectroscopic techniques, such as handheld Near-Infrared Spectroscopic (NIRS) techniques, could be an ideal solution for the issue as it is a rapid, direct, cost-effective, non-destructive investigation using portable devices. The emergence of several handheld NIR spectrophotometers, including some that are remarkably compact, lightweight, and affordable, is revolutionizing NIRS technology. These devices enable measurements to be taken in real-world settings, such as during field operations, at the point of delivery, manufacture, sales, purchase, and usage. T.C.M. Pastore et al. recently developed near-infrared spectroscopy with a soft independent modelling class analogy (SIMCA) method to authenticate rosewood essential oil [3]. In 2021, De Oliveira Moreira A.C. et al. authenticated Copaiba oil with a handheld NIR spectrometer [4].

There have been scientific curiosity and research attempts to apply NIRS to agarwood oil and extractive quality and geographical authenticity [5-7], but no research work has been noticed in the public domain that has tested market-circulated agarwood oil products with handheld NIRS. This research examines the application of handheld

NIRS by comparing the chemometric data of Aquilaria Crassa agarwood oil samples directly sourced from the distiller's factory with commercially available similar products sourced from two significant international trading spots.

2. Material and Methods

Both commercial oil and directly sourced oil were claimed to be distilled from cultivated *Aquilaria Crassna* species by trade experts and plantation owners. The group one samples of All twenty samples were claimed to be plantations harvested and grown around the border regions of Thailand, Laos, and Cambodia. The wood was chopped and ground to make a homogeneous powder with industrial machinery.

Table 1 were examined at the factory premises in southern Thailand except for sample eight. Sample eight was distilled at a nearby border region of Cambodia at the distiller's Thailand office. The group two samples were collected and measured at the high street (Soi Sukhumvit 3/1) perfumery shops in Bangkok, Thailand. Group three samples were measured at high street shops in Daira, Dubai, UAE. All twenty samples were claimed to be plantations harvested and grown around the border regions of Thailand, Laos, and Cambodia. The wood was chopped and ground to make a homogeneous powder with industrial machinery.

Table 1 Agarwood Oil sample sources

Number	ID	Group	Supplier	Number	ID	Group	Supplier
1	Av_S	1	AVS	11	A_CN	2	ABR
2	Av_C	1	AVS	12	Ab_L	2	ABR
3	Av_St	1	AVS	13	A_CO	2	ABR
4	Av_D	1	AVS	14	An_ST	3	ANF
5	DG	1	DGW	15	AIm_T	3	ALM
6	Sm	1	SOM	16	AIm_C	3	ALM
7	Sm_D	1	SOM	17	AM_C	3	ABM
8	Nd_C	1	NAD	18	It_L	3	ITR
9	DG_D	1	DGW	19	Anf_T	3	ANF
10	P_T	2	PHI	20	Anf_P	3	ANF

In this experiment, two spectrometers were used for agarwood oil measurement. The specifications of the NIR instruments are handheld NIRvascan Smart Near Infrared Spectrometer Transmissive Model T1 (900nm to 1700nm) and Transmissive Model T11 (1350nm to 2150 nm). All oils were placed in a 3.5 ml glass vial for NIR measurement. All measurements were taken three times. The raw data were pre-processed with Origin Lab software, and Principal Component Analysis PCA analysis was performed with SIMCA 14.1.

3. Results

All preprocessed and normalized data of NIRS measurements is presented in Figure 1a. From raw NIR spectra, only two spectra can be seen as single out from others, which are visualized as light blue coloured (ID: A_CN) at around 900nm to 1200nm and another dark chocolate coloured (ID: A_CO) at around 1600nm to 1650nm. However, when those spectra are chemometrically analyzed with PCA, the differences and similarities among the three groups of samples are better separated and visualized in Figure 1b.

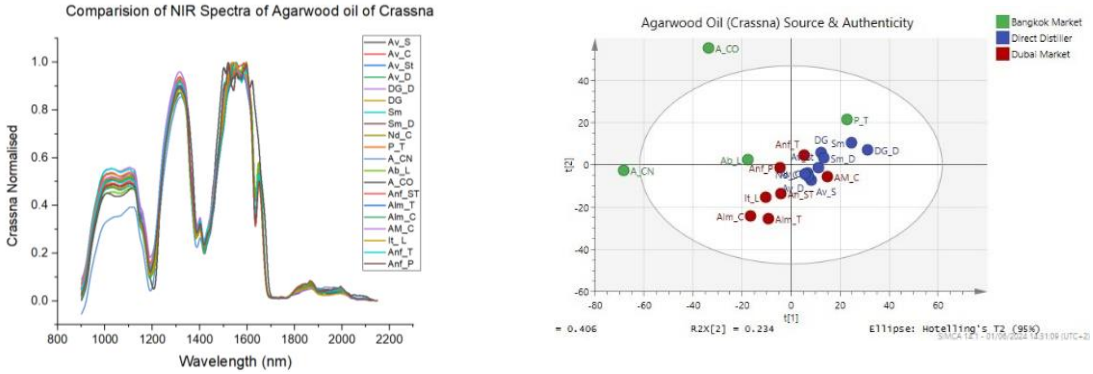

Figure 1 (a) Preprocessed NIR spectra of all twenty samples A. Crassna agarwood oil samples. **(b)** Score plot of PC(1&2) of all NIR spectra. The green, blue, and red circles indicate the sample source type of the marketplace.

The samples that are visually seen as different in the NIR spectra in Figure 1a also become separated from other data points at PC1&2 as outliers (Figure 1b). The samples sourced from distillers are more closely distributed in PCA than samples collected both from Dubai and Bangkok. In Figure 2a, the PC1&2 data are coloured according to supplier or trader's source. Here, it can be seen that except for samples of supplier ABR *(*highly adulterated*)*, the rest of the supplier samples are grouped in nearby spaces. A further PCA is performed on second-order derivative sample signals (excluding two outlier samples) to reveal deeper features of the NIRS data set. The PC 1&2 of second-order derivatized data is shown in Figure 2and Figure 2b represents PC1&3 of the same dataset.

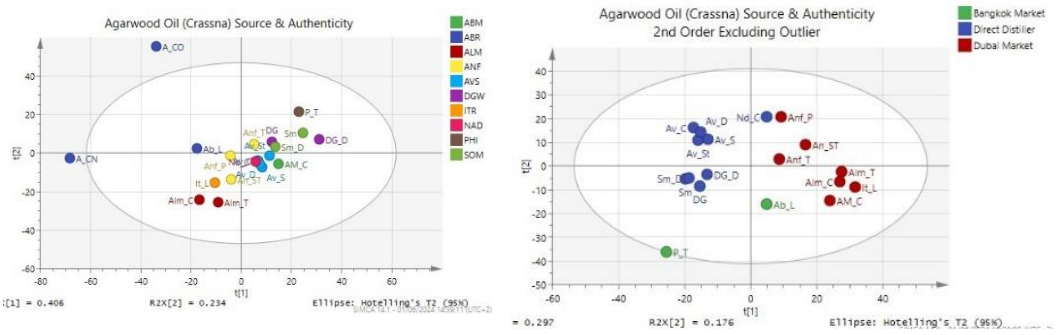

Figure 2 (a) PC (&,2) score plot of all samples. The colour code indicates samples sourced from a specific supplier. **2(b)** PC(1,2) Score plot of all NIR spectral second derivative lines excluding two outliers samples (ID: A_CO and ID: A_CN).

The PCA of 2nd-order derivatized data sounds more informative than the PCA of the normalized NIR dataset of *A. Crassna* oil samples. The oil sample groups are further separated from each other in this way. PC 1&3 is more distinguishable than PC 1&2 of the 2nd order derivative NIR dataset.

4. Discussion and Conclusions

A few distinct characters are revealed through this experimentation. The handheld NIR spectrometer can successfully capture such distinguishable features of complex agarwood oil samples that it is able to identify variations in sample datasets among distillers and different adulteration practices in the samples at different marketplaces. The Principal Component Analysis (PCA) of the NIR data and the PCA of the second derivative of the NIR data show a further clustering of the same supplier, potentially due to the different distillation types and practices of each distiller or the variations in adulteration practices of each trader. To further verify the hypothesis of this experimental outcome, future sample sets need to be examined with a specific experimental design. Nonetheless, it is clearly advantageous to use a handheld NIR spectrometer for such a future experiment due to the convenience of taking the instrument directly to a designated spot. Law enforcement agencies, essential oil supply chain personnel, and designated labs can easily identify specific species of oil using handheld NIRS instruments and a chemometrics calibration model from a large number of authentic sample datasets. The overall implementation of such a technique will ensure sustainably sourced agarwood oil verification in the trade route and help regain consumers' faith in the marketed authentic agarwood oil products. This research not only establishes a foundation and an efficient approach for assessing the quality and verifying the authenticity of agarwood essential oils available on the market, but it also serves as a guide for developing innovative techniques to qualitatively and quantitatively monitor other essential oils.

Conflict of Interest

There is no conflict of interest.

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OL-05

Investigating adulteration in commercial true lavender oils

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Adulteration of essential oils (EOs) is an unethical practice that negatively impacts both consumers and reputable suppliers. Although this issue is widely recognized, few original research articles in the scientific literature address this phenomenon. Therefore, the aim of this study was to investigate the adulteration of commercial essential oils of *Lavandula angustifolia*. A total of 55 oil samples were obtained from various retailers. The oils were characterized using GC-FID and GC-MS, including examination of the chromatographic profile according to ISO and Ph. Eur., the chiral profile, and quantification using an internal standard according to the Cachet method [1].

Out of the 51 essential oils labeled as *Lavandula angustifol*ia, only 51% were confirmed to be authentic. The remaining 49% were found to be adulterated, with 6% containing lavandin oil, 14% containing synthetic substances, and over 29% containing both lavandin oil and synthetics. On average, adulterated products were cheaper than genuine ones, priced at €0.45 and €1.19 per mL, respectively. However, some more expensive products were also found to be adulterated.

The key synthetic adulterants identified included synthetic linalool, synthetic linalyl acetate, 3,5,5-trimethylhexyl acetate, α-terpinyl acetate, and dipropylene glycol. The gas chromatographic profiles of the tested oils were generally consistent with the quantitative results, with only 4 of the 55 samples being diluted. Dilution was not evident in the standard chromatographic profile according to ISO and Ph. Eur. Principal component analysis enabled the differentiation of natural EOs from those with synthetic additives and allowed for the clustering of unadulterated *L. angustifolia* EOs. The chemical components that positively contributed to the product categorization of the natural group, whether true lavender or lavandin, included ocimene isomers, 1-octen-3-ol, 1-octen-3-yl acetate, trans-α-bergamotene, lavandulol, lavandulol acetate, α-santalene, (E)-β-farnesene, borneol, and bornyl acetate. Contrarywise, increased levels of α-pinene, *p*-cymene, limonene, and camphor were found to be correlated with EO adulteration. Isoborneol and isobornyl acetate were exclusively present in adulterated EOs.

Certain products were characterized by a composition strongly different from the natural one, suggesting that they were artificial blends that were not natural essential oils of plants of the *Lavandula* genus. Multivariate analyses revealed differences between EOs with synthetic additives and those of natural origin (both lavender and lavandin, regardless of labeling). Natural EOs were further differentiated into true lavender and lavandin groups, while a group of adulterated oils was completely distinguishable from unadulterated oils.

Industry standards were evaluated to detect true lavender EO adulteration. ISO 3515 was deemed impractical due to difficulties in assigning sample origin and being too stringent. However, Ph. Eur. demonstrated good performance in eliminating adulterated samples without rejecting many genuine samples, excluding only one genuine sample.

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OL-06

Application of Artificial Intelligence in predicting essential oil composition and correlating with biological activity using NMR and IR data

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Keywords: Artificial Intelligence, NMR Spectroscopy, IR Spectroscopy, Essential Oils, Composition Prediction, Biological Activity, Fingerprint Analysis, Machine Learning

Abstract

Essential oils (EOs) are known for their diverse biological activities and therapeutic potential, making them valuable natural products. This study explores the application of artificial intelligence (AI) and explainable AI (XAI) in analyzing nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy data to predict the composition of essential oils and correlate these compositions with their biological activities. Using machine learning (ML) and artificial intelligence (AI) algorithms, we obtained preliminary models that showed good ability to identify essential oil components from IR and NMR data. In addition, NMR and IR spectral profiles of EOs can be used as unique fingerprints instead of chemical composition to develop quantitative spectra-activity relationships (QSpAR) models to predict the activity of freshly extracted EOs with simple spectrophotometric IR analysis. These approaches will improve our understanding of the bioactivity of EOs and support their targeted application in various fields.

Introduction

Essential oils (EOs), derived from plants, since millennia have long been used for their medicinal, aromatic, and therapeutic properties. Their composition complexity and instability pose challenges for traditional analytical methods. Recent advancements in artificial intelligence (AI) offer promising and interesting solutions for the detailed and fast analysis of these natural products. This study focuses on the use of AI to interpret NMR and IR spectroscopic data, aiming to predict essential oil composition accurately and correlate these findings with their biological activities through quantitative spectra-activity relationships (QSpAR) models.

Material and Methods

IR spectral data were obtained with a FT-IR spectrometer Spectrum Two, equipped with a Universal ATR sampling accessory (Perkin Elmer, Milano, Italy). One drop of each sample was deposited on the ATR diamond crystal cell. The spectra were acquired in the spectral range of 4000–450 cm^{-1}, with a spectral resolution of 4 cm^{-1}, averaging 16 scans per spectrum[1]. The number of scans was selected for the optimal signal-to-noise ratio. Regarding the NMR analysis, To prepare the NMR tubes, 50 µL of each mixture were diluted with 550 µL of CDCl3 (0.03% TMS) directly into a WILMAD® NMR tube, 5 mm, Ultra-Imperial grade, 7 in. L, 528-PP (Sigma-Aldrich, Milan, Italy). The preparation of the tubes was performed by considering the exact weight of the mixture and solvent, in order to easily get the final concentration (w/w). All the analyses were carried out on a Bruker FT-NMR Avance III HD 600 MHz spectrometer (Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany). All the experiments were performed at 300 K and non-spinning. The assignments of vegetable oils signals were carried out using standard compounds and by comparison of ^{1}H-NMR and ^{13}C-NMR spectra with literature data in previous work [2]. Similarly to previous works [3-5] machine learning (ML) and AI algorithms, including neural networks and support vector machines, were trained on these spectral datasets to predict the chemical composition of the EOs. The spectral data were also analyzed to establish fingerprints, which were then correlated with biological activity assays performed on the essential oils. The biological activities evaluated included antimicrobial, antioxidant, and anti-inflammatory properties.

Results

The AI models demonstrated high accuracy in predicting the composition of essential oils from NMR and IR data. As previously reported in the development of quantitative composition-activity relationships (QCAR) models

[6,7], preliminary models indicated that the IR and/or NMR spectral fingerprints provided good correlations with the EOs associated biological activities. Essential oils with specific compositions showed distinct bioactivity profiles, highlighting the effectiveness of the AI approach in linking chemical makeup to biological effects.

Monoterpenes identification: accuracy% in real mixtures of monoterpenes

	1,8-Cineole	Geranyl acetate	Camphor	Geraniol	Linalool	Citronellol	Linalyl acetate	Limonene	Citronellal	Citral
NMR	94.839	99.355	91.613	96.129	97.419	97.419	93.548	97.419	92.258	99.355
IR	90.968	81.290	73.548	96.774	87.097	94.839	76.774	85.161	89.677	98.065
NMR+IR	94.194	99.355	87.742	96.129	95.484	96.774	94.839	98.710	92.903	98.065

Monoterpenes identification: accuracy% in the EO of Lavandula and Cymbopogon

	1,8-Cineole	Geranyl acetate	Camphor	Geraniol	Linalool	Citronellol	Linalyl acetate	Limonene	Citronellal	Citral
NMR	75.354	37.980	69.899	88.081	74.141	88.283	81.010	46.465	78.788	87.273
IR	80.202	68.283	71.717	90.505	84.848	84.848	64.242	56.162	69.899	82.020
NMR+IR	73.737	35.152	68.687	90.909	81.414	94.141	77.374	44.848	83.232	87.071

Discussion and Conclusions

The integration of AI in analyzing NMR and IR data represents a significant advancement in essential oil research and analysis. The method here presented offers a rapid, non-destructive means of determining EO's composition and understanding their biological activities. The ability to correlate spectral fingerprints with bioactivity not only aids in quality control but also in the discovery of EO with specific therapeutic properties. These findings underscore the potential of AI-driven spectroscopy in enhancing the application and efficacy of essential oils.

Conclusions.

The study successfully demonstrates the application of AI in predicting essential oil composition and correlating it with biological activities using NMR and IR spectroscopy. This innovative approach provides a powerful tool for the natural products industry, facilitating the development of high-quality, standardized essential oils with targeted biological effects. Future research will focus on expanding the dataset and refining the AI models to further enhance predictive accuracy and application scope.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OL-07

Unfolding seasonal and diurnal variations of leaf aroma volatiles in Indian bay leaf – a potential source of valuable essential oil

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Keywords: Cinnamomum tamala, seasonal and diurnal variations, essential oils, histochemistry, ultrastructure

Abstract

The leaves of Cinnamomum tamala are extensively used in culinary applications due to their rich aroma and therapeutic properties. However, the composition and content of leaf essential oils exhibit variability due to fluctuations in climatic conditions and harvesting time. This study evaluated the impact of seasonal and diurnal variations on the composition and contents of aroma volatiles in mature C. tamala leaves. In summer, the aroma volatile profile was dominated by phenylpropanoids, while in winter, monoterpenes acquired the dominance. The contents of primary metabolites were influenced by the harvesting season and time. Organic acids and sugars showed the highest accumulation in leaves harvested during summer evenings and winter mornings, respectively. Histochemical analysis revealed the presence of lipids and terpenes in the secretory cells, as shown by Sudan III and NaDi staining. Ultrastructural study elucidated the ontogeny of secretory oil cells that accumulate essential oils.

1. Introduction

The aromatic leaves of *Cinnamomum tamala* are widely utilized as spices in the Asian subcontinent and have extensive applications in traditional medicine due to their medicinal properties (Sudan et al. 2013). These perennial trees are distributed across tropical and subtropical regions worldwide. Their characteristic aroma is attributed to essential oils, which find wide use in the food and flavoring industry (Sudan et al. 2013). Environmental factors and seasonal variations significantly impact the content and composition of these oils, a trend observed in other members of the Lauraceae family as well (Xavier et al. 2020). Essential oil synthesis in Lauraceae predominantly occurs in secretory oil cells (Saha et al. 2023), whose formation and chemical content in *C. tamala* leaves require further investigation. This study delves into understanding the influence of seasons and harvesting times on leaf aroma volatiles, as well as seasonal and diurnal effects on primary metabolites and enzyme activities. Additionally, it aims to elucidate the chemical composition of essential oils in secretory oil cells and describes their ontogeny in *C. tamala* leaves.

2. Material and Methods

The analysis of volatiles internal pool in different growth stages of leaves were performed in monsoon season using GC-MS (Saha et al. 2023) and optimum mature growth stage was identified. Then, the comparative profile of volatiles internal pool was investigated during five different seasons and the highest and lowest volatiles yielding seasons were determined. The diurnal variations (06-00h in morning and 18-00h in evening) of volatiles internal pool, primary metabolites (Roessner et al. 2001) and some related enzymes (Das et al. 2019) as well as essential oil analysis (Saha et al. 2024) were performed on these two seasons. Histochemical investigation was carried out to analyze the anatomy of the secretory cells and chemical nature of the oil present inside (Saha et al. 2023). Ultrastructural analysis was done to understand the ontogeny of the secretory oil cells (Maiti et al. 2017).

3. Results

Chemical analyses

The 40-50 days old leaves were identified as the optimum mature stage and used for further investigations. Among five different seasons studied, summer accumulated highest volatiles while winter accumulated the lowest amount of volatiles (Figure 1a). A total of 37 volatile aromatic compounds were identified tentatively and grouped into six chemical classes namely, monoterpenes, monoterpenoids, phenylpropanoids, sesquiterpenes, sesquiterpenoids and alcohols. Diurnal study in summer and winter seasons showed the highest amount of volatiles internal pool accumulation in summer evening and the lowest in winter morning. Besides, organic acids and sugars showed highest accumulation during summer evening and winter morning (Figure 1b). Enzymes related to primary metabolism, also showed higher activity during summer evening. Highest yield of essential oil was also seen in summer evening. The composition of oil was mostly dominated by phenylpropanoid compounds during summer evening while a good content of monoterpenes was present in morning oil.

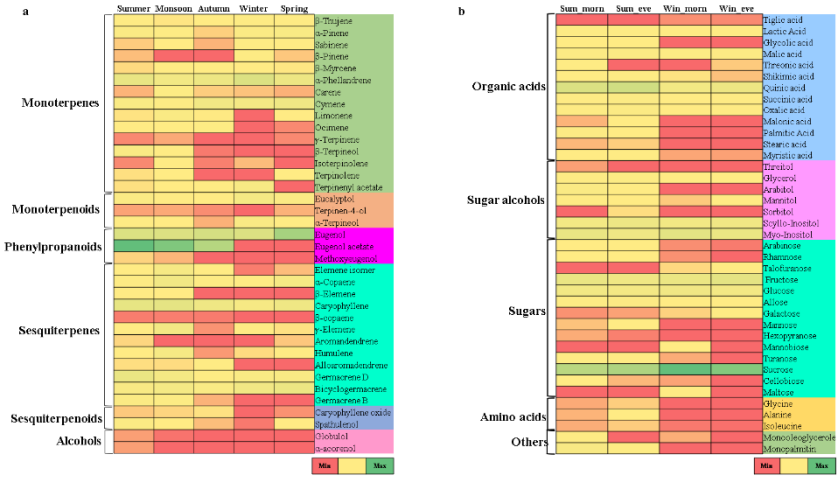

Figure 1: Heatmap showing the diversity of aroma volatiles internal pool present in the mature leaves of *C. tamala* during different seasons. Hexane extracts were analysed by GC-MS (a). Colour codes indicate relative abundance of individual compound as was calculated on the basis of ethyl hexanoate, the internal standard used in this study. Green and red colours denote highest and lowest group abundances, respectively. Heatmap showing the diurnal diversity of primary metabolites present in the mature leaves of *C. tamala* during highest (summer) and lowest (winter) yielding seasons. Derivatized metabolites were analysed by GC-MS **(b).** Colour codes indicate relative abundance of individual compound as was calculated on the basis of total peak area. Green and red colours denote highest and lowest group abundances, respectively.

Microscopic analysis

Histochemical analysis revealed the presence of lipids and terpene compounds in the content of the secretory oil cells whereas lignin, pectin and suberin components were deposited on the wall of the secretory cells (Figure 2a-e).

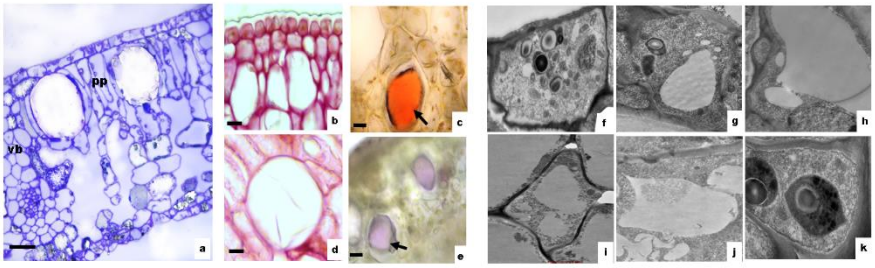

Figure 2: Histochemical analysis of oil cells stained with toluidine blue O, ruthenium red, sudan III and NaDi stains (a-e); arrowheads showing oil cells. Ultrastructural development of secretory oil cells (f-k). Scale bars: 50 µm (a); 30 µm (b-e); 2 µm (f, g, i); 1 µm (h, k); 500 nm (j). [pp: palisade parenchyma; vb: vascular bundle]

Phenolics and starch granules were also detected on the cortical region of petiole and parenchymal region of leaf lamina. Anatomical investigation revealed that the secretory cells are present in the cortex region of petiole and mesophyll region of leaf blade. The oil cells were solitary, isolated and distinguishable by their content, shape, size and thickening of cell walls from the neighbouring cells (Figure 2a). Ultrastructural study elucidated the development of a mature oil cell showing the transformation of a young idioblast cell with a large central vacuole and several other cellular organelles to a mature secretory oil cell with oil filled cavities and no cellular organelles (Figure 2f-k).

4. Discussion and Conclusions

Environmental factors strongly influenced the metabolite content and composition in plants. This study focused on how seasonal and diurnal changes affect metabolite profiles in *C. tamala* leaves. Climatic parameters like temperature, rainfall, and humidity were monitored to evaluate their effects on the yield and composition of internal pool of volatile metabolites. The aroma volatile profile was dominated by phenylpropanoids except in autumn and winter, with temperature significantly affecting phenylpropanoid synthesis. Eugenol and its derivatives varied with temperature, showing higher eugenol acetate in warmer seasons. Monoterpenes and monoterpenoids were more abundant in winter. Diurnal variations showed accumulation of organic acids were higher in summer evenings, while sugar levels were higher in winter morning. Winter leaves showed higher sugar accumulation for osmotic protection. Overall, understanding these variations helps determine optimal harvesting times for quality control. Secretory oil cells, common in at least 20 genera of Lauraceae, were identified as the primary site for essential oil accumulation in *C. tamala*. These cells, usually spherical with suberized walls, contain lipids and terpenes, while phenolics, aldehydes, and pectic substances are found on the cell walls, indicating chemical compartmentalization. We observed that very young idioblast cells, potential future oil cells, initially resemble surrounding cells but contain a central vacuole and lack osmiophilic substances. As they develop, these cells show increased electron density and the cell wall thickened by suberin deposition and tertiary wall formation. The developmental stages of oil cells did not correlate strictly with leaf maturation, as different stages were observed within the same leaf. However, leaves aged 40-50 days had a higher number of mature oil cells, suggesting the highest oil yield. The study also elucidated that during oil cell development, the endoplasmic reticulum reorganized in parallel arrays, and plastids were involved in essential oil synthesis. Oil droplets fused with the oil cell cavity contributing to larger oil accumulations. The plastids presumably took part in oil deposition by direct fusion with the oil cell cavity or by exuding its content to the surrounding vacuole, aligning with previous literature.

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Conflict of interest: The authors declare no conflicts of interest.

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OL-08

Effects of *Alpinia zerumbet* essential oils on human olfactory receptors and depressive symptoms of postmenopausal women

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Keywords: *Alpinia zerumbet*, shell ginger, olfactory receptor, postmenopausal women, depression

Abstract

Alpinia zerumbet is a plant of the ginger family (*Zingiberaceae*) used in folk medicine and is widely distributed in subtropical and tropical regions. There are mainly two types, *Alpinia zerumbet* (Pers.) Burt & Smith) (AZ) and *Alpinia zerumbet* (Pers.) B. L. Burtt & R. M. Sm. var. *excelsa* Funak & T. Y. Ito) (AZe) in Okinawa, which are genetically distinct subspecies and have very different components. Thus, the present study was conducted to investigate the effects of these two AZEOs on human olfactory receptors and depressive symptoms of postmenopausal women. A comprehensive analysis on the effects of two essential oils on human olfactory receptors revealed that AZEO and AZeEO each exhibited different activation patterns among the receptors, and that only one receptor was commonly activated among the top 22 receptors by both AZEOs. In the human study, aromatherapy using two AZEOs improved depressive symptoms in postmenopausal women. There were no symptoms in common that were improved between the two AZEOs., and the effect of AZEO was higher that of AZeEO, suggesting that the difference in the effects of these two AZEOs is due to differences in their constituents and the olfactory receptors that the constituents activate. Our findings suggested that aromatherapy using AZEO is recommended for improving depressive symptoms of postmenopausal women.

1. Introduction

Olfactory receptors, which detect scent components, are the largest gene family among G protein-coupled receptors (GPCRs) with approximately 400 types of human olfactory receptors. It is believed that diverse scents are recognized by the various patterns of olfactory receptor activated by scent molecules. Essential oil components have also been reported to activate olfactory receptors. *Alpinia zerumbet* is a plant of the ginger family (*Zingiberaceae*) used in folk medicine and is widely distributed in subtropical and tropical regions. There are two types, AZ and AZe in Okinawa, which are genetically distinct subspecies and have very different components. It has been reported that inhalation of shell ginger essential oil induces neuropsychiatric behavioral changes and has an anxiolytic effect in mice [1,2]. Several essential oils were known to have positive effects on QOL, insomnia and psychological symptoms of postmenopausal women. We found that the essential oils improved the stereotypic and other overt behaviors induced by ovariectomy. Thus, the present study was conducted to investigate the effects of AZEO and AZeEO on human olfactory receptors and on depressive symptoms of postmenopausal women.

2. Material and Methods

The essential oils of AZ and AZe were prepared from their leaves using steam distillation, and the main component contents are as follows: AZEO contained p-cymene (19.25%), 1,8-cineole (15.50%), α-pinene (10.48%), limonene (10.10%), camphene (5.18%). and AZeEO contained terpinene-4-ol (21.70%), 1,8 cineol (18.42%), γ-terpinene (13.19%), sabinene (11.63%), *p*-cymene (7%). HEK cells were transfected with 389 types of human olfactory receptor genes and the reporter plasmid cAMP-dependent luciferase (pGloSensor), which emits light in response to cAMP concentration. The cells were treated with AZEO and AZeEO in a vapor state and the increases in intracellular cAMP accompanying the activation of olfactory receptors was evaluated by measuring the levels of luminescence [3]. Human study was approved by the Ethics Committee of Kitakami Central Hospital (No. 2023-01). The subjects included 40 menopausal or postmenopausal female volunteers experiencing depression. Aromatherapy was performed by inhalation three times a day (10 minutes each time) for two weeks. On day 1, before aromatherapy, subjects were asked about their date of birth, current medical history, duration of illness, and

past medical history, and their height, weight, blood pressure, and pulse were measured. They were asked to fill out a questionnaire, including the Quick Inventory of Depressive Symptoms (QIDS-J), the Quick Occupational Stress Questionnaire, and the level of depression. In addition, blood samples were taken to measure plasma catecholamines, serotonin, and cortisol.

3. Results

When the activation of olfactory receptors by vapor treatment with AZEO and AZeEO was sorted by activation, it was found that there were no common receptors among the top three olfactory receptors, and even among the top 22, there was only one common receptor (Figure 1). This result was due to the difference in the scent components of each AZEO and may also contribute to the difference in physiological effects in cell culture level and in vivo.

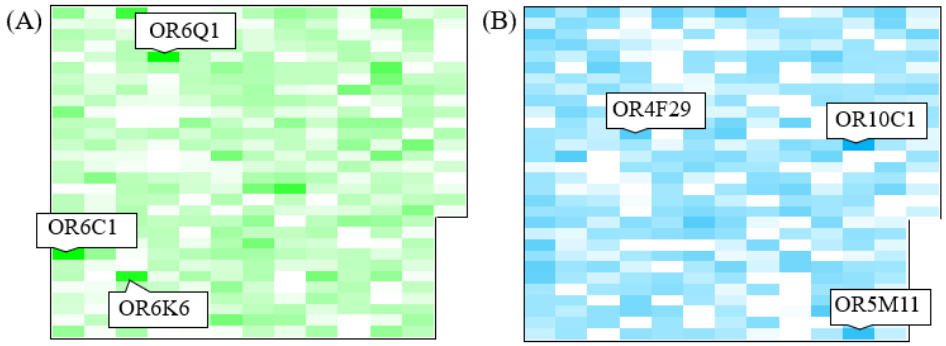

Figure 1 Reaction intensity of each human olfactory receptor to AZEO (A) and AZeEO (B). The relative luminescence intensity (minus the mock control value) is expressed as color intensity.

Because preclinical studies have shown that EO ameliorated abnormal behaviors induced by ovariectomy in a postmenopausal animal model, we decided to conduct clinical trials in postmenopausal volunteers. The average age at the start of the two groups was 53 to 55 years old, and there were no differences between the groups in age, height, weight, diastolic blood pressure, pulse rate, or level of depression. The level of depression, which was the main evaluation item, significantly decreased in both groups after aromatherapy: in AZeEO group, from an average of 5.0 to 3.7, and in AZEO group, from an average of 4.5 to 3.0. (Table 1). There was no difference between the groups in the total score of the Quick Inventory of Depressive Symptoms (QIDS-J), but the burden level of the Simple Occupational Stress Questionnaire was significantly (p=0.024) higher in the AZEO group (5.0±2.1 points) than in the AZeEO group (3.7±1.8 points) (Table 2). After the treatment, the subjects' impressions of improvement in their depression and overall health were both "slightly improved" by an average of about 2 points, with no difference between the two groups.

Table 1 The effect of aromatherapy on post-menopausal depression

	AZeEO		AZEO	
	before	**after**	**before**	**after**
Age (years)	54 ± 5		55 ± 7	
Height (cm)	158 ± 5		157 ± 6	
Weight (kg)	57 ± 11		55 ± 12	
Systolic blood pressure (mmHg)	127 ± 17	122 ± 19	113 ± 16	112 ± 17
Diastolic blood pressure (mmHg)	72 ± 12	70 ± 12	69 ± 8	68 ± 12
Pulse (/min)	75 ± 10	71 ± 9	78 ± 8	76 ± 7
Severity of depression (0-10 points)	5.0 ± 1.9	3.7 ± 1.9*	4.5 ± 1.7	3.0 ± 1.1***
Number of aromatherapy sessions (times/day)		3.2 ± 0.5		3.3 ± 0.6
Degree of improvement in depression		2.1 ± 0.9		1.6 ± 0.8
Degree of improvement in overall health		2.2 ± 0.8		1.9 ± 0.9

(*p<0.05, **p<0.01, ***p<0.001 vs before)

On the simplified depression symptom scale of QID-J, 0-5 points is normal, and 6-10 points is mildly depressed. Before aromatherapy, participants in the AZEO and AZeEO group were in a mildly depressed state, with an average of 7.1 points and 7.7 points (Table 2), respectively. After aromatherapy, five items (concentration, self-outlook,

suicidal ideation, involvement, energy) in the AZEO group and two items (sleep disturbance and appetite/weight change) in the AZeEO group improved significantly, and the total scores in both groups improved significantly, to an average of 4.1 points and 5.4 points, respectively, to within the normal range. In the simple scoring method of the Occupational Stress Simple Questionnaire, no items improved significantly in the AZEO *and* AZeEO group. The average values of all blood test items in the two groups before aromatherapy were within the normal range, and no items changed significantly after aromatherapy.

Table 2 The effect of aromatherapy on post-menopausal depression

		AZeEO		AZEO	
		before	**after**	**before**	**after**
Simple Depressive Symptoms Scale QIDS-J	Sleep disturbance	2.0 ± 0.8	1.4 ± 0.8*	1.9 ± 0.7	1.8 ± 0.7
	Sad mood	0.7 ± 0.6	0.6 ± 0.6	0.5 ± 0.6	0.3 ± 0.4
	Appetite/weight change	1.2 ± 0.9	0.7 ± 0.9*	1.0 ± 0.9	0.6 ± 0.8
	Concentration	0.6 ± 0.5	0.4 ± 0.5	0.5 ± 0.5	0.1 ± 0.2***
	Self-outlook	0.8 ± 1.0	0.6 ± 0.9	0.7 ± 0.6	0.3 ± 0.4**
	Suicidal ideation	0.5 ± 0.7	0.2 ± 0.4	0.4 ± 0.6	0.1 ± 0.3*
	Involvement	1.0 ± 1.1	0.8 ± 1.1	1.1 ± 1.1	0.3 ± 0.5**
	Energy	0.8 ± 0.6	0.5 ± 0.5	0.8 ± 0.6	0.3 ± 0.4**
	Psychomotor change	0.5 ± 0.6	0.3 ± 0.6	0.5 ± 0.8	0.3 ± 0.5
	Total	7.7 ± 3.5	5.4 ± 3.9*	7.1 ± 3.8	4.1 ± 2.4**
Occupational Stress Simple Questionnaire (simple scoring method)	Workload	3.7 ± 1.8	3.9 ± 2.1	5.0 ± 2.1	4.6 ± 2.1
	Job control	1.2 ± 1.1	0.9 ± 1.1	1.2 ± 1.2	1.0 ± 0.9
	Interpersonal relationships	0.9 ± 1.1	0.7 ± 0.7	0.5 ± 0.8	0.5 ± 0.8
	Compatibility	0.5 ± 0.8	0.4 ± 0.8	0.2 ± 0.5	0.2 ± 0.5
	Psychological stress	7.8 ± 4.3	7.7 ± 4.4	6.0 ± 4.4	5.2 ± 3.3
	Physical stress	4.4 ± 2.7	4.4 ± 2.5	3.4 ± 2.6	2.9 ± 2.2
	Workplace support	2.7 ± 2.1	2.7 ± 2.3	3.3 ± 2.2	3.4 ± 2.2
Blood test	Adrenaline (ng/mL)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.02
	Noradrenaline (ng/mL)	0.43 ± 0.11	0.42 ± 0.10	0.45 ± 0.09	0.42 ± 0.11
	Dopamine (ng/mL)	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.01
	Serotonin (ng/mL)	120.9 ± 42.6	119.2 ± 41.0	112.5 ± 41.2	116.3 ± 40.4
	Cortisol (μg/mL)	8.18 ± 2.57	7.49 ± 2.72	8.93 ± 3.82	8.23 ± 2.30

(*p<0.05, **p<0.01, ***p<0.001 vs before)

1. Discussion and Conclusions

The findings showed that aromatherapy using AZEO *and* AZeEO has improvement effects on depressive symptoms in the perimenopausal or postmenopausal women. The effect of AZEO was greater than that of AZeEO, and this difference in effect is thought to be due to the differences in the components contained in AZEO and AZeEO and probably their effects on olfactory receptors.

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Conflict of Interest

Je Tae Woo and Aki Yamano are employees of YL Okinawa Research & Development Center. *G.K.* Hsueh Kung and Mark Bartlett are employees of Young Living Essential Oils.

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OL-09

Aromatogram testing: Standardization of the clinical broth microdilution test to support the development of appropriate integrated antimicrobial therapies and address antibiotic resistance

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Keywords: Aromatogram, clinical test, standardization, broth micro-dilution test, antimicrobial activity

Abstract

The "Aromatogram" is the clinical microbial test used to evaluate the antimicrobial effectiveness of Eos against pathological microbial strains. To date, this test is conducted with different laboratory methods without any standardization and reproducibility. This prevents the spread of this microbiological method among clinical tests. The aim of this work was to standardize the Aromatogram test (AT) according to the EUCAST international guidlines. The AT was planned using 15 essential oils already monographed by the European Medicine Agency or by national or international organizations. The standardization was planned in two phases. Phase 1: Standardization of the test using microbial reference strains. Phase 2: Data collection using microbiological clinical strains. Parameters set during Phase 1 were the following: (i) most appropriate surfactant, (ii) Dilution range of Eos, (iii) durability of a preloaded kit. Standardization has made it possible to define a reproducible, sensitive and reliable test but, at the same time, easy to perform and interpret. Standardization of AT is important because, like antibiotics, EOs do not always show the same activity against pathogenic strains belonging to the same species. Furthermore, in the era of antibiotic resistance, it is important to have clinical methods capable of providing effective therapeutic options, to be used alone or together with antibiotics in an integrated protocol, in order to enhance antimicrobial therapy and counteract the emergence of resistance.

1. Introduction

"Aromatherapy" is a branch of Phytotherapy which uses essential oils (EOs) to treat various pathologies including those of microbial origin. Recently, the scientific community strongly accredited the traditional use of EOs as antimicrobial treatments but, unfortunately, the medical use has not experienced the same growth due to the lack of diagnostic tools capable of supporting physician's therapeutic choices. Until now, the "Aromatogram", which is the clinical test used to evaluate the antimicrobial effectiveness of EOs, was carried out with different non-standardized laboratory methods. This variability, on the one hand, does not make it possible to obtain reproducible results between laboratories (generating confusion) and, on the other hand, does not bring added value to practitioners who prefers to work empirically, basing his therapies on data obtained from literature or traditional uses. This work aimed to standardize the Aromatogram test (AT) according to the clinical laboratory method used to test the sensitivity of pathogenic strains to antibiotic drugs.

2. Material and Methods

The standardization of the AT was planned in two phases. Phase 1: Standardization of the test with microbial reference strains. Phase 2: Data collection using microbiological clinical strains.The phase 1 was developed by using the broth micro-dilution test according to EUCAST international guidlines. Three Gram negative and two Gram positive reference strains were chosen for the standardization (*Klebsiella pneumoniae* ATCC700603, *Pseudomonas aeruginosa* ATCC060127853, *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC29213, *Enterococcus faecalis* ATCC29211). 15 EOs (Table 1) already monographed by the European Medical Agency (EMA) and/or by international or national organizations such as ISO and AFNOR. All EOs used for the standardization were chemotyped in order to check their congruence with international standards. 96-well round

bottom plates were used. The latter is a small variation compared to EUCAST guidelines [1,2] made to facilitate the visual reading of the test. Three controls were placed in duplicate on each plate. Specifically, a positive control without surfactant, one with surfactant at the higher concentration and one negative. An adhesive film generally used for ELISA tests was used to seal wells to prevent the volatile components from influencing each other. The following parameters were set during the standardization: (i) most appropriate surfactant. It was decided to study two surfactants: DMSO (because it is the most indicated in international clinical protocols) and Tween80 (because it is the most indicated in the literature as a surfactant for the AT). (ii) Dilution range of EOs. For each essential oil, 6 scalar dilutions were tested starting from a maximum concentration of 8% v/v. (iii) Storage of the pre-loaded 96-well plate. Tests were developed to evaluate the durability of pre-loaded plates stored at -20°C. Tests were monitored for 24 months. Phase 2 was developed by testing the standardized protocol in Phase 1 against clinical strains including *S. aureus*, *E. coli*, *Pseudomonas* spp, *K. pneumoniae*, *E. faecalis*, *Candida* spp, *S. pyogenes*.

Table 1. List of essential oils used for standardization

N°	Binomial nomenclature	Vulgar name	Chemotype (*)
1	*Thymus vulgaris*	Thyme	Thymol
2	*Origanum vulgare*	Origan	Carvacrol
3	*Syzygium aromaticum*	Geranium	Eugenol, eugenil acetate
4	*Cinnamomum zeylanicum*	Cinnamon	Cinnamaldehyde
5	*Coriandrum sativum*	Coriander	Linalool, 2-decenal
6	*Melaleuca alternifolia*	Tea-tree	Terpineol, 1,4-terpinene
7	*Cymbopogon martinii*	Palmarosa	Geraniol, geranyl acetate
8	*Mentha piperita*	Peppermint	Menthol, menthone
9	*Cymbopogon citratus*	Lemongrass	Neral, geranial
10	*Cuminum cyminum*	Cumin	Cuminaldehyde
11	*Illicium verum*	Star anise	Trans-anethole
12	*Citrus limon*	Lemon	Limonene
13	*Juniperus communis*	Juniper	Alpha-pinene, sabinene
14	*Eucalyptus globulus*	Eucalyptus	1,8 cineole
15	*Carum carvi*	Caraway	Limonene, carvone

Note. (*) compound(s) present at a higher concentration(s) are indicated.

Results

The method has been standardized according to the international European EUCAST guidelines in order to obtain a laboratory test that was reproducible, sensitive and reliable but, at the same time, easy to perform and interpret. Tween80 has proven to be the best surfactant for EOs solubilization. Both the most powerful EOs and the easily oxidizable ones (Lemongrass) were tested setting the higher concentration tested equal to 2% v/v. Pre-loaded plates can be stored at -20°C for a year without risk of altering the effectiveness of the test. Until today, the standardized Aromatogram has been used to evaluate the sensitivity of approximately 400 clinical strains, refining the method towards fungal or bacterial strains, both aerobic and anaerobic.

Discussion and Conclusions

The standardization of the AT is important because, like antibiotics, EOs do not always show the same activity towards pathogenic strains belonging to the same species. Therefore, empirical treatments do not always have positive outcomes. For this reason, the development of a standardized test to support the choice of the correct therapy is of fundamental importance. Furthermore, in the era of antibiotic resistance, it is becoming increasingly important to have effective treatment options, to be used alone, in cases of minor infections to reduce the use of antibiotics, or toghether with antibiotics in integrated protocol in order to enhance antimicrobial therapy. With the introduction of the standardized aromatogram in clinical laboratories, pathogenic strains isolated from biological samples of muco-cutaneous origin can be analyzed for their sensitivity to EOs offering an additional weapon in the fight against antibiotic resistance.

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Conflict of Interest

Authors (M.D.V., M.D.M., M.M., M.S. and F.B.) declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. A.Z. had no role in the collection, analyses, or interpretation of data.

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OL-10

Synergistic solutions: Essential oil compounds with conventional antimicrobials for Skin Infections

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Keywords: antimicrobial, dermatology, essential oil compounds, cytotoxicity, anti-inflammatory, interactions, antibiotics

This study delves into the efficacy of combining essential oil compounds with conventional antimicrobials against various skin pathogens, aiming to elucidate interactive profiles, toxicity, and anti-inflammatory properties. Six essential oil compounds (α-pinene, γ-terpinene, ±linalool, eugenol, carvacrol, and cinnamaldehyde) and eight conventional antimicrobials (amoxicillin, ciprofloxacin, erythromycin, gentamicin, meropenem, tetracycline, miconazole, and nystatin) were tested against common skin pathogens (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606, *Cutibacterium acnes* ATCC 11827, and *Candida albicans* ATCC 10231). Minimum inhibitory concentrations (MIC) were determined, and synergistic interactions were assessed, where eight synergistic interactions were identified, primarily against Gram-positive bacteria. Combining selected compounds reduced toxicity, with the amoxicillin-eugenol combination showing the lowest toxicity (LC50 = 1081 µg/mL) and highest selectivity index (14.41) when combined in a 70:30 ratio with the antibiotic in the higher ratio. Further analysis evaluated cytotoxicity and anti-inflammatory effects. The combination of ciprofloxacin and cinnamaldehyde demonstrated the lowest cytotoxicity (88.42% ± 3.72 cell viability; combination index of 0.12) and the highest reduction in nitrite production (77.42%; ΣFa = 0.44). Ibuprofen was also tested in the combinations, revealing synergistic interactions with conventional antimicrobials. Higher order combinations involving the synergistic combinations were tested and three synergistic interactions were identified against *C. acnes* and two against *A. baumannii*. Overall, combining selected essential oil compounds with conventional antimicrobials shows promising therapeutic options. Cinnamaldehyde-containing combinations exhibited both antimicrobial and anti-inflammatory properties, warranting further investigation. Additionally, combining ibuprofen with these compounds and conventional antimicrobials may offer advantages in managing skin infections.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

OL-11

Influence of water supply on essential oil of annual caraway (*Carum carvi* var. *annuum* L.)

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Keywords: climate change, food industry, d-carvone, irrigation, evapotranspiration

Abstract

Annual caraway has been used for thousands of years and is now a popular spice in the food industry. It can be found in salted products of meat industry and bakery due to its flavouring and preservative effect. In our experiment, the aim was to determine the influence of the water supply on essential oil content (EOC) and quality of annual caraway. The investigations were carried out in lysimeters in 2022 and 2023 in Szarvas, Hungary. Three irrigation treatments were conducted (Irr0: non-irrigated control, Irr1: irrigated by micro sprinkler with 90 mm water, Irr2: 100% ET). According to results, the amounts of EOC were noted between 4.26±0.06 and 6.29±0.60 ml/100 g dry matter. The most EOC was caused by 100%ET treatment (Irr2) in 2022. The EOC were less due to the harmful effect of natural precipitation in the irrigated lysimeters in 2023compared to 2022. The main components were the d-carvone and the limonene, but there were not significant treatment effects in these cases. Significant differences were found among the treatments on beta-mircene, cis-dihydrocarvone, trans-dihydrocarvone, trans-carveol, germacrene-D and caryophyllene-oxide in 2023. There was strong positive correlation between the water supply and the EOC in 2022. In the case of d- carvone and limonene significant correlation could not be proved. But positive correlations were between water supply and beta-mircene, furthermore negative correlation were between water supply and cis-limoneneoxide, cis-dihydro-carveol and charyophyllene-oxide independent of crop year, respectively.

1. Introduction

Essential oils are used in the food industry mainly due to their antimicrobial effect and fragrance. Caraway has a wide range of uses: it is also used in the meat industry, the canning industry, the backing industry and cosmetics industry. But the caraway essential oil is not directly suitable for flavouring soft cheese, because it gives a too intense, unpleasant taste compared to ground caraway seed. The caraway has excellent antispasmodic and carminative effects. The essential oil has antiseptic influence, so it can also be used against *Esherichia coli* and *Staphyllococcus aureus*, among others [1–4]. The preservation ability of annual caraway, which has been known since ancient times, plays an important role in food safety nowadays, too. The essential oil content and composition of annual caraway were influenced by row space and nutrient supply: the least essential oil content was resulted by 48 cm row space; nitrogen supply caused decrease and N_0K_{80} treatment caused the highest amount of essential oil content. Furthermore, increased potassium level enhances the amount of d-carvone in the oil [5] . Drought increased the essential oil yield. The main essential oil constituents were d-carvone and limonene. Due to water deficit, the proportion of limonene of essential oil increases. Thus, The fatty acid content was decreased and an the essential oil yield was increased by water deficit [5–7]. Weather conditions have not a significant influence on the content of essential oil in caraway seeds. However, the weather conditions and the production site had a great effect on the yield of seed and essential oil yield [8]. The ratio of d-carvone was decreased by drought [6,9]. The natural precipitation before harvest has harmful effect for essential oil content of annual caraway, while an increase in the number of hot days results essential oil content increase [9].

Caraway does not germinate without irrigation in drought-prone spring caused by climate changes, so the development of irrigating cultivation technology is also justified in Hungary. The aim of our investigation was to determine the effect of different irrigation amounts on essential oil content and quality of annual caraway.

2. Material and Methods

Production site: The experiments were carried out at the Lysimeter Station in Szarvas in 2022 and 2023. Eight compensation lysimeters and eight gravitation lysimeters were used during the investigations. The first season was characterized by severe drought conditions, while the second season received nearly double the precipitation from May to August (2022:55,10 mm, 2023:107,70 mm). *Plant material:* We used seeds from common cultivation. The seeds were sown in 3 rows 4.6 g/ m^2, the row space was 36 cm. The sowing time were 21 March, 2022 and 14 March, 2023. Harvest time:03 August,2022 and 01 August, 2023. *Treatments:* There were 3 treatments: Irr0 was the non-irrigated control, Irr1 was irrigated by micro sprinkler system with 90 mm water (6 times 15 mm), Irr2 was the 100% ET (The water level was kept in 30 cm soil depth in the lysimeters without limitations). The irrigation water was originated from the local Körös oxbow lake.

The essential oil content was determined according to VII. Hungarian Pharmacopeia with hydrodestillation, and the composition was determined by GC-MS method [10]. The tests were performed 3 repetitions per treatment.

3. Results

As for essential oil content, significant treatment effects were noted in 2022. The most amount of essential oil was caused by Irr2. But the most EOC was noted from Irr0 in 2023 (Table1).

Table 1 Essential oil content (EOC) (ml/ 100 g dry matter) of annual caraway according to influence of different water supply

	Irr0	Irr1	Irr2
2022 (Dry year)	4.31±0.15 [a]	5.16±0.18 [b]	6.29±0.16 [c]
2023 (Rainier year)	6.02±1.6 [a]	4.64±0.11 [a]	4.26±0.06 [a]

Different letters (a, b, c) mean significant differences among the treatments at the $p \leq 0.05$ according to Tukey multiple range test.

The main components were the d-carvone and the limonene of essential oil, but there were not significant treatment **effects in these cases. Significant differences were obtained among the treatments on beta-mircene, cis** dihydrocarvone, trans-dihydrocarvone, trans-carveol, germacrene-D and caryophyllene-oxide in 2023 (Table 2).

Table 2 Effect of different water supply on essential oil constituents of annual caraway (Szarvas, 2023)

	Irr0	Irr1	Irr2
Beta-mircene	0.54±0.02 [a]	0.52±0.03 [a]	0.63±0.05 [b]
Limonene	41.65±2.77 [a]	37.31±2.24 [a]	36.85±1.73 [a]
Cis-dihydrocarvone	0.18±0.03 [b]	0.17±0.02 [b]	0.10±0.02 [a]
Trans-dihydrocarvone	0.39±0.03 [a]	0.66±0.03 [b]	0.43±0.03 [a]
Trans-carveol	0.50±0.13 [b]	0.43±0.06 [ab]	0.28±0.05 [a]
D-carvone	52.32±3.72 [a]	52.52±4.59 [a]	56.81±1.51 [a]
Germakrene-D	0.22±0.03 [a]	1.18±0.58 [b]	0.48±0.87 [ab]
Charyophyllene-oxide	0.21±0.01 [b]	0.17±0.03 [b]	0.07±0.02 [a]

Different letters (a, b, c) mean significant differences among the treatments at the $p \leq 0.05$ according to Tukey multiple range test.

There was strong positive correlation between the water supply and the EOC in a drought year. In the case of d-carvone and limonene significant correlation could not be proved, but relationship was found between water supply and other essential oil components independent of crop year (Table3).

Table 3 Results of Pearson's correlate between the water supply and essential oil content and its components

		Essential oil content	Beta mircene	P-cimene	Limonene	Cis-limonene-oxide	Cis-dihydro -carveol	Trans-carveol	D-carvone	Charyo-phyl-lene-oxide
2022 (Dry year)	Water supply	0.970**	0.936*	-0.905*	-0.619	-0.903*	-0.898*	-0.918*	0.827	-0.894*
2023 (Wetter year)	Water supply	-0.590	0.797*	-0.608	-0.565	-0.702*	-0.861**	-0.787*	0.574	-0.960**

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4. Discussion and Conclusions

The main components are the d-carvone and limonene, in agreement with [5,6]. According to our results limonene was increased by water deficit, same to[6,7].

As for conclusion, adequate water supply has favourable effect on the essential oil of annual caraway, but further tests are required to determine the optimal seasonal irrigation norm of caraway to best amount and quality of essential oil.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OL-12

Does the availability of water affect the biomass and secondary compound production in *Ocimum basilicum* L.'Genovese'?

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Keywords: biomass, essential oil; polyphenol; water supply

Abstract

In medicinal and aromatic plants, drought is a major abiotic stressor that impacts growth and bioactive compound accumulation. These effects are primarily caused by changes in metabolic functions and morpho-biochemical characteristics. Hence, the objective of this study was to examine the effects of drought on *Ocimum basilicum* 'Genovese' under open-field and greenhouse pot experiments in 2022. The results showed that although there was a slight increase in essential oil content, there was a significant reduction in both biomass and essential oil yield under drought stress conditions. Additionally, the ratios of essential oil components changed, influenced by factors such as the growing environment, drought severity, and the specific essential oil component. Therefore, it is often misleading to generalize that drought stress enhances the accumulation of secondary compounds in medicinal and aromatic plants, as this process is influenced by multiple factors. In conclusion, to achieve higher biomass production and essential oil yield, *O. basilicum* 'Genovese' requires a continuous water supply of at least 70% soil water capacity in protected cultivation and supplemental irrigation (twice a week) in outdoor cultivation.

1. Introduction

The genus *Ocimum* is part of the tribe *Ocimeae*, subfamily Nepetoideae, and family *Lamiaceae* (Chowdhury *et al*., 2017). It includes over 60 species of perennial and annual herbs and shrubs, each with unique characteristics (Gurav *et al*., 2022). These species are naturally distributed across diverse regions such as Asia, Africa, and the Americas (Paton *et al*., 1999). Notable species within the genus include *Ocimum basilicum*, *Ocimum gratissimum*, *Ocimum sanctum*, and *Ocimum americanum* (Gurav *et al*., 2022). Aromatic essential oils and polyphenols extracted from sweet basil cultivars (*O. basilicum*) are used in flavoring, fragrance, cosmetics, aromatherapy, and pharmaceuticals (Pandey *et al*., 2014). These compounds have antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory properties. However, water supply greatly influences their biomass production and the accumulation of secondary compounds. Despite *Ocimum basilicum*'s water-intensive nature, little is known about how drought stress affects secondary compound accumulation. Thus, the experiment was designed to investigate biomass production as well as biochemical changes in *Ocimum basilicum* 'Genovese' in an open field and in a greenhouse pot experiments.

2. Materials and methods

2.1. Plant material and experimental design

In 2022, a study was conducted at the Experimental and Research Farm of the Hungarian University of Agriculture and Life Sciences (MATE) in Budapest-Soroksár. These studies tested *O. basilicum* 'Genovese' under various conditions, including irrigated (20 mm m^{-2}) and non-irrigated open field settings, as well as three different water supply levels (70%, 50%, and 30% of Soil Water Capacity) within a greenhouse. The experimental design followed a randomized complete block layout with three replicates. Throughout the experiment, the mean air temperature and relative humidity levels were as follows: 20°C with 64% humidity in the open field and 29°C with 47% humidity in the greenhouse. The soil media mixture composition and properties for each experiment are shown in Table 1.

Table 1. Soil characteristics of the soil media mixture

pH (H_2O)	Humus (%)	NO_2+NO_3-N (mg/kg)	P_2O_5 (mg/kg)	K_2O (mg/kg)	Mg (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	SO_4 (mg/kg)
Openfield									
7.6	1.7	11.4	544.4	177.4	377.9	58.5	4.9	2.5	71.6
Greenhouse									
6.9	6.2	300.3	565.4	160.7	631.6	34.8	9.3	2.4	11179.6

2.1. *Measurement of parameters*

Biomass measurement*:* The fresh herb weight (g plant^{-1}) was measured immediately after harvesting, and the dry herb weight (g plant^{-1}) was measured two weeks after drying in a well-ventilated room.

Essential oil content determination was done following the guidelines outlined in the Hungarian Pharmacopoeia (1986). the detailed procedure is indicated in our recent publication Mulugeta *et al.* (2023).

Essential oil composition analysis: It was determined by Gas Chromatography-Mass Spectrometry (GC-MS). GC analysis was carried out with an Agilent Technologies 6890 N instrument coupled with an Agilent Technologies MS 5975 detector. The comprehensive procedure is indicated in our recent publication (Mulugeta and Radácsi, 2022).

2.2. Data analysis: the data were evaluated using a one-way analysis of variance (ANOVA). Significant differences in means were investigated using Tukey's HSD. All statistical analyses were conducted utilizing IBM SPSS version 29.

3. Results and discussions

3.1. Water supply on biomass production

Water supply had a significant ($p<0.01$) effect on the biomass production of *O. basilicum* 'Genovese' in both open-field and greenhouse growing conditions (Table 2). Consequently, irrigated plants in open fields produced higher fresh and dry herb yields compared to non-irrigated plants, which had 30% and 35% lower fresh and dry herb yields, respectively. Similarly, basil plants grown with a higher soil water capacity (70%) achieved the highest fresh (161 g plant^{-1}) and dry herby yield (74.1 g plant^{-1}) in the greenhouse. In contrast, basil plants subjected to severe drought stress showed over 80% reduction in both fresh and dry herb yield. Similar results have also been reported in studies where basil is cultivated in drought conditions (Radácsi *et al*., 2020; Mulugeta *et al*., 2023).

Table 2. Effect of water supply on biomass production of Ocimum basilicum 'Genovese' in open field and greenhouse pot experiment.

	Treatment	Fresh herb yield (g plant^{-1})	Dry herb yield (g plant^{-1})
Open field	Irrigated	273.8±53.0 A	51.0±10.2A
	Non-irrigated	192.2±64.6 B	32.9±9.8 B
Greenhouse	70%	161.0±37.4 a	74.1±8.2 a
	50%	55.7±10.9 b	16.5±5.8 b
	30%	29,5±2.3 b	7.5±0.8 c

Values are presented as Mean ± SD. Different letters are for significantly different groups. Capital letters to differentiate between irrigation treatment under open-field and small letters are used to differentiate drought stress under greenhouse.

3.2. Water supply on secondary compound production

The essential oil content of *O. basilicum* 'Genovese' varies between 0.5% and 0.7% as indicated in Table 3. Water supply had a heterogenous effect on the secondary compound accumulation depending on the specific traits (Tables 2 and 3). Thus, the shortage of water slightly enhanced the content of the essential oil but the essential oil yield decreased due to its dependence on biomass production.

Table 4. Effect of water supply on secondary compound accumulation of *O. basilicum* 'Genovese' under the greenhouse pot experiment

Treatment	Essential oil content (%)	Essential oil yield (mL plant^{-1})
Open field		
Irrigated	0.5 ± 0.2^{B}	0.3 ± 0.2^{A}
Non-irrigated	0.7 ± 0.0^{A}	0.2 ± 0.0^{A}
Greenhouse		
70%	0.5 ± 0.0^{a}	0.4 ± 0.0^{a}
50%	0.6 ± 0.1^{a}	0.1 ± 0.0^{b}
30%	0.7 ± 0.1^{a}	0.1 ± 0.0^{b}

Values are presented as Mean ± SD. Different letters are for significantly different groups. Capital letters to differentiate between irrigation treatment under open-field and small letters are used to differentiate drought stress under greenhouse.

In each experiment, over 50 compounds were identified in the essential oil. As shown in Table 5, the main essential oil compound of *O. basilicum* 'Genovese' was linalool with ratios ranging from 30.7 to 51.9%, along with 1,8-cineole, *trans*-α-bergamotene, and tau-cadinol. The water supply slightly altered the essential oil composition ratios. Accordingly, moderate drought stress significantly lowered the linalool ratio by 31%. On the contrary severe drought stress increased the linalool ratios by 17%. In addition, severe drought stress increased the 1,8-cineole by 188% compared to the control. The heterogeneous effect observed could be attributed to several factors like the high temperature, lower relative humidity, and growing media, in addition to the drought treatment.

Table 5. Effect of water supply on essential oil composition of *O. basilicum* 'Genovese'

			Open-field		Greenhouse		
Component	RT	LRI	I	NI	70%	50%	30%
1,8-cineole	8.44	1034	8.9	10.9	8.0	9.0	23.1
linalool	10.88	1097	47.2	50.2	44.5	30.7	51.9
camphor	12.69	1144	0.8	0.6	1.0	2.4	0.5
α-terpineol	14.55	1189	1.1	1.3		1.8	1.2
isobornyl acetate	18.52	1284			3.3	4.0	5.3
eugenol	21.49	1361	1.4	1.8	3.5	3.8	0.6
ß-elemene	22.92	1391	1.6	1.4	0.5	1.3	0.1
trans-α-bergamotene	24.69	1437	5.4	6.8	4.8	7.5	4.0
α-humulene	25.38	1454			0.9	1.4	0.1
germacrene D	26.49	1482	2.7	2.1	2.9	3.4	0.9
α-bulnesene	27.48	1506	2.4	2.6	2.4	2.5	0.3
cis-γ-cadinene	27.80	1515			2.6	2.8	0.9
palustrol	29.77	1562			1.5	1.7	
tau-cadinol	32.62	1644	7.9	7.9	9.5	10.0	5.0
others (<1%)					12.0	12.6	3.0
Total			97.6	98.5	98.3	97.0	97.4
Monoterpenes			1.9	2.1		2.6	2.8
Oxygenated monoterpenes			61.3	66.4		49.5	82.6
Sesquiterpenes			18.0	18.1		25.8	6.3
Oxygenated sesquiterpenes			1.0	10.4		14.9	5.1
Phenylpropanes			13.2	2.4		4.7	0.6

RT – retention time. LRI – linear retention index relative to C9-C23 n-alkanes on an HP-5MS capillary column. I- irrigated and NI- non-irrigated (control).

4. Conclusion

Water supply significantly affects the biomass production and accumulation of secondary compounds in *O. basilicum* 'Genovese'. Water shortage restricted the growth and development of the sweet basil plants, thereby reducing biomass production and EOY. However, water shortage slightly enhanced the EOC. Additionally, water supply also modified the EO composition ratios. To optimize biomass and essential oil yield, supplemental irrigation in open-field conditions and maintaining at least 70% soil water capacity in protected cultivation is recommended for *O. basilicum* 'Genovese' in Hungary and similar environments.

Acknowledgement

This work was supported by the Research Excellence Programme of the Hungarian University of Agriculture and Life Sciences.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OL-13

Repellent and attractant activity of ten plant essential oils and their compounds against the pea aphid

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Keywords: essential oil, volatile compound, repellency, attractivity, pea aphid

Abstract

The pea aphid, *Acyrthosiphon pisum* Harris, is a significant pest of Fabaceae crops, causing extensive damage and economic losses. Managing this pest is challenging, and plant essential oils (EOs) offer a natural alternative to chemical pesticides, potentially serving as repellents or attractants in a push-pull strategy. This study assessed the effects of ten plant EOs and their chemical compounds on the pea aphid using choice bioassays. The results demonstrated that EOs from Chinese cinnamon, peppermint, anise, basil, spearmint, and dill were highly repellent, while EOs from coriander, rose geranium, bitter orange, camphor tree, and southern blue gum were attractive. A machine learning model suggested that high levels of transcinnamaldehyde, trans-anethole, menthol + menthone, estragole, and carvone contributed to the repellency, whereas linalool, citronellol, or linalyl acetate were linked to attractivity. Therefore, the bioactivity of these EOs and their compounds could offer a more sustainable method for controlling the pea aphid in the future.

Introduction

The pea aphid, *Acyrthosiphon pisum* Harris, is a significant pest of Fabaceae crops, causing severe damage that can result in substantial economic losses [1]. The conventional control method is to spray synthetic chemical pesticides, which is not efficient and dangerous for the environment and health [2,3]. Institutions like the European Union advocate reducing pesticide use and promoting Integrated Pest Management (IPM). The push-pull IPM strategy consists to use stimuli as natural repellent or attractant products to maintain a balance between pests and their natural enemies [4]. In this regard, plant-derived natural products, particularly essential oils (EOs), offer numerous advantages due to their biodegradability, wide availability, non-toxicity to mammals, and effectiveness against specific targets [5]. In the past twenty years, there has been a growing focus on the bioactivity of EOs against aphids, although relatively few studies have specifically targeted the pea aphid [6]. In this study, we assessed the bioactivity of ten EOs against the pea aphid. Additionally, we conducted a chemical analysis of these oils using gas chromatography-mass spectrometry (GC-MS) and correlated their chemical composition with their bioactivity. As a result, we could identify potential volatile organic compounds (VOCs) involved in their effectiveness against the pea aphid.

Material and Methods

Plant essential oils: The ten EOs presented in Table 1 were selected according to their bioactivity reported in the literature against various crop pests.

Olfactory two-choice bioassay: Pea aphids, synchronized as one-day-old apterous nymphs (N1), were obtained from LL01 line alate adults reared on *Vicia faba* L. 'Aquadulce' plants. Aphids were placed in the center of a tube (8.33 cm^3) with an artificial diet at each end. One side of the tube had filter paper with ethanol (control), and the other had filter paper with ethanol mixed with 0.12 µl EO (treatment) (Figure 1). After one day, the number of aphids on each side was counted. This test was repeated 18 times with 5 aphids per tube, totaling 90 aphids. Pairwise Chi^2 tests were conducted to compare EO bioactivity based on aphid distribution. *Chemical composition related to bioactivity*: The chemical composition of the ten EOs was analyzed using GC-MS equipped with a DB-5 MS capillary column. EOs were diluted in hexane (1:100), and 1 µl samples were injected. The column temperature was increased from 60 °C to 220 °C at a rate of 6 °C/min and held for 2 min. Chemical components were identified by comparing retention indices and MS spectra with database references. Concentrations were calculated based on their relative peak area percentages. To identify potential VOCs contributing to bioactivity, a machine learning model was developed. It classifies compounds based on their impact on bioactivity and employs

clustering to handle compounds present only in specific EOs. A RBF-kernel SVM Regressor was trained to model bioactivity as a function of EO composition, and Kernel Shapley values Explainer was used to measure the contribution of each cluster to bioactivity of EOs.

Table 1. Identification of the ten plant essential oils tested.

Scientific name	Common name	Plant organ	Mode	Production	Country	Source
Anethum graveolens L.	Dill	Seed	SD	Conventional	Hungary	a
Cinnamomum camphora (L.) J. Presl	Camphor tree	Bark	SD	Conventional	Central Asia	a
Cinnamomum cassia (L.) J. Presl	Chinese cinnamon	Aerial parts	SD	Organic	China	a
Citrus aurantium L.	Bitter orange	Leaf	SD	Organic	Paraguay	a
Coriandrum sativum L.	Coriander	Seed	SD	Conventional	Moldova	a
Eucalyptus globulus Labill.	Southern blue gum	Leaf	SD	Organic	Portugal	b
Mentha piperita L.	Peppermint	Aerial parts	SD	Organic	India	a
Mentha spicata L.	Spearmint	Flowering top	SD	Organic	India	c
Ocimum basilicum L.	Basil	Flowering aerial parts	SD	Organic	India	d
Pelargonium graveolens L'Hér.	Rose geranium	Aerial parts	SD	Organic	Egypt	a

SD = Steam Distillation. [a] Voshuiles (Nevers, France), [b] La Drôme Provençale SA (Saillans, France), [c] Aroma-Zone (Paris, France) and [d] La Compagnie des Sens (Lyon, France).

Figure 1. Olfactory two-choice bioassay tube. Groups of five aphids are placed in the center of the tube and have one day to choose the control side with ethanol-impregnated filter paper or the treatment side with a mixture of ethanol and EO and to settle on the artificial diet.

Results and discussion

According to Figure 2a, the EOs of Chinese cinnamon, dill, peppermint, spearmint, and basil exhibited high repellency against pea aphids, with a fixation rate on the control side exceeding 80 %. In contrast, the EOs of southern blue gum, bitter orange, camphor tree, rose geranium, and coriander attracted the aphids, with only a 2035 % fixation rate on the control side. While peppermint, spearmint, basil, and coriander oils showed similar results against other aphid species in previous studies, coriander was repellent in those cases [7,8] but attractive to pea aphids in our study. This highlights the variation in EO effects depending on the targeted aphid species, emphasizing the importance of identifying effective oils against specific chosen targets.

The variation in aphids' responses to EOs may be attributed to differences in their chemical compositions, as depicted in Figure 2b. Model calculations indicate that specific clusters of compounds predominantly influence the bioactivity of EOs (Figure 2c). On one hand, high levels of trans + 2-methoxy-cinnamaldehyde in Chinese cinnamon, carvone in dill and spearmint, menthol + menthone + menthyl acetate + neomenthol in peppermint, as well as estragole in basil, may contribute to the high repellency of these EOs. These VOCs are commonly used as active ingredients in biopesticides [9]. On the other hand, various combinations of compounds like limonene, eucalyptol, sabinene, citronellol, trans-geraniol, linalyl acetate, and linalool found in southern blue gum, bitter orange, camphor tree, rose geranium, and coriander oils may be responsible for their attractivity. It is known that oligophagous aphids are attracted to compounds characteristic of their host plants [6]. Linalool, present in broad beans, the host plant of the pea aphid, was discovered to attract the black bean aphid *Aphis fabae* Scopoli [10], which is another aphid species known to infest Fabaceae crops. Furthermore, a previous study has demonstrated that limonene contributes to the attractive effect of EOs on the pea aphid [11].

Conclusions

This study investigating the bioactivity of various EOs on the pea aphid demonstrated that EOs from Chinese cinnamon, peppermint, anise, basil, spearmint, and dill were highly repellent, while EOs from coriander, rose geranium, bitter orange, camphor tree, and southern blue gum were attractive. A machine learning model suggested that high levels of trans-cinnamaldehyde, trans-anethole, menthol + menthone, estragole, and carvone contributed to the repellency, whereas linalool, citronellol, or linalyl acetate were linked to attractivity. These findings mark an initial step toward exploring the impact of plant EOs and their VOCs on pea aphid as an alternative to conventional pesticides. Future research should explore the effects of pure EO compounds and different doses in

plant interactions and field conditions. Additionally, their phytotoxicity on Fabaceae crops and potential side effects on the natural enemies of the pea aphid should be examined to integrate these oils into an IPM strategy.

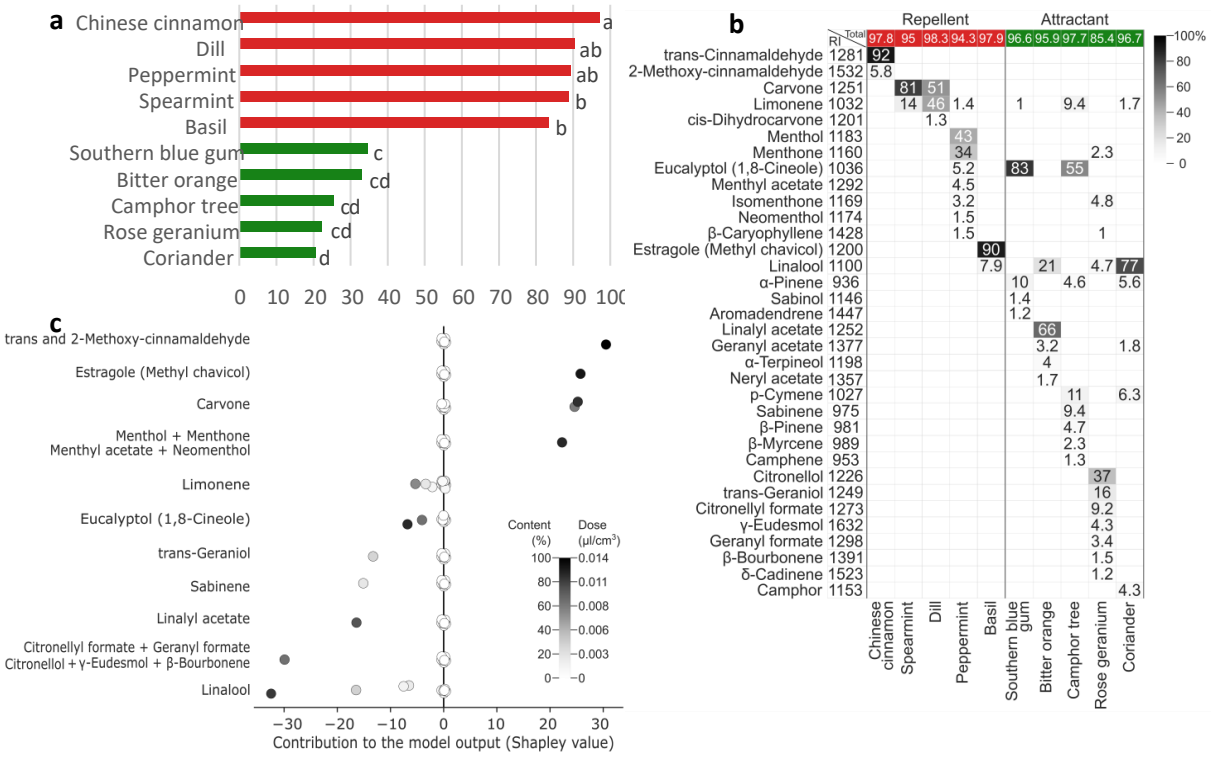

Figure 2. a. Repellent (red) and attractant (green) activity of ten EOs against the pea aphid represented by the percentage of aphids settled on the control side of the two-choice test (Chi² statistical test (P≥0.05), letters as significant differences); b. Relative percentages of chemical compounds identified in the ten plant EOs with a threshold of 1% (GC–MS). RI = Retention Index; c. Clusters of EO compounds with the highest bioactivity against pea aphid according to the machine learning model. For each cluster, ten points are displayed corresponding to the ten EOs. The greyscale of the points indicates the relative content of the cluster in each EO, while their position on the Shapley value axis shows the cluster's contribution to repellency (positive values) or attractivity (negative values) of the EO.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OL-14

How sublethal concentrations of *Origanum vulgare* L. essential oil affect the growth potential of the pathogen *Listeria monocytogenes* in food environments

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Keywords: Origanum vulgare, growth potential, Listeria monocytogenes, carvacrol, NaCl

Abstract

Research on the antimicrobial effect of essential oils (EOs) as food preservatives is in the spotlight, nevertheless few details are available about the effect of EOs sublethal concentrations on the cell physiology and growth dynamics. Therefore, aim of the work was to assess the adaptive response of the foodborne pathogen *L. monocytogenes* to EOs using Phenotype Microarrays technology. *L. monocytogenes* ATCC7644 cells were exposed to the sublethal concentration of 1.25 µL/mL *Origanum vulgare* L. essential oil (OEO, Zuccari srl) for 1h at 30°C. Afterwards, the cells were inoculated in GenIII microplates (Omnilog, Rigel) containing different substrates and conditions (i.e., pH, salt content, etc.) commonly encountered in food products. The growth dynamics and parameters were obtained by fitting the obtained values, represented as Omnilog Units, by the Baranyi and Roberts model, thus allowing the comparison of treated and untreated cells. Interesting results were observed and, in particular, the growth dynamics of treated cells were modified by a single exposure to OEO, revealing a stressing effect determined by the sublethal OEO concentration, that lasts even after OEO is removed. The cells exhibit notable alterations in their growth behaviour, particularly in the extension of the lag phase and the reduction of the maximum growth value, fundamental parameters for food preservation approaches. Interestingly, the exposure to OEO, also restored the cells sensitivity to antimicrobial compounds and antibiotics. These significant findings should be further explored for possible uses of essential oils in health and food production.

1. Introduction

Research into the use of natural food preservatives is being fueled by the growing need for innovative and sustainable substitutes for conventional food additives. While the antimicrobial activity of essential oils is well known, few details are available on the microbial response to sublethal concentrations. Nevertheless, comprehending the behaviour of bacteria in sublethal conditions is essential for optimising antimicrobial treatments, especially in the food processing industry. These data are even more necessary for foodborne pathogens such as *Listeria monocytogenes*, an hubiquitous bacterium, tolerant to salt concentrations and low temperatures, able to contaminate a wide variety of food products. It is one of the most serious foodborne pathogen under surveillance in European countries due to its 18.1% death rate [1].

Within this framework, the study's objective was to investigate the impact of sublethal concentrations of *Origanum vulgare* L. essential oil on *L. monocytogenes* cells. This was accomplished by utilising a cutting-edge methodology based on Phenotype Microarray Technology, which allowed the assessment of the cells' adaptive response to OEO under various conditions encountered in foods and food processing environments.

2. Material and Methods

The reference strain *Listeria monocytogenes* ATCC 7644 in early stationary phase was exposed to 1.25 µL/mL *Origanum vulgare* L. essential oil (Zuccari srl) for 1h at 30°C. The main OEO constituents were carvacrol (68%), o-cymene (6.0%) and thymol (3.8%), while the applied OEO concentration was previously determined as sublethal for the strain (Minimal Inhibitory Concentration equal to 2.50 µL/mL) After exposure, the cells were washed twice in PBS and resuspended in IF-a fluid to a transmittance of 95%, then inoculated in GenIII Microplate (Omnilog, Rigel Process and Lab, Rome, Italy) and incubated at 30°C in the Omnilog Reader for 96 hours. The grade of colour development generated by the NADH reduction of a tetrazolium-based redox dye is used in OmniLog Phenotype Microarray to evaluate and quantify the substrate utilisation [2]. After calculating the mean of the three biological repetitions, the data were processed by using the DMFit programme (available at www.combase.cc) to

fit the growth curve using the Baranyi and Roberts models. The growth parameters (lag phase, growth rate and final growth value) were also calculated.

3. Results

The most interesting result obtained regards the effect of a single exposure to OEO on the growth dynamics of *L. monocytogenes* cells. In fact, a lag phase extension was clearly observed for the greater part of the substrates. Even for glucose (figure 1) which is a common substrate for bacteria, after exposure to OEO, the lag phase went from about 6 to 26 hours. Results even more evident were observed for other sugars, such as rhamnose (Figure 1), as not only the lag phase extension, but also the reduction of the maximum growth value was revealed.

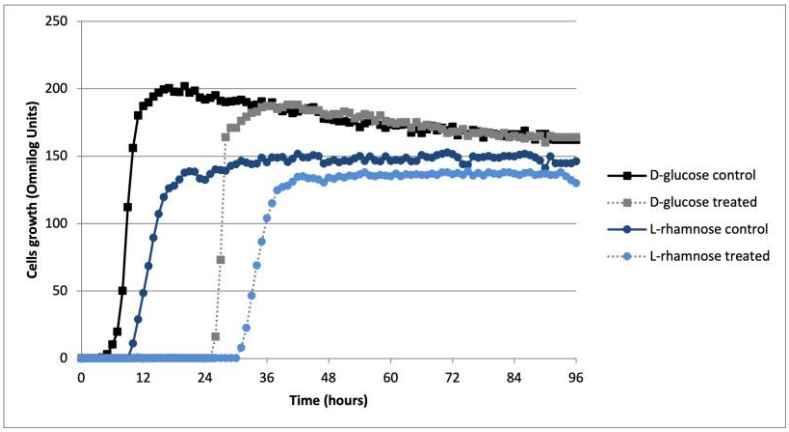

Figure 1 Growth dynamics of control and treated *L. monocytogenes* ATCC 7644 cells in presence of glucose and rhamnose during 96 hours of incubation at 30°C. The results are expressed as a mean of three repetitions.

Conditions normally encountered in food products include small quantities of salt (e.g. about 1%), nevertheless *L. monocytogenes* is halotolerant and can easily grow in presence of concentrations up to 8%, as demonstrated in Figure 2. Also in this case, the lag phase extension was the main effect of the pre-exposure to OEO. In detail, iwhile the maximal growth value stayed almost constant, the lag phase was prolonged more by the pre-exposure to 1.25 µL/mL of EO and the concentration of 1% NaCl (20.6 hours) than by 8% NaCl alone (5.8 hours). A significant lag phase extension was observed also in substrates at pH 6.0, while at pH 5.0, which normally allows the growth of the pathogen, the growth was inhibited (data not shown). Again, the results underline the stressing effect of sublethal concentrations of OEO on the microbial cells and the impact on their growth capabilities. Moreover, the effect of OEO on the growth potential was noticed also when the cells were cultivated in presence of antibiotic compounds, such as vancomycin and troleandomycin (data not shown). In this case, after OEO exposure, *L. monocytogenes* was not able to grow, and therefore the EO seems to enhance the effect of the antibiotic compounds.

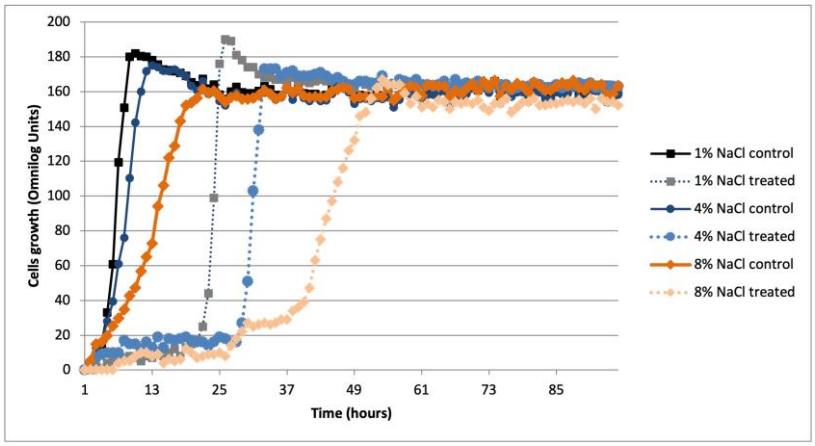

Figure 2 Growth dynamics of control and treated *L. monocytogenes* ATCC 7644 cells in presence of 1%, 4% and 8% NaCl during 96 hours of incubation at 30°C. The results are expressed as a mean of three repetitions.

4. Discussion and Conclusions

After 1 hour of exposure to a sublethal concentration of *Origanum vulgare* L. essential oil, the growth potential and dynamics of *L. monocytogenes* cells were significantly affected. In particular, since the lag phase is widely considered as an adaptive and preparatory phase for microorganisms [3, 4], its prolonged extension indicates that the exposure to OEO hampers the growth of *L. monocytogenes*. The cells' growth was hindered as a result of the stressful events determined in the cells by OEO. The main compound of OEO was carvacrol, which is characterized by a hydroxyl group and a system of delocalized electrons. Carvacrol's ability to function as a proton exchanger, thanks to these molecular configurations, can disrupt the transmembrane gradient, depleting the ATP pool and collapsing the proton motive force, often determining the cells death. The sublethal concentration applied was probably not sufficient to inactivate the cells, rather, it likely lowered the transmembrane gradient and hindered energy production, leaving the cells with reduced ATP levels that required an adaptation to stressful circumstances [2]. In addition, the exposure to OEO slows down and reduces the growth potential in conditions normally permissive for the cells, such as the presence of 1% of NaCl, common in many food products. From a food preservation standpoint, the elongation of the lag phase is a significant parameter, and the results obtained after only 1 hour of contact of cells and OEO could suggest superficial food treatments followed by OEO removal. Finally, other authors have reported the combination of essential oils and antibiotic to restore antibiotic susceptibility by means of different mechanisms of action [5]. Nevertheless, our study demonstrates that it is not necessary for the EO and the antibiotic to be present at the same time, and that the cells were inhibited by some antibiotics after being previously only exposed to the EO.

Understanding the behaviour of bacteria in sublethal conditions is crucial for improving antimicrobial treatments, particularly in the food processing industry, and these results add important insights about the potential of OEO in promoting modifications in the growth potential of *L. monocytogenes* in conditions resembling food environments. *Origanum vulgare* L. essential oil, chemotyped to carvacrol, has been confirmed as one of the most effective in counteracting the growth of *L. monocytogenes.* Moreover, only 1 hour of contact in sublethal concentrations with the bacterial cells could effectively reinstate the antibacterial efficacy of several antimicrobial compounds, and the result deserves deeper studies to comprehend the impact of essential oils on the response of pathogenic bacteria, to reduce their resistance.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OL-15

Insights into the chemo-profiling of rhizome essential from Northeast Indian *Zingiber officinale* germplasm

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Keywords: *Zingiber officinale*, essential oil, Northeast India, GC/MS, Zingiberene

Abstract

Introduction: Ginger is an important cash crop commonly used both in fresh and dried form[1]. Being a biodiversity hotspot the Northeast India is a rich hub of ginger with great diversity which could lead to promising lines of ginger with high essential oil yield [2]. The demand for ginger is ever-growing and being a seasonal crop, good variety of ginger with high essential oil yield would be economically more profitable. Objective: The objective of this work was to evaluate the chemical composition of 343 germplasm of giner (*Zingiber officinale* Rosc.) rhizome essential oil from Northeast India for essential oil yield and compositional variation. Methods: For extraction of essential oil, 300 g of shade dried ginger rhizome were hydro distilled for a period of 8½ h using the Clevenger apparatus of 3L capacity as it is the optimum period for complete isolation of ginger essential oil. The GC/MS analysis was carried for evaluation of the chemical composition of the ginger germplasm essential oil. Results: The essential oil yield ranged from 0.03% to 0.40% (w/w) based on the fresh weight of the rhizome. A total of 19 chemical markers were identified in the ginger essential oil. Based on the average area percentage of the compounds for the 343 germplasm of ginger the The markers broadly belonged to monoterpene hydrocarbon (MH), oxygenated monoterpene (OM), sesquiterpene hydrocarbon (SH) and oxygenated sesquiterpene (OS). α-Pinene (MH), camphene (MH), 1,8-cineole (OM), linalool (OM), 1-borneol (OM), α-terpineol (OM), citronellol (OM), Z-citral (OM), geraniol (OM), E-citral (OM), geranyl acetate (OM), ar-curcumene (SH), zingiberene (SH), α-farnesene (SH), β-bisabolene (SH), β-sesquiphellandrene (SH), nerolidol (OS), zingiberenol (OS), 7-epi-γ-eudesmol (OS) were the identified constituents. Conclusions: Quantitative variation was observed in the sample set, indicating a moderate variability in the levels of oleochemicals and the major compounds were E-citral and Z-citral. Based on the study some high essential oil yielding germplasm of ginger were identified, *viz*- Z-7, Z-152, Z-193 and Z-566 which would be of great significance in ginger breeding program. Moreover, the germplasm Z-81 was identified as the highest Zingiberene containing (23%) germplasm which has immense potential for the pharmaceutical applications.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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YOUNG SCIENTISTS' ORAL LECURES

YOL-01

The combination of gas chromatography based analysis and spectroscopy techniques for a thorough characterization of the essential oil of *Piper gaudichaudianum* Kunth from Brazil

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Keywords: preparative gas chromatography, chiral GC, multidimensional gas chromatography, *Piper*, spectroscopy analysis

1. Introduction

This study aimed to characterize the essential oil obtained from the leaves of *Piper gaudichaudianum* Kunth from Brazil by means of gas chromatography based approaches. After GC-MS analysis, a component consituting 27% of the essential oil was not identified, needing a proper analytical strategy to collect and identify the unknown component in reasonable times. In parallel, the main chiral terpenes were investigated by means of enantio-selective multidimensional gas chromatography.

2. Material and Methods

Leaves of *Piper gaudichaudianum* were collected in Brazil and hydrodistilled in a Clevevenger-type apparatus to obtain the essential oil. While GC-MS analyses were carried out to characterize the main essential oil components, prep-GC, GC-FTIR and NMR were mandatory to collect and characterize the unknown component, respectively. Enantio-selective multidimensional gas chromatography was carried out to define the enantiomeric ratios of the main terpenes investigated.

3. Results

In a first step, GC-MS analysis was carried out, allowing the identification of 76 components. Notably, a component constituting 27% of the entire sample was not identified, requiring preparative gas chromatography for collecting sufficient quantities. After that, structural elucidation studies, consisting of NMR, GC-MS and GC-FTIR, allowed the unambiguous identification of the para-phenol substituted component. In parallel, to gain more information about the chemical composition of the EO, the enantiomeric ratios of eight terpenes were assessed by exploiting an MDGC approach in heart-cut mode.

4. Conclusions

The essential oil of *Piper gaudichaudinaum* Kunth from Brazil underwent comprehensive characterization using gas chromatography based approaches and spectroscopy methods. The combination of these analytical methods allowed a reliable characterization of the main volatiles.

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Conflict of Interest

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YOL-02

Chritmum maritimum L., an edible plant. Chemical composition, PCA analysis and food safety.

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Keywords: Apiaceae; *Crithmum maritimum* L.; limonene; dillapiole; statistical analysis; food safety

Abstract

In this research, the volatile profile of essential oils of aerial parts of four *Crithmum maritimum* L. accessions, not previously investigated, collected in Isola delle Femmine (Italy), Croatia, Montenegro, and Israel were evaluated by GC-MS and 38 compounds were identified. All the samples analyzed show a composition characterized essentially by monoterpene hydrocarbons (94.0-97.6%), with limonene, *γ*-terpinene, *β*phellandrene, *α*-pinene, and *p*-cymene as the principal compounds. In addition, a comprehensive review of the composition of *C. maritimum* essential oils that have been studied thus far was conducted. To evaluate the similarity between samples, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were utilized. To assess the safety of dietary of *C. maritimum*, a matrix plot analysis of the content of dillapiole, a toxic constituent, in the samples was performed. The results of the statistical analysis show the presence of six clusters indicating some differences between *C. maritimum* accessions from different locations. Regarding dillapiole content, the four accessions discussed in this paper showed dillapiole values of less than 2%, suggesting the healthiness of sea fennel from these locations.

1. Introduction

Crithmum maritimum L. (Apiaceae) is an edible perennial halophyte plant. This plant, commonly found on rocky coastal areas, piers, and breakwaters and rarely on sandy beaches, is known by several names including rock sapphire, sea fennel, and marine fennel [1]. This wild plant grows all along the Mediterranean coast it is also found along the Atlantic coast in Portugal [2]. The plant has fleshy, hollow stems that can grow up to 50 cm tall and small green or yellowish-green flowers that bloom in summer [3].

Crithmum maritimum has a unique fennel-like aroma and flavor and is used in culinary dishes as a flavoring herb, but also it is used in traditional medicine and cosmetics. The medicinal application of *C. maritimum* varied depending on the used part [4], specifically, aerial parts are used in form of infusion, decoctions, and juices to prevent or alleviate many diseases such as gastrointestinal disorders, [5] inflammatory and skin problems [6,7] and liver and genitourinary diseases [5,7,8]. The edible use of *C. maritimum* is typical in many countries, where the fresh leaves and young branches of sea fennel are pickled and used as a condiment in salads, sometimes replacing capers [8]. It is a very versatile dish, typically paired with fish. Sea fennel can be used both fresh and dried. The fresh leaves of the sea fennel are used as an aromatic herb in addition to soups, sauces, and salads. Essential oils showed the presence of many bioactive compounds responsible for different biological properties such as antibacterial, antioxidant, insecticidal, acaricidal, anti-tumor, antiinflammatory, mosquicidal, vasodilatory, cholinesterase inhibitory [9-12]. In this research, the essential oils of four accessions of C. maritimum were studied. We performed statistical analysis to find similarity between our samples and those reported in the literature. Furthermore, in order to improve knowledge on the food safety of this plant, the content of the unsafe metabolite dillapiole was analyzed in all samples.

2. Material and Methods

2.1. Plant material

The flowering aerial parts of four accessions of *C. maritimum* were collected on the beach at different localities: Isola dell Femmine, Palermo, Italy, (38°11'01" N 13°14'06" E 3 m s/l) in August 2023 (S43); Sakarum Beach,

Dugi Otok, Croatia (44°08'03" N 14°52'24" E 1 m s/l) in July 2023 (S44); Drobni Pijerak, near Budva, Montenegro (44°08'03" N 14°52'24" E 3 m s/l) in July 2023 (S45); Caesarea (Israel), (32°29'58 "N 34°53'27 "E 7 m s/l) in June 2023 (S46).

2.2. Analysis of volatile components

The fresh samples were ground in a Waring blender and then subjected to hydrodistillation for three hours. The oils were dried over anhydrous sodium sulphate and stored in sealed vials under N_2 at -20°C, ready for GC-MS analyses. Samples S43, S44, S45, and S46 yielded 0.85%, 0.14%, 0.24%, and 0.1% oil (w/w), respectively. GC-MS analysis of essential oil was performed according to the procedure reported by Porrello et al. [13].

2.3. Statistical analysis

Principal component analysis (PCA) was performed on the dataset (46 samples) containing five variables: monoterpene hydrocarbons (MH), oxygenated monoterpenes (MO), sesquiterpenes hydrocarbons (SH), and oxygenated sesquiterpenes (OS) and others (O) and, based on variance/covariance matrix. Similarly, Hierarchical Cluster Analysis (HCA) was used to test the similarity among the different samples in relation to the contents of their chemical constituents.

3. Results and Discussion

Hydrodistillation of *C. maritimum* aerial parts collected in Isola delle Femmine (S43) gave a pale-yellow oil. Overall, twenty-three compounds were identified, representing 97.6% of total components according to their retention indices on a DB-5MS column and classified into five classes based on their chemical structures. Monoterpene hydrocarbons (72.0%) were the principal metabolites with γ-terpinene (49.0%), *p*-cymene (13.6%), and β-phellandrene (4.1%) as the main ones. Oxygenated monoterpenes was the second most abundant class (25.4%), totally represented by thymol methyl ether (24.5%).

The oil of the other population, collected in Croatia (S44) also gave a pale-yellow oil. In this case, twenty-eight compounds were identified, representing 97.3% of total components. Also in this case, monoterpenes hydrocarbons represent the main class (95.1%), but the composition of this oil is quite different from that of the previous sample (S43). In fact, limonene is the major compound (79.0%), followed by β-phellandrene (8.0%) while γ-terpinene accounts for only 1.4% of the total composition.

The chemical composition of *C. maritimum* essential oil from Montenegro (S45) was identified for 97.2% (Table 1), showing twenty-five compounds. The metabolites occurring in this oil are very similar to those of S44, although their percentages vary somewhat. In fact, in S45 the main class was represented by monoterpene hydrocarbons (87.8%) with limonene (50.0%) and β-phellandrene (28.3%) as principal constituents of the oil. The amount of γterpinene (4.0%) is slightly higher with respect to S44 (1.4%). Oxygenated monoterpenes was the second most abundant class (7.5%) being terpinen-4-ol as the principal metabolite (5.7%).

Hydrodistillation of *C. maritimum* aerial parts collected in Israel (S46) gave a pale-yellow oil. Overall, twenty-six compounds were identified, representing 94.6% of the total components. Similar to the other essential oils analyzed in this paper, monoterpene hydrocarbons represent the main class (90.6%) of S46 with limonene (43.1%), γterpinene (27.0%), α-pinene (15.3%) as principal metabolites.

For the *C. maritimum* essential oils, as shown in the graph (Figure 1), all variables affected PC1 and PC2. In fact, PC1 (72%) was represented mainly by MH in the positive score, and in a minor contribution by OM, O, SH, OS in negative scores; meanwhile, PC2 (23%) was represented mainly by a positive score of MH, OS, and O and in negative score by OM and SH.

HCA based on the Euclidean distance between groups indicated six cluster (from A to F, Figure 1) identified by their essential oil chemotypes with a similarity ≤ 25.

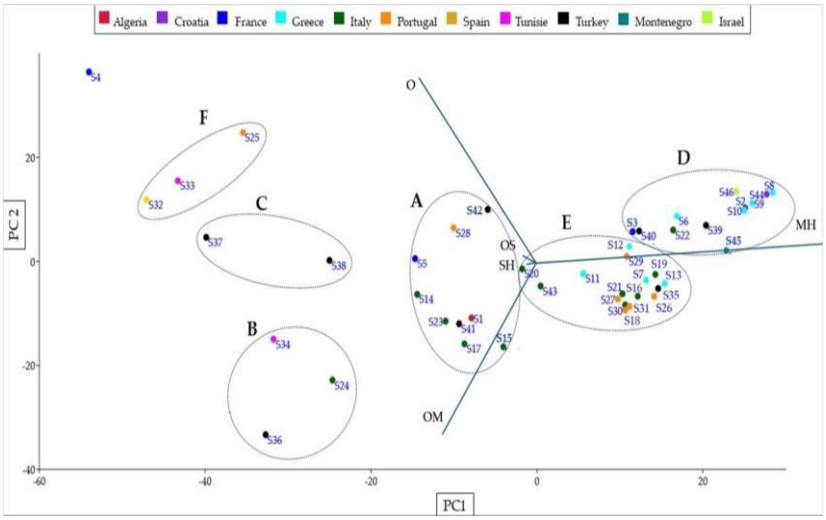

Figure 1 Principal component analysis (PCA) of the essential-oil composition of various accessions of *C. maritumum* based on the principal classes of compounds. The vectors displayed represent the eigenvectors of the covariance matrix.

Conclusions

In the present work, the essential oil chemical composition of four accessions of *Crithmum maritimum*, not previously studied, was investigated. The volatile profile of all samples was characterized by a large amount of monoterpene hydrocarbons. The edible use of *C. maritimum* suggests the need to expand knowledge on the composition of this plant, to highlight the presence of compounds that are harmful to humans. In this study, the presence of dillapiole, a toxic compound, was assessed in plants harvested from different locations in Europe and the Middle East. It is advisable to ingest sea fennel that contains minimum dillapiole values. PCA analyses based on the different chemical classes and other statistical analyses are useful tools for a comprehensive investigation, also leading to an understanding of the diversification of sea fennel from different countries.

Conflict of Interest

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YOL-04

Exploring the anxiolytic potential of *Cupressus torulosa* essential oil: An *In vivo* approach

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Keywords: *Cupressus torulosa*, essential oil, anxiolytic effect, biological activity

Abstract

Cupressus torulosa D. Don is an evergreen conifer tree widely distributed throughout the Himalayan range. This study aimed to explore the anxiolytic effect of *C. torulosa*'s needles derived essential oil (CTEO). Qualitative and quantitative analysis of the hydro distilled CTEO was conducted using GC-MS and GCFID techniques. The anxiolytic-like behavior of CTEO was evaluated in mice using two behavioral models: the actophotometer test and the open-field test. Mice received CTEO at doses of 10 and 30 mg/kg body weight (b.w.), while a standard drug, diazepam, was administered at 2 mg/kg b.w. Terpinene-4-ol (39.38%), totarol (5.50%), sabinene (4.37%), and sempervirol (4.08%) were identified as major constituents of CTEO. In the actophotometer test, mice exhibited reduced movement for up to 3 hours postadministration, akin to standard diazepam, suggesting CNS depressant behavior of CTEO. Similarly, in the open-field test, mice displayed exploratory behavior indicative of the anxiolytic nature of CTEO at 1 hour post-administration, particularly at the dose of 30 mg/kg b.w. The observed anxiolytic behavior of CTEO may be attributed to its major component, terpinene-4-ol, possibly modulating neurotransmitter activity in the brain. These findings highlight *C. torulosa* needles essential oil as a potential natural candidate for treating anxiety-related issues, pending further investigation into its mode of action and clinical trials.

1. Introduction

Essential oils are renowned for *their* myriad therapeutic benefits, including their ability to alleviate anxiety and promote relaxation. The anxiolytic effects of certain essential oils stem from their aromatic compounds, which can influence the limbic system, the emotional center of the brain. Inhalation or topical application of essential oils such as lavender, sandalwood, and ylang-ylang have been shown to reduce symptoms of anxiety, calm the nervous system, and induce feelings of tranquility [1]. Noteworthy are the stress-relieving properties associated with essential oils derived from Cupressaceae trees [2]. Cypress oil, derived from *Cupressus sempervirens* and widely available commercially, has already found application in aromatherapy [3].

Cupressus torulosa D. Don is an evergreen coniferous tree belonging to the *Cupressaceae* family, one of the largest genera in its family. Commonly known as the 'Himalayan cypress' or 'Surai', *C. torulosa* thrives across the Himalayan regions [4]. The essential oil extracted from the needles of *C. torulosa* (CTEO) has been previously documented for its anti-inflammatory, antioxidant, and antimicrobial properties [5, 6]. Traditionally, dried cones and young branches of the plant are believed to possess various therapeutic properties, including anthelmintic, antipyretic, antirheumatic, antiseptic, astringent, balsamic, vasoconstrictive, and antifungal.

Despite the numerous traditional uses, there is a scarcity of scientific studies investigating the biological potential of the needles derived essential oil of *C. torulosa*. This study aims to investigate the anxiolytic properties of the essential oil derived from *C. torulosa* needles (CTEO) for the very first time, filling a significant gap in scientific knowledge. By exploring its potential benefits, particularly in healthcare and wellness, the research seeks to enhance the acceptance and utilization of CTEO.

2. Material and Methods

2.1. Collection of plant material and isolation of CTEO

The needles from *C. torulosa* trees were collected (~2 Kg) from the Botanical Garden (30°20’30.4“ N, 78°0’4.2” E; 693 meters) within the premises of the ICFRE-Forest Research Institute in Dehradun, India. Fresh needles were subjected to hydro distillation using Clevenger-type apparatus for 4 hours. The essential oil was separated from the aqueous distillate and dried over anhydrous Na_2SO_4.

2.2. GC-FID and GC-MS assisted analysis of CTEO

The chemical composition of the CTEO was determined using GC-MS to identify its individual components. Quantification was carried out by employing methyl octanoate as an internal standard with GC-FID, following established procedures outlined in previous study [6]. Identification of compounds present in CTEO was achieved by comparing retention indices (RIs) and mass spectra with established databases, including NIST17 and F&F libraries, NIST chemistry webbook, and relevant literature.

2.3. Anxiolytic studies of CTEO

2.3.1. Ethical considerations

All animal experiments were approved by the Institutional Animal Ethics Committee (Registration number 1156/PO/Re/S/07/CPCSEA, approval number DITU/IAEC/21-22/07-04) and carried out in accordance with the Institutional Animal Ethics Committee's ethical guidelines.

2.3.2. Animals

Equal number of male and female Swiss albino mice were procured from NIBL, Noida, India. The animals were kept in a controlled environment with a 12-hour light and 12-hour dark cycle at a room temperature of 22±2°C and were provided with a standard rodent diet with access to water ad libitum.

2.3.3. *In vivo* studies

Two different behavioral models, the actophotometer test and the open-field test, were employed to evaluate the anxiolytic effect of the CTEO *in vivo*.

Male and female mice were equally distributed into four groups, each consisting of six animals. The groups received intraperitoneal doses of the following substances: CTEO at 10 and 30 mg/kg body weight (b.w.), DMSO (control) at 5 mL/kg b.w., and diazepam (standard drug) at 2 mg/kg b.w.

3. Results

3.1. Chemical composition of CTEO

The chemical composition of CTEO has been previously reported in our study [6]. In summary, the oil exhibited a greenish-yellow color with a yield of 0.60 ± 0.07%. A total of 29 compounds were identified and quantified, collectively constituting 91.18% of the total oil. Oxygenated monoterpenes represented the predominant class, accounting for 49.93% of the total oil, followed by monoterpene hydrocarbons at 21.47%. Key components included terpinene-4-ol (39.38%), totarol (5.50%), sabinene (4.37%), and sempervirol (4.08%).

3.2. Anxiolytic effect of CTEO

The anxiolytic behavior of CTEO was assessed using two behavioral models: the actophotometer test and the open-field test. For the open-field test, a device comprising a smooth field of squares alternately painted in black and white, resembling a chessboard, was utilized. Each mouse was placed at the center of the board and observed for 5 minutes at 0, 30, 60, 120, and 180 minutes post-dose administration, with the time spent in black and white squares recorded. Increased time spent in the bright white squares indicated anxiolytic behavior induced by the drug. Both doses of CTEO (10 and 30 mg/kg b.w.) elicited similar exploratory behavior towards the bright white squares compared to standard diazepam. In the actophotometer test, mouse movements were digitally recorded by the device, with decreased locomotive activity serving as an index of central nervous system (CNS) depression. The results demonstrated a doseand time-dependent activity of CTEO in both models. Specifically, robust activity of CTEO was observed up to 180 minutes in the actophotometer test at the dose of 30 mg/kg b.w (Figure 1), while significant activity was observed at 60 minutes post-administration at same dose in the open-field test (Figure 2).

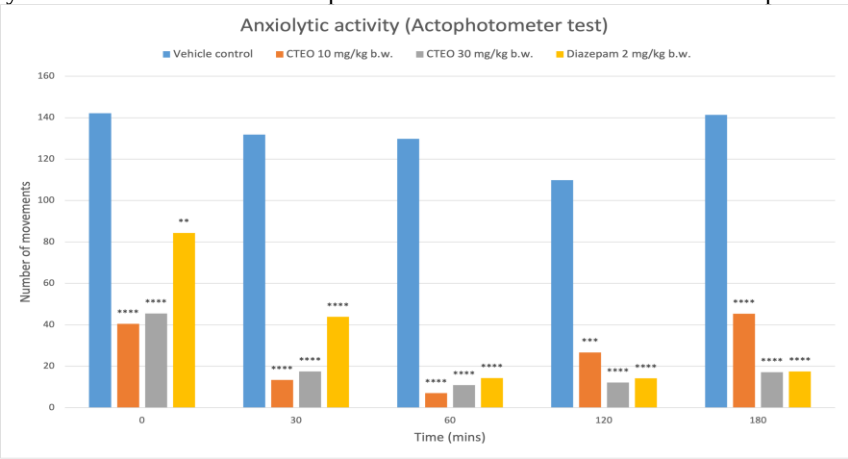

Figure 1. Activity score of CTEO in actophotometer test

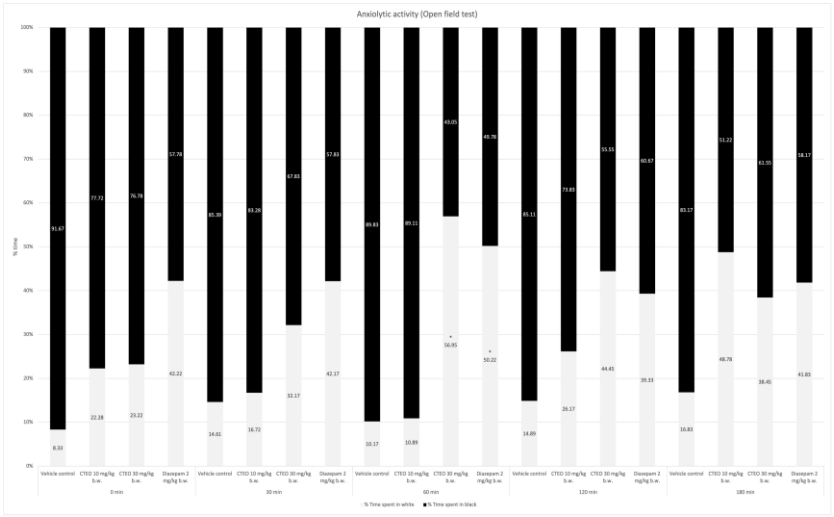

Figure 2. Activity score of CTEO in open-field test

4. Discussion and Conclusions

The present study demonstrated that CTEO, at both tested doses, reduced the locomotive behavior of mice, akin to the standard sedative drug diazepam. The open-field test, utilized to gauge the effect of drugs on gross general behavior and nervous excitability in a novel environment, revealed that CTEO exhibited anxiolytic behavior similar to diazepam. In a new environment, animals typically exhibit exploratory behaviors; reduced ambulation, exploration, and normal grooming behaviors, coupled with increased defecation, signify anxiety and fear. Anxiolytics induce disinhibition, enhancing these exploratory behaviors [7]. The observed anxiolytic activity of CTEO suggests a potential role in alleviating anxietyrelated behaviors.

Terpenes and their oxygenated derivatives have been widely reported as potent anxiolytic agents, contributing to the significant activities of essential oils. These compounds primarily modulate neurotransmitter activity in the brain, particularly serotonin (5-HT), dopamine (DA), glutamic acid, and gamma-aminobutyric acid (GABA), to mitigate anxiety symptoms and promote a sense of calmness [1]. While the exact mechanism of action of CTEO remains unknown, it is hypothesized that terpinene-4-ol, the major component, along with synergistic interactions with other constituents, may underlie its anxiolytic effects. Nevertheless, the observed anxiolytic activity of CTEO warrants further investigation using additional models and a deeper exploration of its mechanism of action, ultimately positioning the oil of *C. torulosa* within the realm of complementary and alternative medicine.

ACKNOWLEDGEMENTS

Financial assistance from the National Authority CAMPA, MOEF&CC for the project "Bioprospecting for Industrial Utilization of Lesser-Known Forest Plants" under the scheme "Strengthening Forestry Research for Ecological Sustainability and Productivity Enhancement" is gratefully acknowledged.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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YOL-05

Interplay of drought and salt stress on the yield and composition of essential oil in *Thymus pannonicus*

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Keywords: *Thymus pannonicus*, monoterpenes, sesquiterpenes, thymol, plant stress.

Abstract

This study aimed to investigate the effects of drought stress and salt stress, applied individually or in combination, on the yield and essential oil composition of *Thymus pannonicus*. The experiment took place at the Hungarian University of Agriculture and Life Sciences, Budapest, in 2023. Treatments included control (70 % SWC), drought stress (40% SWC), salt stress (60 mM NaCl + 70% SWC), and combined drought and salt stress (60 mM NaCl + 40% SWC). Essential oil extraction and GC-MS analysis were performed, revealing significant decreases in essential oil composition under drought stress, either individually or combined with salt stress. Thymol and its precursors (mainly α-terpinene and γ-terpinene) exhibited a similar response pattern to the essential oil yields, while oxygenated monoterpenes and sesquiterpenes showed opposite trends. Notably, 1,8-cineole, borneol, thymol methyl ether, β-Caryophyllene and β-Bisabolene, increased significantly under drought stress conditions. However, salt stress alone did not significantly affect essential oil yiels and composition, possibly due to the salt tolerance of the species. This study provides insights into the biochemical changes in *Thymus pannonicus* under drought and salt stresses, suggesting potential consequences for plant physiology and volatile compound production.

1. Introduction

Today, plants face numerous abiotic stresses as a consequence of climate change, including altered water availability, extreme temperatures, soil fertility, salinity, and soil composition. Drought conditions lead to water deficits for plants, while widespread soil salinity worsens these challenges, often subjecting plants to combined stress scenarios. These stresses can have harmful effects on plant yield, morphology, physiology, and biochemistry. Particularly affected is *Thymus pannonicus*, which grows in the Pannonian plain and deals with the dual challenges of drought and salt stress. These environmental pressures can significantly impact the essential oil content and composition of this species of thyme.

2. Material and Methods

The experiment was conducted in the experimental greenhouse situated at the Buda Campus of the Hungarian University of Agriculture and Life Sciences in Budapest. The treatments included a control group maintained at 70% of Soil Water Capacity (SWC), drought stress conditions at 40% SWC, salt stress conditions with 60 mM NaCl added to 70% SWC, and combined drought and salt stress at 40% SWC with 60 mM NaCl. Each treatment consisted of 25 replicated of *Thymus pannonicus* plants. The experiment lasted for about 7 weeks, following which the plants were collected and subjected to drying. Essential oil extraction was performed using a Clevenger-type apparatus following the procedure outlined in Pharmacopoeia Hungarica 8th edition [1]. Gas chromatography-mass spectrometry (GC–MS) analyses were carried out, to quantify the various volatile components present in the essential oil of *Thymus pannonicus*, following the methodology described by Pluhár et al. [2]. Statistical analyses were performed using IBM SPSS version 27, employing one-way analysis of variance (ANOVA) for all data sets, followed by Tukey HSD test with a significance level set at ($p \leq 0.05$).

3. Results

The impact of different treatments applied in the essential oil of *Thymus pannonicus* is presented in Figure 1. Under drought stress conditions, both individually and combined with salt stress, the essential oil composition (EOC) decreased significantly ($p<0.05$) by 46.00 % and 42.38 %, respectively, compared to the control condition, which yielded 0.913 ml/100g DW. However, the salt stress condition did not show any significance in comparison to other treatments.

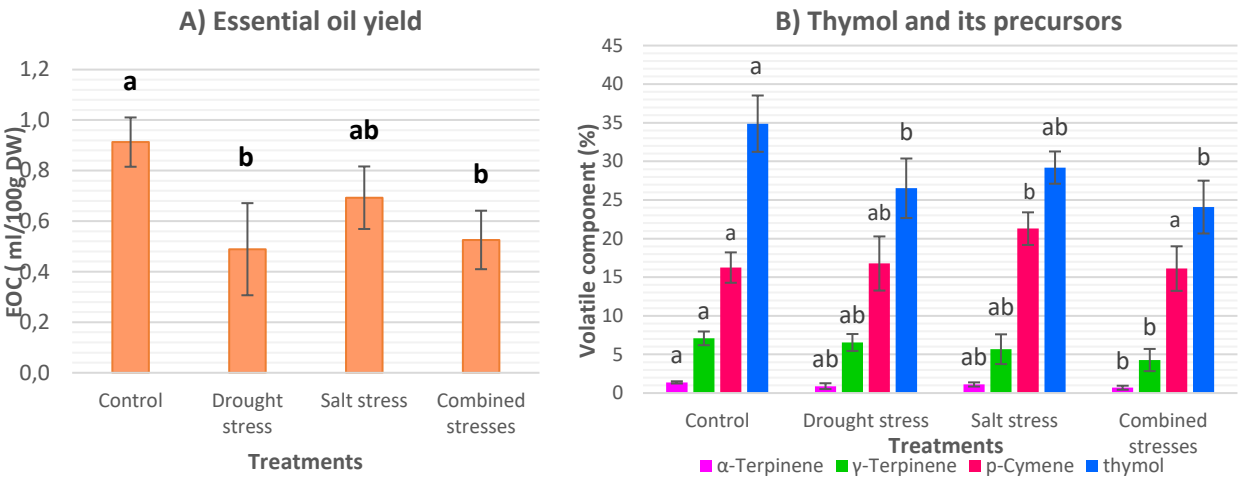
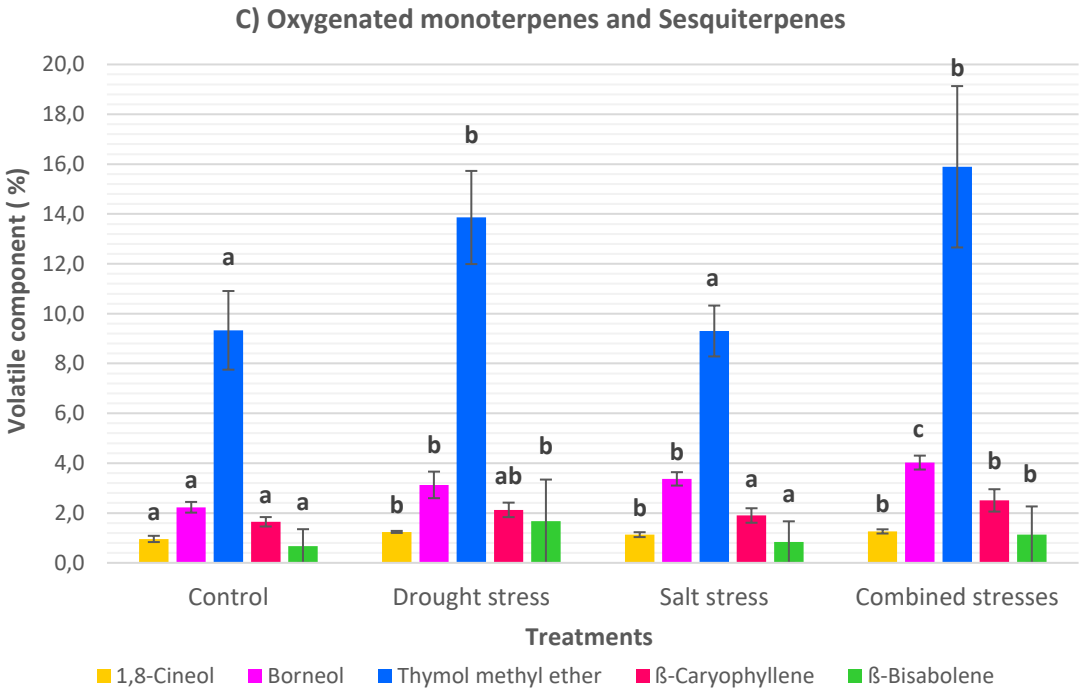


Figure 1. The effect of different treatments on the essential oil concentration (EOC) and composition of *Thymus pannonicus*. The data shown as means ± SD. Different letters on the bars indicate significant differences among the means of the treatments ($p<0.05$).



The GC-MS results, also illustrated in Figure 1, affirm the effect of the different stresses applied, on the composition of the essential oil. A distinguishable trend was noticed in the yields of the different detected terpenes, allowing us to distinguish two groups according to their responses to the stresses: one encompassing thymol and its precursors and the other including the rest of the studied terpenes, such as oxygenated monoterpenes and sesquiterpenes. Thymol, considered the main volatile component in *Thymus pannonicus* (35% for the control), presented a similar response to the different treatments as shown in EOC with its precursors, even though with slight differences between them. In particular, α-terpinene and γ-terpinene decreased significantly in the combined stresses (0.69% and 4.28%, respectively) when juxtaposed to the control (1.38% and 7.09%, respectively). In contrast, the studied oxygenated monoterpenes and sesquiterpenes show an opposite result; in all stressed conditions (either drought, salt individually, or combined), both 1,8-cineol and borneol show a notable and statistically significant ($p<0.05$) increase compared to the control. Similarly, thymol methyl ether, as well as the sesquiterpenes β-Caryophyllene and β-Bisabolene, demonstrate a statistically significant ($p<0.05$) increase (15.89%, 2.50%, and 6.79%, respectively) only under the combined stress treatment in comparison to the control (9.33%, 1.65%, and 4.79%, respectively).

4. Discussion and Conclusions

The analysis of essential oil yield and composition in *Thymus pannonicus* underscores the adverse effects of drought stress, whether individually or in combination with salt stress, on essential oil production and composition, including the levels of thymol and its precursors such as α-terpinene and γ-terpinene. Notably, this stress condition positively impacts certain oxygenated monoterpenes (1,8-cineol, borneol, and thymol methyl ether) as well as sesquiterpenes like β-Caryophyllene and β-Bisabolene.

These findings align with previous research by Bahreininejad et al [3]., which demonstrated a decrease in essential oil yield in *Thymus carmanicus* with increasing SWC. Similarly, Llorens-Molina et al. [4] reported a significant increase in the proportion of 1,8-cineole in *Thymus vulgaris* under drought stress, consistent with our observations. Additionally, Leicach et al. [5] also noted an increase in oxygenated monoterpenes under water deficit conditions in *Eucalyptus* camaldulensis essential oil. Our findings in accordance with those of Alavi-Samani et am. [6] and Fazeli-Nasab et am. [7], who observed a decrease in thymol content under drought stress in *Thymus vulgaris* and *Thymus daenensis* essential oils. In fact, drought stress alters gene expression in *Thymus* species, potentially influencing enzymes like pyruvate decarboxylase-1 (PDC-1) and histone deacetylase-6 (HDA-6), further impacting thymol production [8]. Interestingly, our results do not support the findings of Amiri et am. [9], who suggested an increase in thymol content while oxygenated monoterpenes decreased at 20% and 40% SWC in *Thymus eriocalyx*.

Our research confirms the biochemical changes in *Thymus pannonicus* under drought stress conditions (40% SWC) or a combination of drought and salt stresses. While the impact of salinity appears minimal at a concentration of 60mM, it is conceivable that higher salt concentrations may have a more discernible effect on *Thymus pannonicus*.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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YOL-06

Exploring the selective inhibitory activity of essential oils on the Alzheimer associated enzymes AChE and BChE: a comprehensive screening based on a bio-guided fractionation approach. Focus on *Mentha* sp. essential oils

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Keywords: acetylcholinesterase inhibition, butyrylcholinesterase inhibition, essential oils, bioguided fractionation, *Mentha* x *piperita* L.

Abstract

Essential oils (EOs), complex mixtures of volatile compounds obtained from individual plant species via steam distillation, dry distillation or mechanical processes, have attracted scientific interest due to their traditional use. Their biological effects, including effects on the central nervous system, have become the focus of research. In this work, we have conducted an extensive screening of numerous essential oils and identified those that have a promising inhibitory activity on two different enzyme targets involved in Alzheimer's disease: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Further investigation focused on the trend that most EOs exhibit selectivity towards one enzyme. The EOs found to be selectively active for AChE are primarily composed of 1,8-cineole, a compound already known in the literature for this activity [1], and it was confirmed to be solely responsible for the activity of these EOs. The same is true for *Origanum vulgare* L. EO, related to the compound carvacrol. Conversely, *Mentha* x *piperita* L. and *Mentha arvensis* L. were EOs with selective activity toward BChE, but the activity of the main compound, menthol, didn't explain completely that of the total essential oil. Therefore, a bio-guided fractionation of the EOs was performed to finally identify the compounds to which this activity might be attributed. In addition, preliminary *in vitro* studies were carried out on mice cellular models (NE-4C culture purchased from ATCC) to investigate the activity of *Mentha* x *piperita* L. EO on BChE, with the aim of improving our understanding of its interesting biological effects.

1. Introduction

Due to the complex composition of EOs and their widespread use in various fields such as cosmetics, food and pharmaceuticals [2], extensive research has been carried out to investigate the biological properties of the essential oils. Particular emphasis has been placed on studying their effects on enzyme activity, especially enzymes involved in critical human diseases such as Alzheimer's disease (AD) [3].

Currently, drugs that target the enzyme AChE play a central role in the AD treatment, such as galantamine, rivastigmine, and donepezil. While many studies have focused on AChE inhibition, other potential targets, such BChE, are often overlooked [4]. Therefore, the aim of our study was to investigate the effects of EOs on both AChE and another interesting target for AD treatment, BChE.

2. Material and Methods

All essential oil samples were subjected to colorimetric AChE and BChE *in vitro* assay according to the Ellman method. The protocol used in this study is that of Rhee et al. with slight modifications [5].
Fractionation was performed using a PuriFlash 450 column chromatography (Sepachrom, Italy), equipped with both a UV and Evaporative Light Scattering detector (ELSD). Petroleum ether and ethyl acetate were used as solvents for the mobile phase; Sphera 50 µm silica cartridges (Sepachrom, Italy) were selected. The eluent flow was maintained at 25 ml/min and the volume of essential oil injected was 1 ml.
The chemical characterization of the essential oils and their respective fractions was performed by GC-FID and GC-MS analysis.

Preliminary *in vitro* studies were carried out on mice cells using NE-4C cell culture purchased from ATCC. Cells were treated with the essential oil for thirty minutes (t1) or one hour (t2). The cells were then lysed, and the

resulting cell lysates were subjected to the Ellman's assay [6].

3. Results

This study began with a comprehensive screening of the inhibitory activity of about 50 EOs on the enzymes AChE and BChE, both of which are involved in Alzheimer's therapy. The active EOs showed preferential activity on only one of the two enzymes. Therefore, an investigation of this selective activity of the EOs on these enzymes was warranted. Initially, the EOs with predominantly selective activity for AChE, *Eucalyptus globulus* Labill. EO, *Melaleuca viridiflora* Sol. ex Gaertn. EO, *Melaleuca cajuputi* Maton & Sm. ex R.Powell EO, *Rosmarinus officinalis* L. EO, *Cinnamomum camphora* (L.) J.Presl EO, *Myrtus communis* L. EO, *Salvia officinalis* L. EO, were analysed. These essential oils are mainly composed of 1,8-cineole, a compound that has been previously studied and is known in the literature for its inhibitory activity on AChE [1]. Subsequently, the experimental inhibitory activity of these essential oils was compared with the theoretical activity calculated as if 1,8-cineole alone was responsible for the inhibitory activity. The results, shown in **Figure 1**, indicate that the inhibitory activity of these EOs, which are mainly composed of 1,8-cineole, is exclusively due to the activity of 1,8-cineole. A similar approach was adopted to *Origanum vulgare* L. EO, focussing on the compound carvacrol, leading to comparable results as shown in **Figure 1**.

The study then turned to the EOs that act selectively on BChE, in particular those of *Mentha* x *piperita* L. and *Mentha arvensis* L. Here, the experimental inhibitory activity was compared with the theoretical inhibitory activity calculated for L-menthol alone. However, the experimental inhibitory activity was greater than the calculated theoretical inhibitory activity for menthol, as shown in **Figure 2**, indicating the presence of other active compounds besides menthol. Therefore, a bio-guided fractionation approach was adopted, in which the hydrocarbon and oxygenated fractions of the essential oil were separated, with only the oxygenated fraction showing activity.

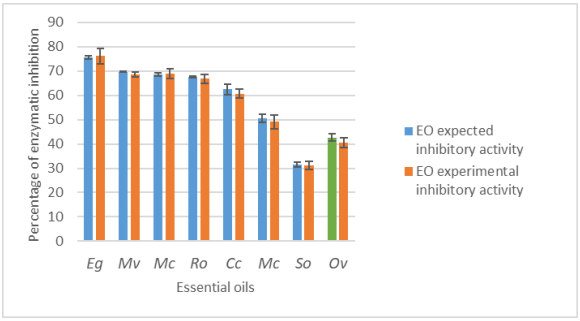

Figure 1 Comparison of the percentage of experimentally measured enzymatic inhibition against AChE (5 batches tested for each EO) and the expected enzymatic inhibition for 1,8-cineole as the only bioactive compound in the essential oils. The comparison for *Origanum vulgare* L. EO refers to carvacrol as the bioactive compound. Legend: *Eg: Eucalyptus globulus; Mv: Melaleuca viridiflora; Mc: Melaleuca cajuputi; Ro: Rosmarinus officinalis; Cc: Cinnamomum camphora; Mc: Myrtus communis; So: Salvia officinalis; Ov: Origanum vulgare.*

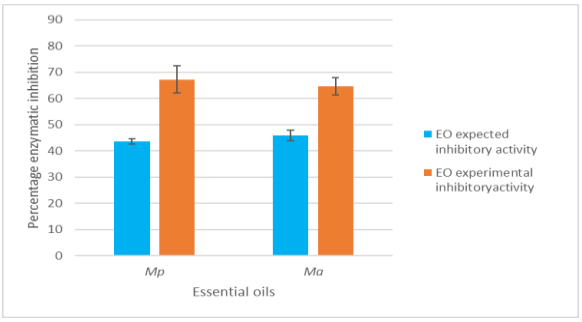

Figure 2. Comparison of the percentage of experimentally measured enzymatic inhibition against BChE (5 batches tested for each EO) and the expected enzymatic inhibition for menthol as the only bioactive compound in the essential oils. Legend: *Mp: Mentha* x *piperita; Ma: Mentha arvensis.*

In addition, a preliminary study was carried out with NE-4C cells from mice treated with *Mentha* x *piperita* L. The cells were treated for two different durations, 30 minutes and one hour, followed by evaluation using the Ellman assay. Interestingly, a 30-minute treatment with peppermint EO did not lead in inhibition, while the one-hour treatment showed a significant inhibitory effect (percentage inhibition 38.0±2.9, n=6).

4. Discussion and Conclusions

In this study, our aim was to investigate the inhibitory activity of a number of EOs from plants belonging to different botanical families on two cholinesterase enzymes: AChE, a key target in current Alzheimer's disease, and

BChE, an emerging target of research interest. Some EOs showed remarkable activity on these enzymes and exhibited selectivity towards one of the two enzymes. Further investigation of the compounds responsible for this activity revealed that 1,8-cineole is the active compound in the essential oils that acts selectively on AChE, which is consistent with data already published in the literature [7]. In contrast, menthol in *Mentha* x *piperita* L. and *Mentha arvensis* L. EOs targeting BChE showed an experimental inhibitory activity lower than the calculated theoretical inhibition, leading to a bio-guided fractionation approach. Subsequent tests identified the oxygenated fraction as the only active fraction, suggesting the presence of other active oxygenated compounds. The activity of *Mentha* x *piperita* L. EO was also confirmed in NE-4C cells from mice treated for one hour. Future research will focus on thoroughly identifying the other compounds responsible for the inhibitory activity of *Mentha* x *piperita* L. and *Mentha arvensis* L. EOs against BChE, as well as developing selective essential oil blends that target both enzymes and thus have a dual activity. In conclusion, this study suggests that the essential oils investigated are promising for further research as adjuvants to conventional Alzheimer's therapy.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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YOL-07

Exploring synergistic and antagonistic interactions: Investigating antimicrobial and biofilm inhibitory activity in the oral cavity

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Keywords: essential oils, formulations, antibiofilm activity, synergism, oral pathogens

1. Introduction

The oral biofilm, composed of pathogens like *Porphyromonas gingivalis* and *Streptococcus mutans*, drives oral diseases, allowing direct entry into the bloodstream, contributing to systemic illnesses [1]. Commercial oral hygiene products containing synthetic antibacterial agents may induce cytotoxic effects to fibroblasts with extended use [2]. Discovering natural alternatives to inhibit oral biofilm formation is crucial. Essential oils (EOs) are suggested for their biological properties and ability to inhibit oral pathogens [3,4]. This study aimed to prepare EOs - formulations based on synergistic and antagonistic studies and examine their antibiofilm activity.

2. Material and Methods

The experimental process is presented in Figure 1.

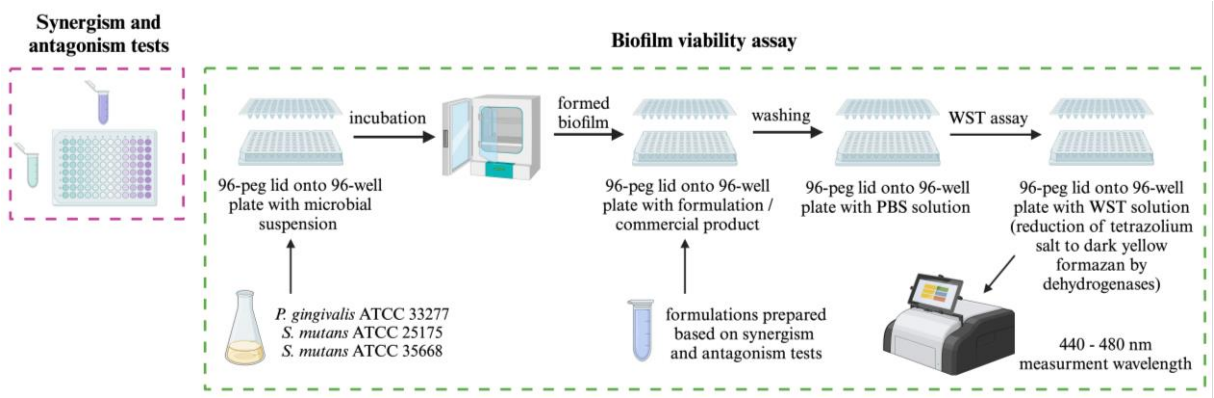

Figure 1. Scheme of experimental research.

3. Results

Synergism studies have found the best synergistic pairs for each strain (Table 1.). Details on the antibiofilm activity will be presented.

Table 1. Summary of the best synergism pair for each microorganism

S. mutans ATCC 25175	*S. mutans* ATCC 35668	*P. gingivalis* ATCC 33277
Hiba EO (*Thujopsis dolaborata*) MIC = 1.56 µg/mL x **Vetiver EO** (*Chrysopogon zizanioides* syn. *Vetiveria zizanioides*) MIC = 25 µg/mL **FICI = 0.31**	**Nerolina EO** (*Melaleuca quinquenervia*) MIC = 12.5 µg/mL x **Vetiver EO** (*Chrysopogon zizanioides* syn. *Vetiveria zizanioides*) MIC = 25 µg/mL **FICI = 0.31**	**Hiba EO** (*Thujopsis dolaborata*) MIC = 1.56 µg/mL x **Hydro vetiver EO** (*Chrysopogon zizanioides* syn. *Vetiveria zizanioides*) MIC = 100 µg/mL **FICI = 0.27**

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4. Discussion and Conclusions

In this study, combinations of natural materials were tested to enhance their antibacterial activity against oral pathogens. It is reasonable to use such combinations in formulations as an alternative to antibacterial and antibiofilm products. The results significantly expand the knowledge base in the area of combining EOs.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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YOL-08

Innovative approach for improvement of essential oil yield and quality in patchouli (*Pogostemon cablin* (Blanco) Benth.) through coloured shade net cultivation

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Keywords: Leaf volatiles, Patchouli essential oil, Patchouli alcohol, Photoselective shade net, Sesquiterpenes

Abstract

Pogostemon cablin (Blanco) Benth., a shade loving aromatic member of Lamiaceae is known to produce essential oil with rich aroma and huge medicinal values. In an attempt to introduce photoselective shade netting - a modern agricultural practice to protect crops from various biotic or abiotic hazards, it was revealed that not only different spectral conditions, but also seasonal variations affect morphology as well as the essential oil profile. Vigorous growth in terms of leaf count, leaf size and fresh and dry weight, was shown to be **highest under green nets monsoon** (August to October), under **blue and brown nets in winter** (November to January) and under brown **net in summer** (April to June). Pigment profile of the plant fluctuated under each shade net in three seasons but the **highest amount of total chlorophyll and carotenoids were found under black shade net in all the seasons**. GC-MS analysis of the internal pool of volatile compounds showed different degree of **variation in concentrations of major sesquiterpenoid patchouli alcohol** along with other compounds like α-pinene, β-pinene, caryophyllene, seychellene and squalene. This suggests alteration of physiology of the plant under different spectral treatment in different seasons. The essential oil (EO), isolated by hydrodistillation was greatly impacted by both the seasonal and spectral variations. **Green and brown coloured shade nets in all three seasons recorded higher EO content and EO yield** than the other conditions. Upon GC-FID analysis of the essential oil, **significant varitation in the concentration of patchouli alcohol** was noted in under each photoselective shade net in every season.

1. Introduction

Patchouli [*Pogostemon cablin* (Blanco) Benth.] is an industrially-valued tropical perennial aromatic herb. Along with a rich woody and spicy fragrance, the oil has excellent fixative properties which made this plant more valuable in fragrance industries for production of soaps, detergents, body lotions and perfumes (Swamy and Sinniah, 2016). Presence of a very diverse range of phytochemicals, majorly terpenoids, are reported to have potential therapeutic properties including antimicrobial, antioxidant, antiinflammatory, antiemetic, antimutagenic, antidepressant, analgesic, fibrinolytic, and cytotoxic activities and many more.

Various biotic and abiotic factors are known to impact plant growth and development as well as their secondary metabolite profile. Both light intensity and the spectral quality is known to be one of the major role player among these factors. Photoselective shade netting is innovative technique in such a way that it provides plants a protection against several biotic hazards meanwhile providing them altered solar spectra for improved yield. This current study aims to introduce this technique for robust patchouli cultivation throughout the whole year in Indian agricultural context for enhanced essential oil yield.

2. Material and Methods

Experimental design: Four different coloured shade nets viz. green, brown, black and blue are purchased from local merchants in India with shading percentage of 85% (±5). The spectral specification and PAR at the target of all the shade net were monitored using a light meter (Gigahertz-optik, MSC 15, Germany). A variety of patchouli (*Pogostemen cablin*), CIM-Samarth, cultivated and distributed by CIMAP, Lucknow were brought and vegetatively propagated in IIT Kharagpur. The experiments were conducted in three different seasons viz. monsoon (August-October), winter (November-January) and summer (April-June).

Plant growth and photosynthetic pigment assessment: After an experimental period of 90 days different growth parameters were measured and the plants were harvested. Spectrophotometric estimation of total chlorophyll and carotenoids were carried out with the leaf extracts prepared in 80% acetone.

Assessment of internal pool of leaf volatiles: The GC-MS analysis was done with n-hexane leaf extract of the 90 days old leaves. The GC was equipped with a split mode injector and a TG-5MS capillary column. An injection volume of 1 µl with a split ratio of 10:1 was maintained for GC analysis. Content of each endogenous volatile compound were evaluated in nmol using the peak area of each compound and the known amount of ethyl hexanoate.

Isolation of essential oil: The essential oil was extracted through the hydro-distillation method in the Clevenger apparatus for 4 h. Essential oil yield and essential oil content were formulated for each condition by the formula given by Oliveira et al., 2015.

Quantification of patchouli alcohol: The GC equipped with a split mode injector and a TG-5MS capillary column. An injection volume of 1 µl with a split ratio of 10:1 was maintained for GC analysis. The concentration of patchouli alcohol was quantified against a standard curve prepared with different known concentration of patchouli alcohol analytical grade standard procured from Sigma-Aldrich.

3. Results

Spectral quality under photoselective shade nets:

Table 1. Photosynthetic photon flux density under different photoselective shade nets in three seasons

	Open Field ($\mu mol\ s^{-1}m^{-2}$)	**Green** ($\mu mol\ s^{-1}m^{-2}$)	**Blue** ($\mu mol\ s^{-1}m^{-2}$)	**Brown** ($\mu mol\ s^{-1}m^{-2}$)	**Black** ($\mu mol\ s^{-1}m^{-2}$)
Monsoon	2070	361.1	247.48	387.78	200.56
Winter	1265	201.48	91.54	224.48	77.05
Summer	1863	299	162.54	322	184

Analysis of Plant growth paratmeters and photosynthetic pigments:

Plants grown under shaded conditions, compared to the experimental control (open field), appeared to be taller and slender with larger and greener leaves depicting vigorous growth while open field grew patchouli rather bushier appearance. With varying seasons, greater biomass was generated during summer compared to other seasons for every experimental conditions. Significantly higher leaf leangth and breadth were observed in all the shaded conditions in summer compared to other seasons. Interestingly, under open field the thickest leaves were found in all three seasons. Also lower accumulation of photosynthetic pigments e.g. chlorophyll a, chlorophyll b and carotenoids was recorded in open field rather than in all the shaded conditions in all seasons, black nets showing the highest accumulation in monsoon, winter and summer.

Status of internal pool of leaf volatiles:

A diverse range of terpenoid compounds majorly sesquiterpenoids were identified to dominate the internal pool of volatiles in patchouli leaves. Patchouli alcohol and γ-curcumene are the major sesquiterpenoids that are present along with many others like caryophyllene, seychellene, α- patchoulene, β- patchoulene, α-bulnesene, β-bisabolene etc. Overall volatile accumulation was found to be positively influenced by green and brown coloured shade nets during both monsoon and winter while brown nets in summer was shown to be most effective in this case. Noticeably, blue coloured shade nets in every season recorded to have significantly lower accumulation. Many of the aromatic sesquiterpenes like β-Bisabolol, γ-Bisabolene, italicene were found to be absent under both blue and black nets in any of the seasons.

Essential oil profile:

Both the essential oil content (%) and essential oil yield (g/plant) got affected by coloured shade net as well as the seasonal variation. A diverse range of 3.48-16.3% and 0.07-1.26 g/plant in EO content and EO yield (**Figure 1**.) were recorded respectively in patchouli under photoselective shade nets through the whole year. EO content was found to be relatively higher in each treatment during monsoon while the lowest values for the same were noted during winter. Summer crops yielded the highest mass of essential oil under green and brown shade nets. These two coloured shade nets also performed better than the other growing conditions in rest of the seasons. In a contrasting note, the highest concentration of patchouli alcohol in the essential oil was found under blue coloured

nets in winter. In general, a relatively higher amount of patchouli alcohol was found in all the shaded conditions as well as in open environment in winter compared to the other two seasons.

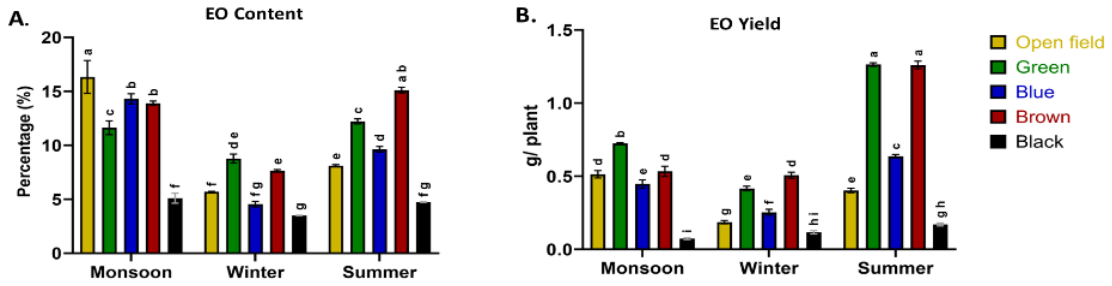

Figure 1. Essential oil parameters under different shade nets in different seasons; A. Essential oil content, B. Essential oil yield.

4. Discussion and Conclusions

The significant increase in stem length under each shaded conditions might be explained as an adaptive strategy developed by the plants to survive in the reduced light intensity under the shade nets. The occurrence of low rates in the growth of plants grown in full sun, relative to plants under shade nets, is a widespread result in the literature (Silva et al., 2016). Previous studies in *Mikania glomerata* and *Aloysia gratissima* also resulted in thicker leaves when grown under full sun compared to the shaded conditions. The decrease in leaf thickness of shaded plants may be attributed to variations in how photoassimilates are distributed and used for leaf growth (Ribeiro et al., 2018). Although light absorbance in the red and far red region is known to be associtaed with higher photosynthate accumulation following production of higher biomass, in this study such observations were not made, rather under green shade nets higher biomass were recorded in monsoon and summer. Similar observation was noted in *Ocimum selloi* (Costa et al., 2010). Higher accumulation of photosynthetic pigments may also be attributed to adaptive response to capture most amount of light under shaded conditions with reduced light intensity. In every season, significantly higher EO content and EO yield were recorded under brown shade nets which can be corelated with the observations in earlier study with patchouli (Ribeiro et al., 2018), where higher values were recorded under red shade nets. Although lower EO content and EO yield in winter were reported, the essential oil from blue net and open field were the richest in patchouli alcohol content. In conclusion, it can be stated that, photoselective shade nets in different seasons indeed have significant role in plant growth and essential oil of patchouli as well as the terpene biosynthesis. Brown coloured nets can be recommended for adapting this technology for patchouli cultivation in Indian tropical climate with a better essential oil yield and richer terpene profile. Meanwhile, green nets are found to be suitable as an alternative in monsoon and summer allowing mass cultivation of patchouli round the year.

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Conflict of Interest

The authors declare no conflict of interest related to this study.

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YOL-09

Essential oils combined with tetracycline restore antibiotic effectiveness against resistant strains of *Salmonella enterica*

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Keywords: antimicrobial resistance, tetracycline, *Salmonella enterica*, essential oils, phytocomplex

Abstract

Antimicrobial resistance (AMR) is a major health concern for both humans and animals. The World Health Organisation has recognized AMR as the "silent pandemic" and has placed it as one of the top ten health risks, expected to kill 10 million people annually by 2050. Hence, innovative therapeutic approaches are required to counteract AMR in pathogens. In this frame, the current study has shown evidence that Essential Oils (EOs) can improve the efficacy of antibiotics in treating *Salmonella enterica*. In detail, eleven *S. enterica* strains isolated from swine were selected for their resistance to tetracycline associated to the presence of the most common resistance phenotype TET. Hence, these strains were treated with tetracycline alone and in combination with *Thymbra capitata* L. (Cav.), *Thymus vulgaris*, *Eugenia caryophyllata*, *Coridothymus capitatus*, *Thymus capitatus* L., and *Thymus serpyllum* EOs, also calculating the Fractional Inhibitory Concentration Index. A restoration of susceptibility of *S. enterica* to the antibiotic treatment was observed, and the synergistic activity of EOs and Tc compounds determined the significant decrease in the Minimum Inhibitory Concentrations (from 256 to 4 μg/mL). Principal Component Analysis allowed to determine the specific compounds in the EOs phytocomplex able to enhance the bioactivity, thus revealing a positive association between Tc and terpenoids, and particularly carvacrol, linalool, sabinene, and thymol. Therefore, the combination between EOs and antibiotic compounds may be a promising strategy to overcome the antibiotic resistance developed in microbial pathogens.

1. Introduction

Improper antibiotic use reduces infection control, raises treatment costs, and spreads drug-resistant bacteria, causing therapeutic failure. Tetracycline compounds are commonly used in livestock production, and resistance-associated genes are prevalent in the swine microbiome [1]. Moreover, *Salmonella* spp. strains isolated from the swine production chain are often resistant to more than three classes of antibiotics [2]. In this scenario, EOs are considered potential strategies to limit bacterial resistance, thanks to the different antimicrobial activities attributed to the phytocomplex. EOs act on multiple targets in bacterial cells, reducing the development of cell response mechanisms without stimulating the development of resistance phenomena [3]. The antimicrobial mechanisms of action resulting from the co-administration of clinical antibiotics and EOs involve a sequential blocking of shared biochemical pathways, the enhanced diffusion of antimicrobial agents within the cell membrane, the inhibition of distinct targets, and the suppression of carrier proteins [4]. For this reason, the objective of this study was to assess the antimicrobial effectiveness resulting from the combination of Tc compounds with *Thymbra capitata* L. (Cav.), *Thymus vulgaris*, *Eugenia caryophyllata*, *Coridothymus capitatus* (CC), *Thymus capitatus* L. (TC), and *Thymus serpyllum* (TS) EOs. Then, the analyses evaluated the effectiveness of the combination of EOs chemotyped to carvacrol (in detail, CC, TC, TS), with Tc against 11 tetracycline-resistant *S. enterica* strains. The FICI was used to evaluate the synergistic effect between Tc and EOs, while PCA assessed the influence of the EOs phytocomplex on antimicrobial effectiveness with Tc treatment

2. Material and Methods

One reference strain and eleven isolates of *Salmonella enterica* were selected from the collection of the Department of Food Inspection at the University of Teramo in Italy (Table 1). *S. enterica* strains were grown on Müeller–Hinton medium (Liofilchem, Roseto degli Abruzzi, Italy) broth at 37°C and were standardised to an optical density of 620 nm to achieve inocula of 10^6 CFU/mL. In a previous study, Lauteri et al. [5] examined the antibiotic resistance profiles of the *S. enterica* strains.

Table 1. *Salmonella enterica* strains under study.

Serovar	Strain	Origin	Resistence phenotype
Monophasic var *S.* Typhimurium	787	Slaughtering environments	AMI, AMP, GEN, PIP, TET, TOB
Monophasic var *S.* Typhimurium	791	Slaughtering environments	AMI, AMP, GEN, PIP, TET, TOB
Monophasic var *S.* Typhimurium	792	Slaughtering environments	AMI, AMP, GEN, PIP, TET, TOB
S. Enteritidis	217	Meat product	AMI, AMP, GEN, PIP, TET, TOB, TRI
S. Enteritidis	208	Meat product	AMI, AMP, GEN, PIP, TET, TOB, TRI
S. Rissen	788	Slaughtering environments	AMI, GEN, TET, TOB
S. Rissen	793	Pig carcass	AMI, CLO, GEN, TET, TOB, TRI
S. Typhi	116	Meat product	AMI, AMP, GEN, PIP, TET, TOB, TRI
S. Typhimurium	117	Meat product	AMI, GEN, TET, TOB
S. Typhimurium	686	Pig carcass	AMI, GEN, TET, TOB
S. Typhimurium	785	Slaughtering environments	AMI, AMP, AMX, CEF, CLO, GEN, NIT, PIP, TET, TOB
S. Enteritidis	ATTC 13076	Type strain	-

Abbreviations: AMI-Amikacin, AMP- ampicillin, AMX- amoxicillin-clavulanic acid, CEF-Ceftiofur, CLO-Chloramphenicol, GEN-Gentamicin, NIT-Nitrofurantoin, PIP-Piperacillin, TET-Tetracycline, TOBTobramycin, TRI-Trimethoprim-Sulfamethoxazole.

Commercially available food-grade *Thymus vulgaris* (TV), *Eugenia caryophyllata* (EC), *Coridothymus capitatus*, *Thymus capitatus* L., *Thymus serpyllum* EOs (Flora S.r.l., Pisa, Italy) and *Thymbra capitata* L. (Cav.) (TL) (Exentiae S.r.l., Catania, Italy) were used for the experiments. The lyophilized tetracycline (Tc, >98%) was obtained from Sigma-Aldrich (Milan, Italy).

The combination between Tc compounds and EOs was tested for 48 hours at 37°C using the Checkerboard assay [6]. First of all, the interaction between Tc and the single EOs was determined by calculating the Minimum Inhibitory Concentrations (MICs) and applying the equation reported by Fratini et al. [6], which allowed the determination of the Fractional Inhibitory Concentration Index (FICI). The FICI value indicates the synergistic, commutative, or indifferent interaction between EOs and Tc compounds. Then, Principal Component Analysis (PCA) was performed to assess the influence of the chemical composition the EOs chemotyped to carvacrol (CC, TC, and TS) on the effectiveness of Tc treatment against *S. enterica* strains. The data was clustered using the XLSTAT 2014 programme (Redmond, WA, United States).

Results

Table 2 shows the results about the interaction between EOs and Tc compounds, which resulted in a significant decrease in the MICs of antibiotics (data not shown). The MIC values were reduced from 256 μg/mL, observed in the individual treatment, to 4 μg/mL when coupled with the EOs. The result is particularly significant, as the concentration returns within the susceptibility breakpoints of ≤ 4 μg/mL specified in the current CLSI standards [7]. The interaction between EOs and Tc exhibited synergy in the presence of EOs characterized by carvacrol, demonstrating notable effects with CC and TS EOs in four strains, and with TC EO in three strains.

Table 2. FICI and effects resulting by the combination between *Eugenia caryophyllata* (EC), *Thymbra capitata L*. (Cav.) (TL), *Thymus vulgaris* (TV), *C. capitatus* (CC), *T. capitatus* (TC) and *T. serpyllum* (TS) EOs with tetracycline (Tc) compounds against *S. enterica* strains.

	FICI			Effects			FICI			Effects		
	EC	**TL**	**TV**	**EC**	**TL**	**TV**	**CC**	**TC**	**TS**	**CC**	**TC**	**TS**
116	1	1	1	C	C	C	0.5	0.1	0.2	S	S	S
117	0.5	1	0.5	S	C	S	0.2	0.5	0.2	S	S	S
208	1	1	0.5	C	C	S	2	1	4	C	I	A
217	1	1	2	C	C	I	10	2	2	A	C	C
686	1	1	2	C	C	I	0.1	0.5	1	S	S	I
785	4	1	1	A	C	C	2	1	1	C	I	I
787	8.1	4.1	4.1	A	A	A	10	1	1	A	I	I
788	1	1	1	C	C	C	1	1	0.2	I	I	S
791	1	1	1	C	C	C	10	1	1	A	I	I
792	1	2	1	C	I	C	5	1	4	A	I	A
793	1	2	1	C	I	C	0.5	1	0.5	S	I	S

MICs values are expressed as μL/mL for EOs and μg/mL for Tc. The standard deviation is not reported, being zero for all replicates. Effects: A, Antagonism; C, Commutative; I, Indifference; S, Synergism. A: FICI value > 2; C: 1 < FICI value ≤ 2; I: FICI value = 1; S: FICI value < 1.

The PCA biplot revealed distinct groups, mostly spread to three clusters associated with the EOs (data not shown), where TS EO had the maximum bioactivity in the presence of Tc chemicals, where 7 of 11 strains had a negative correlation. Furthermore, the PCA biplot emphasized the impact of terpenoid compounds, such as thymol, linalool, 1.8-cineole, sabinene hydrate, β-phellandrene, α-terpineol, and cis-sabinene, on the antimicrobial efficacy of TS EO. This suggests a potential enhancement of antimicrobial properties and underscores the influence of the EO phytocomplex in Tc treatment.

Discussion and Conclusions

This study confirmed the strong antibacterial effectiveness of EOs against *S. enterica*, where the phytocomplex may affect the interactions with the Tc compounds, resulting a strain-specific response of *S. enterica*. This is due to the presence of antibacterial components in EOs, which can improve antibiotic effectiveness and act as adjuvants [8]. The EOs exhibited a significant effectiveness to decrease Tc dose against *S. enterica* and return concentrations to acceptable levels, indicating restored susceptibility. Synergy was noticed between Tc and EOs chemotyped to carvacrol, suggesting potential for reduced administration and increased effectiveness against specific targets. Simultaneously, PCA suggests that the phenolic and terpenoid compounds present in EOs, in particular in TS EO, have the potential to influence the antimicrobial activity mediated by Tc. Terpenoid compounds can enhance the cellular effect and overcome the strain-specific nature of the response by facilitating the transport of carvacrol through the lipid layer of bacterial cells. They can also increase the swelling of bacterial cell membranes, making it easier for the compound to enter the cell [9]. Therefore, the bioactive phytocomplex and Tc may synergistically interact due to the phytocomplex ability to make membranes permeable, resulting in enhanced antibiotic penetration and, consequently, improved effectiveness of antibiotics within bacterial cells.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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YOL-10

Lily of the valley odorants: synthesis and potential applications – preliminary results

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Keywords: fragrance compounds, fragrance industry, synthetic fragrances, aromatic properties, biological efficacy

1. Introduction

In the world of flavors and fragrances, a wide range of ingredients is used to make various products. Due to the small number of compounds with the scent of lily of the valley, limitations related to use, and impact on the environment, new fragrance compounds are still being searched for. The research goal was the synthesis of lily alcohol derivatives.

2. Material and Methods

The Bargellini reaction was used to synthesize carboxylic acid derivatives as intermediate compounds. These intermediate compounds were then combined with various alcohol mixtures using a combinatorial approach that involved Steglich esterification, catalyzed by DCC and DMAP, resulting in a wide range of esters. After gas chromatography with olfactometric detection (GC-O) analysis, esters showing favorable aromatic fragrances were selected for the next stage of the study: synthesis of individual esters. Oxalyl chloride was added to an acid mixture of a derivative of a known aromatic substance, DMF, and hexane. The mixture of Et_3N, DMAP and selected alcohol in chloroform was then added to the previously prepared crude acid chloride. After the reaction, the compounds were purified and then subjected to gas chromatography-mass spectrometry (GC-MS) analysis.

3. Results

We have synthesized 15 volatile esters. Currently, the biological potential of novel compounds, phenolic derivatives with odor properties, is determined.

4. Discussion and Conclusions

The new approach to preparing lily of the valley odorants, which represents an undeveloped research niche with significant commercial potential, will enable further research to fill existing gaps and generate new knowledge.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

YOL-11

Extraction and fractionation of Azorean *Cryptomeria japonica* female cones essential oil via hydrodistillation: antifungal effects against *Thielaviopsis paradoxa*

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Keywords: Forestry wastes valorisation, *Cryptomeria japonica*, essential oil fractionation, hydrodistillation, antifungal

Abstract

Cryptomeria japonica's wood industry generates large amounts of biomass wastes, including female cones. Azorean *C. japonica* immature female cones (Az–CJIFC) are a particularly rich source of essential oil (EO) with multi-bioactivities. Hydrodistillation (HD), a common process for obtaining EOs, also provides the possibility to fractionate them. The present study evaluated, for the first time, the *in vitro* antifungal activity of Az–CJIFC EO fractions (Frs. 1–6) collected at distinct HD timeframes (HDTs: 0–2, 2–10, 10–30, 30–60, 60–120, and 120–240 min), as compared to the crude EO (0–240 min HDT). The HD process was performed in a Clevenger-type apparatus, and the EO fractions chemical composition was determined by GC–FID and GC–MS. The EO fractions antifungal activity was assessed by the micro-atmosphere method against the phytopathogen *Thielaviopsis paradoxa* (De Seynes) Höhn. Monoterpenes prevailed in Frs. 1–4, while sesquiterpenoids dominated in the later fractions (Frs. 5 and 6). The antifungal activity of Az–CJIFC EO samples decreased as follows: Fr4 > Fr5 > Fr3 > Crude EO > Fr2 > Fr6 > Fr1. In conclusion, HD was found to be an efficient process for obtaining EO fractions with variable and enhanced antifungal activity, due to its differential compositions. Hence, these findings could contribute to increasing the commercial potential of *C. japonica´*s EO industry, namely in agrochemical applications.

1. Introduction

Cryptomeria japonica (Thunb. ex L.f.) D. Don (Cupressaceae) is the most economically important tree species in the Azores archipelago, Portugal. As a result, a huge amount of biomass wastes is locally produced every year, such as cones that can serve as a rich source of essential oils (EOs) with multi-bioactivities, including antifungal activity against *Penicillium chrysogenum* Thom [1]. *Thielaviopsis paradoxa*, another phytopathogenic fungus, causes black rot and stem-end diseases in a range of tropical fruits, such as pineapple or coconut, causing large economic losses [2], including in the Azores. Despite the current control measures relying on synthetic fungicides, the increase of resistance amongst fungi is diminishing their effectiveness. Therefore, EOs could be interesting antimicrobial agents, as previously reported in several studies.

On the other hand, fractionating an EO during HD process is a valuable tool for obtaining EO fractions with enhanced bioactivity. Hence, this study aimed to obtain different EO fractions from Azorean *C. japonica* immature female cones (Az–CJIFC) during the HD process and assess their antifungal properties against *T. paradoxa*, as compared to the crude Az–CJIFC EO.

2. Material and Methods

Essential oil extraction and fractionation by hydrodistillation and chemical composition analysis

EO extraction and fractionation was performed according to Arruda et al. [3]. Fresh Az–CJIFC (400 g) were previously ground in a blender and added to 3 L of water. HD was performed over a period of 4 h, starting from the first distillate drop. EO fractions were collected in the following HD timeframes (HDTs): 0–2, 2–10, 10–30, 30–60, 60–120 and 120–240 min (Frs. 1–6). The experiment was repeated in triplicate. In addition, a control EO

sample was collected from a non-fractionated HD (0–240 min) for comparison purposes. GC-FID and GC-MS analysis were performed as described in Lima et al [1].

Antifungal activity

Antifungal determination was assessed by the micro-atmosphere method according to Yakhlef et al. [4]. In this assay, a Petri dish (90 mm) was filled with 20 mL of potato dextrose agar (PDA) medium, and, after solidification, was inoculated with a mycelium fragment (2–3 mm diameter) taken from the periphery of an actively growing fungal culture. Afterwards, a paper disc was loaded with 20 µL of EO fraction and placed in the inner centre of the lid of the Petri dish. The plates were inverted and immediately sealed with 2 layers of laboratory film to prevent the loss of volatile compounds, being then incubated, lid down, at 25 ± 1 °C for 8 days. Each test was replicated in triplicate, and a negative control was prepared in the same way with the paper disc loaded with distilled water. Mycelium diameter (mm) was measured daily on two opposite sides.

3. Results

EO fractions (Frs. 1–6) collected during the HD process varied significantly in the concentration (%) of their major terpene compounds (Table 1). Particularly, monoterpene hydrocarbons were highest at the beginning of HD (97% in Fr1) and gradually decreased over distillation time. On the other hand, oxygenated sesquiterpenes were the highest in the final EO fractions, accounting for 71% of Fr6. Oxygenated monoterpenes (terpinen-4-ol) hit their maximum concentration on mid-distillation on Frs. 3 and 4, accounting for 18 and 17%, respectively.

Table 1. *Concentration (%) of the major compounds in the fractions (Fr) and crude essential oil (EO) of Azorean C. japonica immature female cones EO obtained by hydrodistillation.*

Compound	RI	Crude EO	Fr1	Fr2	Fr3	Fr4	Fr5	Fr6
α-Pinene	930	21.10 ^{bc}	28.37 ^{a}	22.83 ^{abc}	26.23 ^{ab}	24.20 ^{abc}	13.30 ^{d}	3.53 ^{e}
Sabinene	958	32.63 ^{b}	50.53 ^{a}	36.63 ^{b}	19.90 ^{d}	7.47 ^{e}	3.83 ^{ef}	1.77 ^{f}
Myrcene	975	4.33 ^{c}	5.47 ^{a}	4.97 ^{b}	4.87 ^{b}	3.50 ^{e}	1.73 ^{d}	0.43 ^{f}
α-Terpinene	1002	2.00 ^{c}	1.30 ^{d}	2.63 ^{b}	3.93 ^{a}	3.53 ^{a}	2.07 ^{c}	0.80 ^{e}
γ-Terpinene	1035	3.30 ^{c}	2.17 ^{d}	4.47 ^{b}	6.47 ^{a}	5.83 ^{a}	3.40 ^{c}	1.33 ^{e}
δ-Terpinene	1064	1.43 ^{c}	1.27 ^{d}	1.87 ^{b}	2.30 ^{a}	1.93 ^{b}	1.07 ^{e}	0.37 ^{f}
Terpinen-4-ol	1148	6.77 ^{d}	1.87 ^{e}	9.97 ^{c}	14.50 ^{a}	13.83 ^{a}	7.27 ^{d}	2.93 ^{e}
Elemol	1530	6.00 ^{cd}	0.47 ^{e}	2.90 ^{de}	4.37 ^{cd}	10.23 ^{b}	14.93 ^{a}	13.17 ^{ab}
γ-Eudesmol	1609	3.43 ^{c}	0.07 ^{f}	0.33 ^{e}	0.87 ^{d}	3.73 ^{c}	11.47 ^{b}	22.80 ^{a}
β-Eudesmol	1620	2.10 ^{cd}	0.03 ^{g}	0.33 ^{f}	0.67 ^{e}	2.77 ^{c}	7.53 ^{b}	13.27 ^{a}
α-Eudesmol	1634	2.93 ^{cd}	0.10 ^{g}	0.43 ^{f}	1.00 ^{e}	3.73 ^{c}	9.90 ^{b}	17.53 ^{a}
Phyllocladene	2006	1.63 ^{c}	0.13 ^{e}	0.80 ^{d}	1.13 ^{cd}	2.50 ^{b}	4.63 ^{a}	5.77 ^{a}
Total identified %		98.70	99.90	99.70	99.70	98.20	96.20	94.80
Monoterpene hydrocarbons		71.23 ^{c}	96.53 ^{a}	80.40 ^{b}	71.67 ^{c}	53.13 ^{d}	29.70 ^{e}	9.67 ^{f}
Oxygenated monoterpenes		8.67 ^{d}	2.33 ^{e}	12.70 ^{c}	17.80 ^{a}	17.26 ^{a}	9.13 ^{d}	3.47 ^{e}
Sesquiterpene hydrocarbons		1.63 ^{d}	0.33 ^{f}	1.37 ^{de}	1.70 ^{d}	3.00 ^{c}	4.80 ^{a}	3.63 ^{b}
Oxygenated sesquiterpenes		15.47 ^{d}	0.70 ^{f}	4.43 ^{e}	7.23 ^{e}	21.93 ^{c}	47.10 ^{b}	70.77 ^{a}
Diterpene hydrocarbons		71.23 ^{c}	96.53 ^{a}	80.40 ^{b}	71.67 ^{c}	53.13 ^{d}	29.70 ^{e}	9.67 ^{f}

Values are mean of $n = 3$. Different superscript letters in the same row indicate significant statistical differences ($p < 0.05$). RI: retention index on a DB-1 column.

The antifungal activity of Az–CJIFC EO samples exhibited varying degrees of effectiveness, as illustrated in Figure 1a, decreasing as follows: Fr4 > Fr5 > Fr3 > Crude EO > Fr2 > Fr6 > Fr1. Thus, in comparison to the PDA control plate, which was entirely covered by *T. paradoxa* within three days (reaching a diameter of 90 mm), Fr4 displayed significant suppression of mycelium growth. After three days of incubation, the diameter of *T. paradoxa* mycelium on Fr4 was only 8 mm (as depicted in Figure 1b), and it took eight days for *T. paradoxa* to completely cover the PDA plate under these conditions.

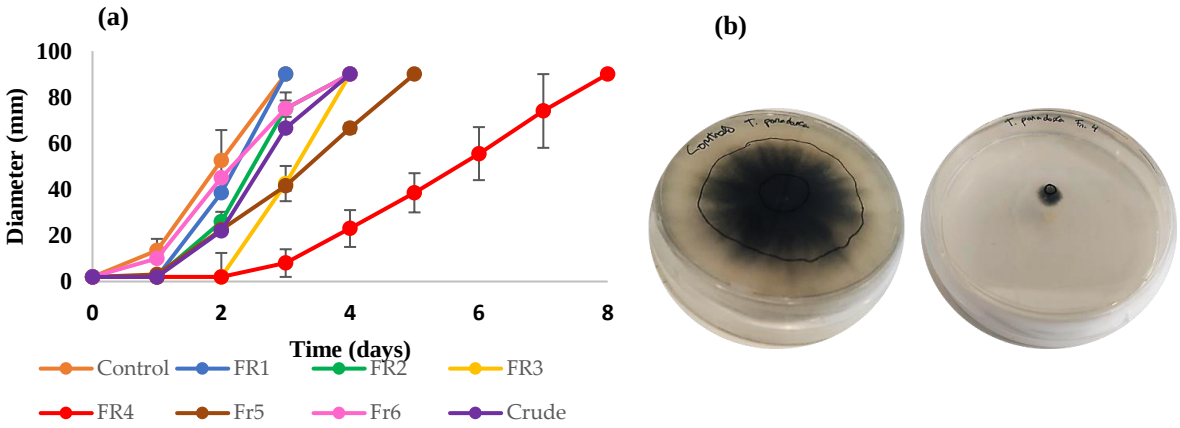

Figure 1: (a) *Thielaviopsis paradoxa* mycelium growth in presence of fractions (Fr) and crude essential oil (EO) of Azorean *C. japonica* immature female cones at 25 °C. (b) Observation of the effect of Fr4 (on the right) on the mycelium growth of *T. paradoxa* on the 3rd day on incubation vs control plate (on the left).

4. Discussion and Conclusions

As a part of our continuous efforts towards the valorization of Azorean *C. japonica* biomass wastes and incentive for local *C. japonica´*s EO industry, we recently found that Azorean *C. japonica* cones EO is a broad-spectrum antibacterial agent [1], thus, we focused our attention on this less studied part and on the fractionation of its EO for enhanced antifungal activity.

The effect of HDTs on the Az–CJIFC EO fractions' composition can be attributed to various factors influencing the distillation order of EOs components during HD, such as (i) volatility: EOCs with lower boiling points and higher vapour pressures tend to be distilled earlier in the HD process; (ii) molecular weight: EOCs with lower molecular weight generally have higher vapour pressure; (iii) chemical structure: EOCs with polar functional groups or hydrogen bonding capabilities may interact with HD medium (water), causing them to be distilled later [3]. Regarding the Az–CJIFC EO fractions' antifungal activity against *T. paradoxa*, the ones collected at mid-HD (Frs. 3 and 4) were the ones that presented higher activity, which seems to be correlated with the oxygenated monoterpenes content (Table 1). In fact, most of oxygenated monoterpenes are well correlated with antifungal properties [5], particularly, terpinen-4-ol, the major oxygenated monoterpene in Az–CJIFC EO fractions, is well known for its antifungal potential.

Therefore, fractionation of Azorean *C. japonica* cones EO by HD method revealed to be an effective method to obtain EO fractions with enhanced antifungal properties, which might be scalable for industrial applications.

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Conflict of Interest

The authors declare no conflict of interest.

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YOL-12

Quantitative activity-composition relationships through machine learning algorithm. Application to essential oil tested as acetylcholinesterase inhibitors

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Keywords: machine learning, essential oils, acetylcholinesterase, database, plants

Abstract

Acetylcholinesterase (AChE), a central enzyme of the serine hydrolase family, modulates the termination of synaptic transmission by degrading acetylcholine (ACh) into choline and acetate, leading to arresting postsynaptic neuron stimulation, which is essential for neuromuscular integrity. In Alzheimer's disease (AD) and other pathological conditions, abnormal AChE expression or insufficient ACh levels need an external modification to restore the necessary ACh effective concentration. Herein, 59 essential oils (EOs) were experimentally evaluated for their AChE inhibitory potential. Incorporating data extracted from literature, a dataset of 69 EOs was used to train machine learning algorithms (RF, GB, SVM, DT, KNN) to build a predictive quantitative activity-composition relationships (QCAR) model to correlate EOs' chemical composition and the associated AChE inhibition ability. The most predictive model was then inspected and revealed key chemical components that were likely to be mainly influencing AChE inhibition, thus facilitating candidate prioritization. Weighted feature importance analysis highlighted compounds that were positively necessary for AChE modulation, such as 1,8-cineole, carvacrol and 1-nitro-2-phenylethane. Our findings underscore the potential of EOs to potentially discover new therapies and highlight the importance of data-driven approaches in natural product research.

Introduction

Acetylcholinesterase (AChE) is a crucial enzyme belonging to the serine hydrolase family. It plays an essential role in terminating synaptic transmission at cholinergic synapses. This is achieved by its rapid hydrolysis of the neurotransmitter acetylcholine (ACh) into its constituent molecules, choline and acetate [1]. The rapid AChE action ensures that acetylcholine does not accumulate in the synaptic cleft, thereby preventing continuous stimulation of the postsynaptic neuron, which could lead to desensitization or overstimulation. By maintaining this balance, AChE is integral to proper neuromuscular function and overall nervous system regulation [2].

AChE has emerged as a highly promising therapeutic target for symptom improvement in Alzheimer's disease (AD), a neurodegenerative disease of the central nervous system [3]. AD appears to be involved in both an abnormal extracellular accumulation of beta-amyloid protein and increased expression of AChE. Inhibiting AChE reduces acetylcholine degradation, thereby improving cholinergic transmission and alleviating some of the symptoms associated with AD [4]. Currently, research into Alzheimer's disease is ongoing, yet only a few drugs on the market act as reversible inhibitors of the AChE enzyme. Consequently, the potential of natural remedies with analogous properties to these pharmaceuticals has attracted considerable interest from researchers over the years. Notable examples include the use of *Ginkgo biloba* extract to enhance memory and various plants from the *Solanaceae* and *Labiatae* families [5], which have been investigated for their antioxidant and neuroprotective effects. These natural alternatives present promising avenues for the development of complementary therapies for the management of Alzheimer's disease and other AChE related pathologies. A further investigation was conducted into the biological characterization of essential oils (EOs), with a series of 59 EOs evaluated for their ability to inhibit AChE activity. In order to construct a predictive quantitative activitycomposition relationship (QCAR) model that can correlate the chemical composition of essential oils (EOs) with their associated acetylcholinesterase (AchE) activity, the number of EOs was implemented with data extracted from the AI4EssOil database (www.ai4essoil.com). The dataset was employed to train a classification machine learning (ML) model. The model thus enabled the identification of molecules with the most significant positive impact on AChE inhibition, thus aiding in the selection of the most promising candidates for further investigation.

Material and Methods

The 59 experimentally tested essential oils (EOs) were obtained from Farmalabor srl in Assago, Italy. Their chemical compositions underwent analysis using gas chromatography–mass spectrometry (GC-MS), following established procedures [6]. For the enzymatic assays, AChE from the *Electrophorus electricus* was employed in a phosphate buffer (pH 7.4) and the inhibitory activity of the oils was evaluated in accordance with the Ellman's method [7]. The analyses were performed using three different essential oil concentrations: 100 µg/ml, 50 µg/ml and 10 µg/ml.

In order to identify the optimal ML model, data pertaining to the efficacy of oils at a concentration of 100 µg/ml were utilized. A further 10 essential oils with related anti-AChE activity, expressed as a percentage of inhibition, were extrapolated from the AI4EssOil online database. The initial dataset comprised 69 essential oils and 376 distinct chemical components. The calculations were conducted using the Python programming language (version 3.10) [8] and the Jupyter Notebook platform to implement an in-house script. A variety of classification algorithms, including Random Forest (RF), Gradient Boosting (GB), Support Vector Machine (SVM), Decision Tree (DT), and K-Nearest Neighbors (KNN), were employed to generate the QCAR models. The predictive ability, accuracy and robustness of the models were evaluated by means of the Matthews correlation coefficient (MCC). The dataset was subjected to a series of random pretreatments, including data levelling, scaling and principal component analysis (PCA). The optimization of ML models involved the removal of infrequently occurring variables that could potentially compromise model accuracy. The evaluation process considered levels ranging from 0 (retaining all variables) to 4 (retaining variables with at least four non-zero entries). The data was subjected to pre-processing using the MinMaxScaler function, which normalized the columns to a range between zero and one. For the Support Vector Machine (SVM) estimator, scaling was consistently applied, as it significantly enhances model performance and convergence speed. Furthermore, PCA was employed for unsupervised dimensionality reduction, with the objective of capturing various percentages of the explained variance. Various cut-offs (thresholds) were used to divide the dataset into active and inactive EOs related to activity expressed as percentage AChE inhibition (%). In order to perform the Stratified K-fold, the dataset was randomly split into an 80% training set and a 20% test set. The most promising machine learning models were selected on the basis of the highest MCC_{pred} values, thereby assessing their predictive capability on the test set. Subsequently, the robustness of these best models was evaluated using leavesome-out cross-validation (LSO-CV). The most successful models were subjected to further analysis in order to determine their feature importance (FI) and partial dependence (PD) using the Skater Python library. To assess the correlation between the presence of a chemical component in the essential oils and its partial dependence, Spearman's correlation coefficient was applied. This approach permitted the calculation of weighted feature importance (FI) values, which reflected the influence of each chemical component.

Results

The data set was randomly searched for the best optimized model. **Table 1** shows the parameters and their coefficients of accuracy, prediction and robustness characterizing this ML model.

Table 1. Best ML models found with random search

Threshold	Algorithm	N° Level	Scaling	PCA	MCC_{fit}	MCC_{pred}	MCC_{CV}	Accuracy
42.716	SVM	0	1	0.0	1.0	0.84	0.60	0.93

The interpretation of the model continued with a Skater analysis, which allowed us to assign an importance value to the components present in the different oils. Next, for the first 15 chemical compounds, the weighted importance was analyzed, which is the product of the importance of the component and its correlation, calculated by the Spearman correlation coefficient. In **Figure 1** are also plotted the first 15 most important chemical components, colored in green if their presence was predicted positively and in red if it was predicted negatively.

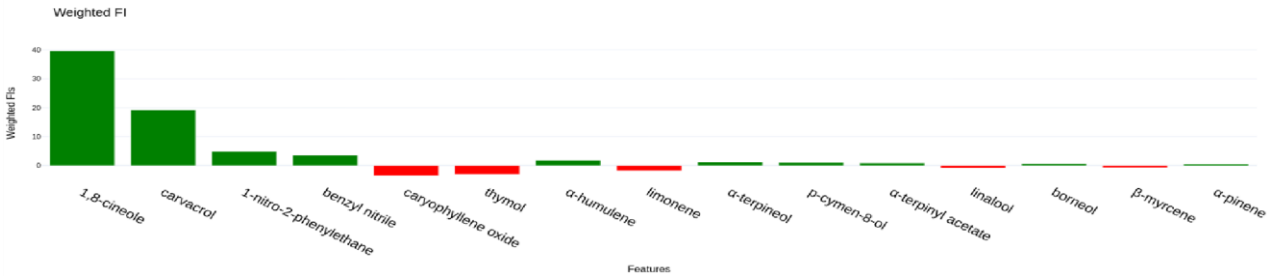

Figure 1. Features Importance (FI) and Weighted Features Importance of the 15 most important compounds

Discussion and Conclusions

The best model was obtained with a threshold value of 42.7% leading to set 24 active oils (i.e. with an inhibition percentage greater than the cut-off) and 45 inactive oils (i.e. with an inhibition percentage less than the cut-off). Following the predictive analysis using the Skater Python library, it was evident that 1,8-cineole (eucalyptol), carvacrol, 1-nitro-2phenylethane, benzyl nitrile, caryophyllene oxide and thymol were the most important components. Among them (**Figure 1**), the first four were indicated to contribute positively to AChE inhibition. It is noteworthy that carvacrol and thymol are isomers but, according to the prediction, only carvacrol was reported active against AChE. A study conducted in 2007 by Jukic et al. specifically analyzed the differential AChE inhibitory activity of some constituents of *Thymus vulgaris* L. essential oil and the oil itself. The study focused on carvacrol, thymol and some of their derivatives including thymohydroquinone and thymoquinone. Despite their structural similarity, carvacrol was found to be approximately ten times more active than thymol [9]. It is not surprising that a molecule like 1,8-cineole is the most important and positive in relation to AChE, as the relationship between this molecule and the enzyme has been analyzed several times in the literature. However, its specific mechanism of action has not yet been elucidated [10-12]. Interestingly, 1-nitro-2phenylethane (NPE), as it only appeared in four EOs with a percentage greater than 60%, was indicated by the model as important. Oyemitan et al [13], tested different essential oils extracted from different parts of the plant *Dennettia tripetala* against AChE. Nevertheless, NPE tested individually showed a lower percentage of inhibition compared to the EO, suggesting that the activity was likely due to some a synergistic activity. In a different report, De Campos et al. found NPE in the EO extracted from *Aniba canelilla* [14]. Interestingly, both EO and NPE effectively reversed spatial learning and long-term memory deficits induced by the muscarinic antagonist scopolamine, comparable to the positive control donepezil. The authors hypothesized that the NPE beneficial effects on memory could be related to the inhibition of AChE. Similarly, as reported [15] for NPE, a molecular docking investigation of the isolated components into the binding pocket of AChE will be soon undertaken. This approach will enable the selection of promising small molecules as potential acetylcholinesterase (AChE) inhibitors, thus paving the way for a parallel study of drug design for new AChE inhibitors.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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YOL-13

Cinnamomum tamala leaf essential oil attenuates lipopolysaccharide-induced inflammation in RAW 264.7 macrophage cells via regulation of the NF-κB signaling pathway

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Keywords: Essential oil, inflammation, lipopolysaccharide, murine macrophages, *Cinnamomum tamala*

Abstract

Objective

Cinnamomum tamala (Buch.-Ham.) T.Nees & C.H.Eberm., commonly known as Indian bay leaf or tejpat, is an important medicinal plant traditionally used for the treatment of inflammation. However, the anti-inflammatory mechanism of action of the leaf essential oil remains unexplored. Therefore, the present research was undertaken for the first time to characterize the chemical composition and to investigate the molecular mechanism underlying the anti-inflammatory activity of *Cinnamomum tamala* leaf essential oil (CTLEO) in lipopolysaccharide (LPS) induced murine macrophage RAW264.7 cells.

Methods

The essential oil was isolated by hydrodistillation method and its volatile profiling was analyzed by gas chromatography mass spectrometry (GC-MS). The cytotoxicity activity of CTLEO was measured by MTT assay. The release of nitric oxide and pro-inflammatory cytokines levels were assessed by Griess and ELISA assay, respectively. Quantitative real time polymerase chain reaction was used to measure the expression of mRNA level and the reactive oxygen species (ROS) level was evaluated by JC-1 assay. Additionally, the mitochondrial membrane potential was evaluated by 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay and the localization of nuclear factor-kappa B (NF-κB) was examined by immunofluorescence assay.

Results

The GC-MS analysis revealed the identification of 48 compounds accounting for 92.31% of the total leaf oil. Phenyl propanoid was found to be principal class of compound with eugenol (52.69%), longifolene (5.92%), caryophyllene oxide (4.47%) and myrcene (2.63%) as the major constituents. Treatment with *C. tamala* leaf essential oil exhibited no cytotoxic effects on RAW 264.7 cells, but at a concentration of 50 µg/mL showed significant reduction in nitric oxide production as compared to LPS-treated group. Essential oil treatment significantly restored the mitochondrial membrane potential in a concentration dependent manner and also reduced the levels of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 in LPS-induced macrophages. The essential oil also decreased the production of intracellular ROS and elevated the levels of endogenous antioxidant enzymes like SOD, GPx, CAT and GSH in LPS-induced macrophages. Pretreament with *C. tamala* essential oil restricted the translocation of the NF-κB dimer and dephosphorylated the p65 protein.

Conclusions

This findinds provide a comprehensive understanding of the molecular mechanisms underlying the anti-inflammatory properties of CTLEO, paving the way for its potential clinical application for treating inflammatory related disorders.

Conflicts of Interest

All authors declare that they have no conflict of interest.

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YOL-14

Exploring natural alternatives for preserving cosmetics: Essential oils as antimicrobial agents

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Keywords: essential oils, cosmetics, alternative preservatives, antimicrobial activity

1. Introduction

Cosmetics often contain preservatives that trigger allergic reactions, especially in sensitive skin products. As demand rises for natural cosmetics, finding alternatives to synthetic preservatives is crucial. One solution might involve integrating natural complex substances (NCSs) like essential oils (EOs), renowned for their potent antimicrobial properties.

2. Material and Methods

Details of the methodology are presented in Figure 1. The following commercially available EOs were selected to create the blend: Petitgrain lemon (*Citrus limon* (L.) Osbeck, BORDAS, Spain), Hiba (*Thujopsis dolabrata* (Thunb. ex L. f.) Siebold & Zucc., BRİSTOL BOTANİCALS, Japan), Clove leaf (*Eugenia caryophyllata* L., JACARADNAS, Madagascar) and Australian Blue Cypress (*Callitris intratropica* Baker & H.G.Sm., ESSENTIALLY AUSTRALIA, Australia).

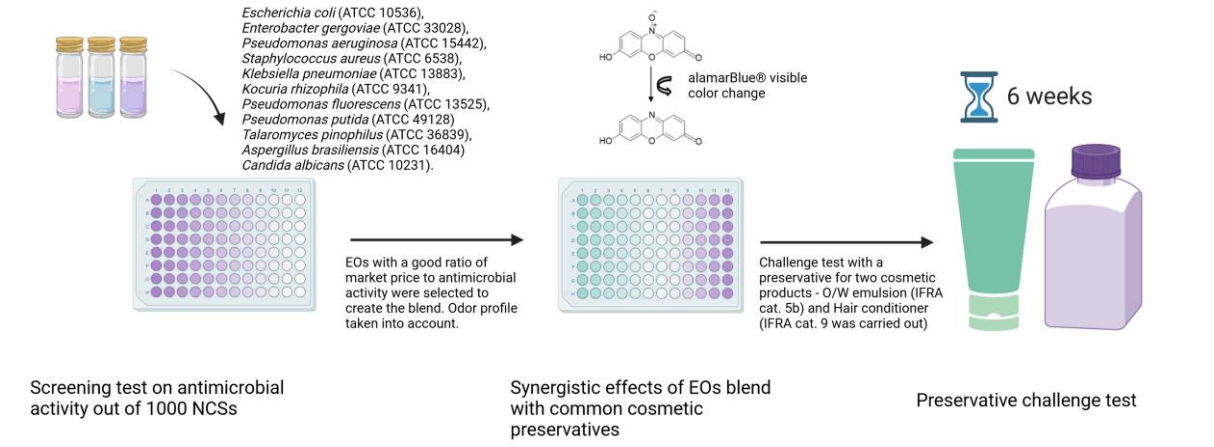

Figure 1 Methodology and research path

3. Results

The research depicted in Figure 1 outlines two formulations developed in compliance with IFRA standards for categories 5b and 9. Synergism trials with natural preservatives showed a substantial boost in antimicrobial effectiveness, reducing Minimum Inhibitory Concentration (MIC) by up to 32-fold. Moreover, challenge test results were encouraging, indicating EOs offer superior infection protection in cosmetics over conventional natural preservatives like *Lactobacillus* ferment or DHA BA (Dehydroacetic Acid, Benzyl Alcohol). Further results will be presented.

4. Discussion and Conclusions

Blending EOs with preservatives allows for lowering their concentration in cosmetics. Optimism surrounds research on EOs' potential as standalone preservatives. Creating pleasing fragrance blends may pose occasional challenges, yet this avenue holds promise for future exploration.

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We would like to thank the producers of EOs for providing us with research material.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

YOL-15

Chemical diversity of essential oils from *Hyptis suaveolens*: implications for management strategies of a global invasive weed

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Keywords: Hyptis suaveolens, Essential oil, GC/MS, Chemotypes

Abstract

Hyptis suaveolens (L.) Poit, an invasive weed in the Lamiaceae family, poses a significant threat to ecosystems. Despite limitations in current control methods, its aromatic biomass and traditional uses offer economic opportunities in aroma and pharmaceutical industries, potentially aiding its management through utilization. This study aimed to analyze the chemical compositions of essential oils extracted from shade-dried aerial parts collected from three locations at varying altitudes. Hydro distillation yielded oils with notable variability in chemical compositions. Maldevta area oil exhibited richness in diterpenoids (68.05%), primarily isopimarol (28.46%). Aamwala uparla oil was dominated by monoterpenoids (75.98%), particularly β-Sabinene (19.86%). Buddhavan forest nursery oil contained significant amounts of mono- (29.80%) and sesqui- (47.79%) terpenoids, with β-caryophyllene (10.17%) and sabinene (9.83%) as chief constituents. Two distinct chemotypes, isopimarol, and β-Sabinene types, were identified. The study's findings highlight the chemical diversity of essential oils from different locations, identifying distinct chemotypes, which can aid in targeted management strategies for this invasive species

Introduction

H. suaveolens(Family Lamiaceae) is known for its extensive ethnomedicinal applications spanning various cultures. Commonly known as "Chan/Wilaiti tulsi," [1], it poses a significant challenge as a fast-spreading invasive weed, wreaking havoc on farmlands and ecosystems. Conventional control methods have proven ineffective due to inherent limitations, prompting the exploration of alternative strategies focused on its utilization. The essential oil derived from the leaves and aerial parts of *H. suaveolens* has been investigated by researchers worldwide, revealing significant variability in content and composition of its essential oil. However, in the state of Uttarakhand, such investigations have been notably scarce. The Hyptis genus, including *H. suaveolens*, is noted for its diverse medicinal properties, ranging from tumorigenic to antimicrobial activities[2]. Particularly, its leaf-derived essential oils exhibit promising antimicrobial and antifungal properties[3,4]. With traditional medicinal uses documented across over 23 countries [5], *H. suaveolens* showcases a rich ethnopharmacological history, employing various plant parts for therapeutic purposes. While the medicinal attributes of *H. suaveolens* are well-documented globally, comprehensive studies on the chemical composition of its essential oils specific to Uttarakhand are lacking. This research gap underscores the need for focused investigation in this region. Research in Uttarakhand highlights a notable gap in understanding due to limited prior investigation. This study pioneers the utilization of freely available raw materials, targeting infestations of this weed in Uttarakhand, offering cost-effective and environmentally sustainable avenues for research aimed at utilization. Given Uttarakhand's geographical diversity, variations in growth patterns and biochemical profiles of *H. suaveolens* are anticipated. Despite its widespread distribution, research elucidating chemical constituents across different locations remains scarce. Therefore, this paper endeavors to compare the chemical composition of *H. suaveolens'* essential oil from three distinct locations in Uttarakhand, India, aiming to unravel the influence of geographical factors on its pharmacological and therapeutic potential.

Materials and methods

Plant material

Aerial parts of *H. suaveolens*were collected from the following locations - Maldevta, Dehradun (Latitude, 30° 18' 46.06713"; Longitude, 78° 6' 2.44841"; Altitude, 648 m); Buddhavan Forest Nursery area, Mohand, Saharanpur (Latitude, 30° 10' 21"; Longitude, 77° 53' 51"; Altitude, 477 m and Aamwala Uparla, Raipur, Dehradun (Latitude, 30° 21' 13"; Longitude, 78° 05' 11"; Altitude, 791 m). The aerial parts collected were then shade dried for 4 days.

Isolation of essential oil

About 700g of shade dried material was subjected to hydro distillation using Clevenger type apparatus for 4h. The oil obtained was separated from the aqueous distillate, dried over anhydrous sodium sulphate and stored at 4°C until examined.

GC-MS analysis of oil

GC-MS analysis of oil was conducted on Agilent 7890B Gas Chromatograph coupled to an Agilent 5977A Mass Spectrometer (electron impact ionization, EI, 70 Ev). Operating conditions were as follows: capillary GC column DB-5ms Ultra Inert (30m x 0.25mm x 0.25µm); carrier gas Helium (flow rate 1 mL/min); injection splitless; injector and detector temperatures, 250°C and 280°C, respectively; oven temperature 40°C (4 minutes), ramped @ 4 °C per minute to 220°C (15 minutes). Mass Spectra were acquired by automatic scanning in the mass range m/z 30-300 at 5.1scan/s. Constituents of the essential oils were characterized using chromatographic (Kovats retention indices. KRIs, comparison with published data) and spectroscopic (mass spectra by comparison of their retention indices (RIs) and mass spectra with established databases, including NIST17 and F&F libraries, NIST chemistry webbook, and relevant literature. The relative amounts of individual components were calculated based on GC integrator peak areas without using correction factors.

Results

Hydro-distillation of aerial parts of *H. suaveolens* collected from three different locations afforded yellowish green essential oils of characteristic odour. The yield of the essential oils was 0.2%, 0.1%, and 0.27% for Maldevta, Buddhavan Forest Nursery, and Aamwala Uparla, respectively. Our results align closely with prior research, with Peerzada [6], Mallavarapu [7], Eshilokun [8], Asekun [4], and Laily Bin Din [9] reporting yields within a similar range, supporting the consistency of our findings. These collective findings underscore the reliability and validity of our research outcomes in understanding essential oil yield from Hyptis suaveolens.

Chemical compositions of the essential oils isolated from the aerial parts of *H. suaveolens* occurring in three different locations are shown in table 1. In *H. suaveolens*oil from Maldevta, Dehradun, 41 compounds comprised 91.62%, with diterpenoids (67.19%) dominating. Notable compounds included kaurene (13.26%), isopimarol (28.46%), and abieta-8,11,13-trien-18-ol (14.01%). Sesquiterpenoids (17.98%) were also present, with β-caryophyllene (4.05%) as a major constituent. In the essential oil from Buddhavan forest nursery area (Mohand), 54 compounds comprising 92.4% of the oil were identified. The oil primarily consisted of sesquiterpenoids (47.79%), with major compounds including β-caryophyllene (10.17%), spathulenol (7.63%), and (Z)-trans-α-bergamotol (4.57%). Monoterpenoids accounted for 29.8% of the total composition, with sabinene (9.83%) being the predominant compound. Diterpenes (11.73%) were also present, with atiserene (6.27%) being the most abundant. The essential oil from Aamwala Uparla, Raipur (Dehradun), comprised 48 compounds, totaling 96.65%. Monoterpenoids dominated at 75.98%, with major compounds including α-pinene (5.40%), β-Sabinene (19.86%), α-phellandrene (9.33%), β-phellandrene (7.94%), γ-terpinene (4.68%), and terpinen-4-ol (7.50%). Sesquiterpenoids constituted 15.53%, with β-caryophyllene (7.50%) as the chief compound. Diterpenoids were present in a minor proportion (0.87%).

Numerous studies have explored the chemical composition of *H. suaveolens* worldwide. Peerzada [6] reported β-Caryophyllene (29%) as a major compound, consistent with findings by Malele et al. [10], Tine et al. [11], and Poonkodi et al. [12]. Earlier reports by Pant et al. [13] and Oliviera et al. [14] highlighted β-caryophyllene and limonene as major components. Similarly, Fitofarmacêutica, Fármacos and Farmácia [15] and Benelli et al. [16] found β-pinene as a major compound. Eshilokun [8] and Noudogbessi et al. [17] observed α-pinene, sabinene, and terpinen-4-ol as predominant components, aligning with our results on the chemical composition of HSEO from Aamwala Uparla, as well as findings from Maldevta and Buddhavan. Four chemotypes including 1,8-cineole, sabinene, β-caryophyllene and fenchonechemotypes are reported to occur in *H. suaveolens* [18]. The presence of 1,8-cineole, a constituent commonly observed in the essential oils of different origins of *H. suaveolens*, has not been detected in the oils under examination.

Conclusion

The analysis of *H. suaveolens*essential oil from three Uttarakhand locations reveals significant variations in yield and composition, suggesting the influence of local environmental factors on metabolite production. HSEO from Maldevta was characterized by a high diterpenoid content (68.05%), whereas HSEO from Buddhavan had dominant sesquiterpenoids (47.79%) along with substantial monoterpenoids (29.8%) and diterpenoids (11.73%). Aamwala Uparla exhibited higher monoterpenoid abundance (15.53%), with moderate sesquiterpenoids and minimal diterpenoids, and boasted the highest count of bioactive compounds among the three locations. With sabinene constituting 19.86% of the oil composition, it delineates the sabinene chemotype.Upon thorough

examination of existing literature, it is evident that *H. suaveolens*essential oil exhibits substantial chemical variability globally. Monoterpenoids and sesquiterpenoids are consistently abundant components, while diterpenoids are typically sparse or entirely absent in most instances. Our results revealed a distinct chemotype present in the essential oil sourced from the Maldevta location, characterized by a significant richness in diterpenoids, comprising 68.05% of the composition which, in contrast with the global trends represents a rare occurrence of a diterpenoid chemotype. These findings imply that ecological conditions, such as soil composition, climate, and altitude, likely shape the chemical profile of *H. suaveolens*essential oil across different locations. The distinct biological properties of the major compounds found within the three essential oils entail divergent applications across various sectors. Understanding the chemical diversity of *H. suaveolens*essential oil across different geographical regions is crucial for its sustainable utilization in various applications, including pharmaceuticals, cosmetics, and aromatherapy. In conclusion, our study not only sheds light on the chemical variability and chemotypic diversity within *H. suaveolens*essential oil but also highlights the intricate interplay between environmental factors and secondary metabolite production. These insights pave the way for further exploration of the aromatic, pharmacological and therapeutic potential of *H. suaveolens*while underlining the importance of sustainable utilization practices in aromatic and medicinal plant research and development.

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Disclosure statement

Authors declare that they have no conflicts of interest.

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YOUNG SCIENTISTS' POSTER ABSTRACTS

P-Y-01

Chemical composition, *in vitro* antioxidant and anti-inflammatory properties of essential oils of sawdust and resin-rich bark from Azorean *Cryptomeria japonica* (Cupressaceae)

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Keywords: Azores, *Cryptomeria japonica*, essential oil, GC/MS, antioxidant and anti-inflammatory properties

Abstract

In Azores, *Cryptomeria japonica´*s timber industry generates large amounts of biomass residues (CJBR) that can provide valuable essential oils (EOs). Here, we evaluated the chemical compositions, antioxidant and anti-inflammatory activities of EOs from Azorean *C. japonica* sawdust (CJS) and resin-rich bark (CJRRB). The CJS and CJRRB EOs, obtained by hydrodistillation (HD), showed both different yields (0.27% vs. 0.80% v/w, dry weight basis) and chemical profiles, as assessed by GC/MS. A total of 64 and 85 components were identified in CJS and CJRRB EOs, representing 95.7% and 96.9% of the total composition, respectively. The major components in CJS-EO were oxygenated sesquiterpenes (mainly α+β-eudesmol, 1-epicubenol and cubebol), while in CJRRB-EO were monoterpenes hydrocarbons including α-pinene, δ-3-carene and limonene (66.6% vs. 6.4% for oxygenated sesquiterpenes and 0% vs. 64% for monoterpenes hydrocarbons, respectively). The antioxidant activity was estimated using (i) two radical-based assays, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity, and (ii) a lipid model assay, the β-carotene-linoleic acid bleaching activity (BCBA). Both CJS and CJRRB EOs exhibited concentration-dependent antioxidant activities, and their DPPH, ABTS and BCBA EC_{50} values were 1107 vs. 1275 µg/mL, 260 vs. 498 µg/mL and 1764 vs. 662 µg/mL, respectively. Regarding the anti-inflammatory activity, the inhibition of bovine serum albumin denaturation test showed the following ranking at 2.21 µg/mL: CJRRB-EO ≥ sodium diclofenac ≥ CJS-EO. The results indicated that both EOs may be sustainable sources for antioxidant and anti-inflammatory compounds. This study expands the chemical and biological knowledge of CJBR, and adds more value to the *C. japonica´*s EO industry.

1. Introduction

Oxidative stress and inflammation are interconnected pathophysiological processes, capable of inducing each other, frequently coexisting in various diseases such as diabetes, atherosclerosis, and cardiovascular disease [1]. Therefore, nowadays, there's a worldwide pursuit for environmentally friendly antioxidant and anti-inflammatory products harnessing natural plant compounds, like essential oils (EOs), valued for their Generally Recognized as Safe (GRAS) status, widespread consumer acceptance, and potential versatile applications [2]. This interest is amplified by growing apprehensions regarding the potentially harmful synthetic additives. In fact, the EOs are ancient therapeutic resources renowned for their diverse pharmacological and biological effects, notably their antioxidant properties. Such antioxidant abilities of EOs depend on their chemical composition, being usually attributable to components with hydroxyl (particularly phenolic) groups or multiple bonds [3].

Cryptomeria japonica (Thunb. ex L.f.) D. Don, or Japanese cedar, introduced to the Azores in the 19th century, dominates the archipelago's timber sector. Logging yields substantial forest residues, presenting an environmental challenge [4]. Yet, these residues, including sawdust and bark, remain untapped resources ripe for conversion into value-added products like EOs. Given the dependency of EO activity on their chemical composition, particularly influenced by plant part, this study aims to compare EOs extracted by hydrodistillation (HD) from Azorean *C. japonica* sawdust (CJS) and resin-rich bark (CJRRB), assessing their antioxidant and anti-inflammatory potentials *in vitro*.

2. Material and Methods

The CJRRB sample was collected in November 2023 from a wound on a *C. japonica* tree located in São Miguel

Island (Azores archipelago, Portugal), at latitude 37° 49' 07.7''N and longitude 25° 21' 33.2''W. The sample was immediately brought to the laboratory, cleaned, and then shade dried at room temperature (20 °C). Following drying, CJRRB sample was pulverized into powder. The CJS sample, obtained from a local carpentry industry on São Miguel Island, was air-dried at room temperature. Then, the CJS and CJRRB EOs were extracted by HD in a modified Clevenger-type apparatus, as described in Janeiro et al. [4]. The EO yield (%, v/w) was calculated on a dry weight (d.w.) basis. The EO` chemical composition was determined by gas chromatography/mass spectrometry (GC/MS) on a Shimadzu GCMS–QP2010 Ultra gas chromatograph/mass spectrometer, equipped with a ZB–5MSPlus (5% phenyl, 95% methyl siloxane) capillary column (Phenomenex Inc., Torrance, CA, USA). Detailed conditions are described in Janeiro et al. [4]. The EO` antioxidant activity was determined using DPPH, ABTS and BCBA assays [4]. The EO` *in vitro* anti-inflammatory activity, i.e. the inhibition of bovine serum albumin (BSA) denaturation assay, was conducted as described by Matotoka et al. [5] with some modifications.

3. Results

CJS and CJRRB EOs yields were 0.27% and 0.80% (v/w, d.w.), respectively. Regarding the chemical composition, a total of 64 and 85 components were identified in CJS and CJRRB EOs, representing 95.7% and 96.9% of the total composition, respectively. The major components (≥ 5.0%) of these EOs are listed in Table 1. The CJS-EO was mainly characterized by oxygenated sesquiterpenes (OS), followed by oxygenated diterpenes (OD), sesquiterpenes hydrocarbons (SH) and diterpene hydrocarbons (DH) (66.6%, 14.8%, 13.3% and 0.9%, respectively). Neither monoterpene hydrocarbons (MH) nor oxygenated monoterpenes (OM) were identified in CJS-EO. The major components (> 5.0%) in CJS-EO were α+β-eudesmol (13.5%), 1-epicubenol (10.7%), cubebol (6.8%), δ-cadinene (6.4%), τ-cadinol (5.9%) and sandaracopimarinol (5.5%). Phytochemical analysis of CJRRB-EO revealed that this EO was dominated by MH (64%), mainly due to α-pinene content (42.7%), followed by limonene (8.9%) and δ-3-carene (6.0%). Concerning the other terpene groups, SH was the second most representative group (19.1%), followed by OS (6.4%), OM (5.4%), OD (1.9%) and DH (0.16%).

Table 1. Composition (≥ 5%) of the essential oils (EO) isolated by hydrodistillation from Azorean *Cryptomeria japonica* sawdust (CJS) and resin-rich bark (CJRRB).

Component	Retention Index	Relative Content (%)	
		CJS-EO	CJRRB-EO
α-Pinene	927	–	42.74
δ-3-Carene	1003	–	6.02
Limonene	1022	–	8.93
δ-Cadinene	1507	6.42	7.23
epi-Cubebol	1486	4.73	0.79
Cubebol	1504	6.76	1.28
1-Epicubenol	1615	10.74	1.93
τ-Cadinol	1632	5.90	–
α+β-Eudesmol	1643	13.54	0.54
Sandaracopimarinol	2253	5.48	–
Grouped components (%)			
Monoterpene hydrocarbons		0.00	63.97
Oxygenated monoterpenes		0.00	5.42
Sesquiterpene hydrocarbons		13.38	19.09
Oxygenated sesquiterpenes		66.64	6.39
Diterpene hydrocarbons		0.86	0.16
Oxygenated diterpenes		14.83	1.89
Identified components (%)		95.71	96.92

The antioxidant activities (EC_{50} values) of the studied EOs using DPPH, ABTS and BCBA assays are shown in Table 2 and compared to the standard antioxidant gallic acid (positive control). Both EOs exhibited weak activity for DPPH and ABTS free radical scavenging activity (FRSA) compared with gallic acid. The CJS-EO presented higher FRSA than the CJRRB-EO in ABTS assay, while no difference in antioxidant activity between the EOs was observed in the DPPH assay. In the BCBA assay, different results emerged, i.e. the CJS-EO exhibited lower antioxidant activity compared to the CJRRB-EO. Overall, when compared to gallic acid, both studied EOs exhibited stronger antioxidant activity in the BCBA assay than in the FRSA assays.

Table 2. Antioxidant activity of the essential oils from Azorean Cryptomeria japonica sawdust and resin-rich bark.

Samples	EC_{50}, µg/mL		
	DPPH	ABTS	BCBA
Sawdust essential oil	1107 ± 94^{b}	261 ± 6^{b}	1764 ± 388^{c}
Resin-rich bark essential oil	1275 ± 347^{b}	498 ± 20^{c}	662 ± 37^{b}
Gallic acid	1.93 ± 0.09^{a}	1.13 ± 0.01^{a}	38 ± 5^{a}

Different letters in the same column indicate statistically significant differences ($p < 0.05$).

The anti-inflammatory activities of the studied EOs using anti-BSA denaturation assay are shown in Table 3 and compared to diclofenac sodium (a reference non-steroidal anti-inflammatory drug). The results showed a percentage of inhibition of 51%, 70% and 59% at a concentration of 2.21 μg/mL, for CJS-EO, CJRRB-EO and diclofenac sodium, respectively. The anti-inflammatory activities of CJRRB-EO and diclofenac sodium were comparable but higher than the CJS-EO.

Table 3. Anti-inflammatory effect of the essential oils (EO) from Azorean *Cryptomeria japonica* sawdust and resin-rich bark in denaturation of bovine serum albumin inhibition assay.

Samples	Percentage protection against protein denaturation concentration (μg/mL)			
	2.21	**4.43**	**8.85**	**17.71**
Sawdust EO	51 ± 9^{c}	67 ± 15^{a}	75 ± 18^{a}	84 ± 15^{a}
Resin-rich bark EO	70 ± 3^{ab}	86 ± 7^{a}	89 ± 9^{a}	92 ± 3^{a}
Dıclofenac sodium	59 ± 10^{bc}	76 ± 11^{a}	84 ± 12^{a}	87 ± 14^{a}

Different letters in the same column indicate statistically significant differences ($p < 0.05$).

4. Discussion and Conclusions

The *C. japonica´*s timber industry, particularly sawmills, produces tons of residues including sawdust and bark, without any or little commercial application. Thus, using these residues to create value-added products, including EOs is imperative. In the phytochemical analysis of sawdust EO sample, a noteworthy revelation is the prevalence of OS, namely α+β-eudesmol, 1-epicubenol and cubebol, which emerged as the principal compounds. In contrast, the investigation into the phytochemical makeup of resin-rich bark EO disclosed the presence of distinct compounds, namely MH, that set it apart from sawdust EO. Notably, α-pinene stood out as the predominant constituent, accounting for a significant proportion of 43% within the EO. Both EOs were able to exert antioxidant activity via different mechanisms of action, as revealed by the different applied tests. However, EOs from *C. japonica* seems to exhibit stronger antioxidant activity in the BCBA assay than in the FRSA assays. Additionally, both EOs protected the BSA against heat induced denaturation. Further research on synergistic effects between potential EO antioxidant and/or anti-inflammatory components is essential. Thus, the results indicated that both EOs, could be alternative raw materials for food, and medicinal products for use in pharmaceutical applications.

Conflict of Interest

The authors declare no conflicts of interest.

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P-Y-02

AI4EssOil: Revolutionizing essential oil insights through artificial intelligence

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Keywords: machine learning, essential oils, QCAR, database, plants

Abstract

Essential oils (EOs) are complex mixtures, often analyzed for their potential in combating a spectrum of pathogens, ranging from bacteria and fungi to viruses, leveraging their properties in several biological systems (antioxidant, antimicrobial, antifungal, antiviral, etc.).

Over the years, there has been a growing endeavor to integrate the therapeutic richness of essential oils into clinical practice, potentially serving as adjunctive therapy to facilitate dosage reduction of conventional medications or combatting multidrug-resistant pathogens. Despite the well-documented efficacy of essential oils, their integration into clinical settings remains limited due to challenges associated with their chemical and physical lack of standardization, thereby hindering the attainment of any formulations. Machine learning (ML) algorithms have recently been developed to shed light on the key chemical components mostly responsible for the EOs biological activity, enabling the developing of quantitative composition-activity relationships (QCAR). The AI4EssOil database, established in 2021, collates extensive experimental data on EOs, enabling the construction of ML classification models. These models, integrated into the database's "Predict" section, allow users to explore existing data and conduct predictive analysis. By manually inputting component data, users can assess the potential efficacy of their essential oils, enhancing accessibility and usability. These advancements represent a pivotal step towards unlocking the full potential of essential oils for various applications.

Introduction

In the last decade EOs have attracted scientists with a constant increase rate of more than 7% as witnessed by almost 5000 articles; numerous biological activities are explored, such as antioxidant or antimicrobial ones [1]. The relationship between EO's chemical composition and its biological activity is rarely analyzed and it represents one of the problems for the clinical use of EOs, especially for their chemical instability. It was exploited the capability of ML algorithms to produce models able to identify the chemical components most responsible for the associated biological activity and then produce the quantitative composition-activity relationships (QCAR). Being able to determine which molecules amplify and promote that biological activity and which ones inhibit it, could be the first step to create a new EO composed of those components that have proved to be beneficial. AI4EssOil is an online database that was established in 2021 with the premise of containing as much experimental data related to essential oils; to date it collects 2806 essential oil compositions, attributable to 1131 different plants and 20979 biological activities, extracted from 1292 scientific publications (www.ai4essoil.com, accessed April 2024). The vast quantity of data available enabled the construction of numerous machine learning classification models, each examining a distinct category of activities (see Table 1 in the Results section). In fact, the database's distinctive feature is its incorporation of AI/ML functions that enable the estimation of the chemical components significance within oils in relation to the activity under examination. In the "Predict" section of the AI4EssOil online database, users are afforded the opportunity to not only explore existing data but also to engage in predictive analysis. By manually inputting component data and their respective percentages, users can assess the potential efficacy of their own essential oils. Furthermore, the integration of machine learning (ML) models within this section enhances accessibility and usability. The platform provides users with a streamlined interface to select their target of interest, access relevant ML algorithm performance metrics, and visualize influential chemical components. thereby providing an indication of the potential activity of that oil and its probability.

Material and Methods

Through the AI4EssOil portal, a series of training sets were composed through the selection of the target of interest (Pathogen Name, Enzyme Name or Antioxidant Activity). All the calculations were performed using the Python programming language (version 3.10) [2] by executing in-house code in the Jupyter Notebook platform [3]. The models were generated with different classification algorithms (RF, GB, SVM, DT and KNN). The models' accuracy was assessed by means of Matthews correlation coefficient (MCC), as a measure of the quality of the ML-based binary classification [4]. The models were optimized in predictive ability and then the best models were cross-validated to analyze their robustness. ML models robustness were evaluated by leave some out cross-validation (LSO-CV) splitting the dataset into two groups. The selection of the best pretreatment parameters and the optimization of the algorithms hyperparameters' was checked through the evaluation of the predictive Matthews correlation coefficient (MCC_{pred}). In those cases with classifiers showing similar MCC_{pred} values ensemble modeling were performed to improve the prediction power and limiting the overfitting. To this the voting classifiers was used combining the predictions of several single classifiers [5]. The best models were then analyzed to evaluate the "feature importance" (FIs) and the partial dependence (PDs) through the Skater python library. The Spearman's correlation coefficient was used to weight the correlation between the presence of a chemical component in the EOs and its partial dependence, obtaining the weighted FI values. This approach enabled the identification of a trend for each chemical component, indicating whether it exerted a positive or negative influence on the activity under examination.

Results

The list of some models that have are included and ready to be used in the www.ai4essoil.com website is provided in the Table 1.

Table 1. ML models published at www.ai4essoil.com

	Target	Activity Type	MCC_{pred}	Accuracy	Positive Components	Negative Components
1.	ABTS scavenging activity	Antioxidant	86.97%	96.67%	• Carvacrol • Thymol • p-cymene • Eugenol	• Limonene • Linalool • Eucalyptol • a-pinene
2.	Acinetobacter baumannii	Antibacterial	68.25%	86.62%	• Linalool • Citral • Carvacrol • Eugenol	• Limonene • Eucalyptol • a-pinene • b-caryophyllene
3.	DPPH Radical	Antioxidant	80.90%	93.46%	• Carvacrol • Eugenol • Thymol • Chrysanthone	• Limonene • Eucalyptol • Linalool • a-pinene
4.	Aspergillus Niger	Antifungal	42.01%	78.18%	• b-pinene • g-terpinene • Camphor • Linalool	• a-pinene • Limonene • Camphene • a-terpineol
6.	FRAP	Antioxidant	61.44%	88.59%	• Borneol • O-cymene • Eucalyptol • b-pinene	• Caryophyllene • Limonene • a-terpineol • b-myrcene
8.	Hydroxyl Radical Scavenging	Antioxidant	89.03%	96.79%	• Linalool • Eucalyptol • Carvacrol • Eugenol	• Limonene • Caryophyllene • b-pinene • Estragole
9.	Linoleic Acid Peroxidation	Antioxidant	95.49%	98.33%	• Carvacrol • Thymol • Eugenol • d-Cadinene	• Caryophyllene • p-cymene • g-terpinene • Linalool
10.	Staphylococcus aureus	Antibacterial	68.79%	83.94%	• Linalool • Carvacrol • Citronellol • Terpinen-4-ol	• a-pinene • Germacrene-d • Eucalyptol • g-terpinene

11.	AChE	Enzymatic	83.78%	92.64%	• Eucalyptol • Carvacrol • 1-nitro-2phenylethane • Benzyl nitrile	• Caryophyllene oxide • Thymol • Limonene • Linalool
12.	3t3-Swiss Cells	Cytotoxic	74.67%	86.92%	• Limonene • Eucalyptol • Linalool • a-pinene	• Caryophyllene • Carvacrol • Geraniol • Eugenol

Discussion and Conclusions

The ML classification models available on the website, given their high accuracy, have provided us with interesting starting points for in vitro experiments capable of demonstrating the actual validity of these predictions. Microbiological assays of minimum inhibitory concentration have already been performed on Acinetobacter baumannii, testing the most important chemical components as indicated by the model. The results indicate a correlation between the positive and negative influences predicted by the model. A series of MTT assays were recently conducted on 3t3-swiss cells, confirming the non-cytotoxicity of a number of molecules, including eucalyptol, limonene, and linalool. Concurrently, promising molecules with antibacterial, antioxidant, and other properties, such as carvacrol and thymol, exhibited a highly significant cytotoxic character, even at dilution percentages > 0.005%. In 2023, we published our work investigating the role of EOs' chemical components investigated for several types of antioxidant activities. The predicted contributions of the compounds agreed well with their experimentally defined biological profiles, demonstrating antioxidant potential and confirming the accuracy of the models [6]. Further investigations and studies of enzymatic activity towards acetylcholinesterase are underway. The ML model was indeed able to detect the positive presence of molecules such as 1-nitro-2-phenylethane, despite its occurrence in only four oils in the database. Other works [7, 8] had already observed the presence of this molecule as a possible inhibitor of this enzyme. For this reason, we are carrying out QSAR and docking studies to evaluate this molecule more thoroughly and possibly attempt in vitro testing for its effective application.

The aim is to develop further models that could enable the design of blended EOs, mainly composed of key components identified by ML models. This iterative process will enable the creation of tailored formulations optimized for specific therapeutic or functional purposes.

In the foreseeable future, AI4EssOil will introduce additional web applications that will extend its functionality. Users will be able to query the database for statistical distribution data, such as the prevalence of a particular chemical component in different plant species. Users can also request the average percentage composition of essential oils (EOs) found to act against specific biological strains. Throughout the development of the project, a series of models will be implemented to facilitate the prediction of biological properties associated with essential oils of known composition. In conclusion the ML and AI modeling applied to EOs data available from AI4EssOil will likely enable to design EOs mixtures reaching a biological standardization that will overcome the lack of EOs chemical standardization.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-Y-03

Fractionation of specific terpene families prior to biological assays exploiting preparative gas chromatography

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Keywords: gas chromatography, preparative gas chromatography, terpenes, biological tests

1. Introduction

The interest on terpene components is evergrowing, strictly related to their multiple biological activities, which made them a valid resource for the pharmaceutical, nutraceutical, and food industries. Among this class of compounds, *β*-caryophyllene (BCP), is of primary importance, due to its antioxidant, anti-inflammatory, and ant-hyperglycemic effects, as well as its dietary availability through consumption of edible plants, including spices. In this research, essential oils having a consistent amount of BCP, obtained from spices, were subjected to preparative gas chromatography to isolate target fractions with and without BCP in order to be assessed in terms of the biological activity.

2. Material and Methods

In this study, a preparative multidimensional gas chromatographic system was exploited, coupled to MS detection, and equipped with wide bore capillary columns. Biological assays were carried out to evaluate the activity of the fractions collected.

3. Results

In a first step, the spice's essential oil and the BCP activity was evaluated. In a second step, target terpenes were isolated through preparative gas chromatography, aimed to evaluate possible synergic actions between target terpene components. Preliminary biological tests are highlighting that the sesquiterpenes fraction is more active than BCP, able to demonstrate possible synergic effects.

4. Conclusions

This study demonstrated the efficacy of a combined approach involving gas chromatography and biological studies in elucidating the relationship between biological activity and specific sample components, isolated from preparative gas chromatography.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

P-Y-04

Annual yield variation of labdanum resin obtained from *Cistus ladanifer* L. plants of different age

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Keywords: rockrose, Mediterranean shrub, oleoresin, ládano, alkaline extraction

Abstract

Labdanum resin is one of the most important perfumery and cosmetics bioproducts obtained from rockrose (*Cistus ladanifer* L.). Spain is the leading producer of this oleoresin, with commercial exploitation centred in southern regions. However, also central Spain possesses significant potential for its production. The aim of this study was to determine the annual yield of labdanum resin obtained from plants of different ages from central Spain. The resin was extracted by immersing the biomass in warm alkaline water for one hour, followed by acid neutralization. The results showed that the labdanum yield varied throughout the year from 2.94 % to 9.22 %, with the highest yields found during autumn.

Introduction

The rockrose, *Cistus ladanifer* L. (Cistaceae), is a prevalent and widely distributed plant resource across the Iberian Peninsula and is also found in southern France and northern Africa [1]. Its leaves and stems secrete a labdanum resin, also known as labdanum gum or ládano [2]. This resin comprises a complex blend of terpenoid and phenolic compounds, primarily characterized by labdane-type diterpenes and methylated flavonoids [2]. Labdanum resin and its extracts are currently used in the perfumery and fragrance industry [3], due to its aromatic and fixative properties and, moreover, hold potential for use as cosmetic ingredients in skincare products [4]. *C. ladanifer* L. is mainly commercially exploited in Andalusia, Southern Spain [5], however other regions like Extremadura and Castilla La Mancha in central Spain hold considerable untapped potential for labdanum production [6].

Objective

This study aimed to determine the annual yield of labdanum resin obtained from plants of *C. ladanifer* of different age from central Spain.

Materials and Methods

Widely grown *Cistus ladanifer* L. (Cistaceae) was manually collected from two locations - Bustares and Hiendelaencina (Guadalajara, Castilla La Mancha, central Spain). The plants were approx. 7 years and 12 years old, respectively. Samples of whole plants, cut at an about 15 cm aboveground, were collected monthly or fortnightly across one year (July 2021-June 2022). The material was cut into pieces of approx. 5-7 cm in size. Labdanum resin was extracted from 100g of fresh plant material (three repetitions per plant age sample) by immersing the biomass in 1L of distilled water with 10 g of Na_2CO_3 that was heated up for 60 min at 60°C. Then, the biomass and impurities were filtered from the solution. Obtained liquid was stored at ambient temperature for 24 hours and then 7 ml of H_2SO_4 were added. 72 hours after the carbonate neutralization, the labdanum gum was collected and dried at ambient temperature. The result for each collection period was expressed as average value of three repetitions in % d.w/d.w. (ratio of initial plant material dry weight and dry resin weight).

Results

The labdanum yields of plants of different ages over one year showed similar trend line pattern (see Figure 1); labdanum yield decrease (below 4% d.w/d.w.) was recorded in August/September and yield increase in September and December and again in June. The yield varied from 2.94 % to 9.22 %. In general, the younger plants produced higher labdanum yields.

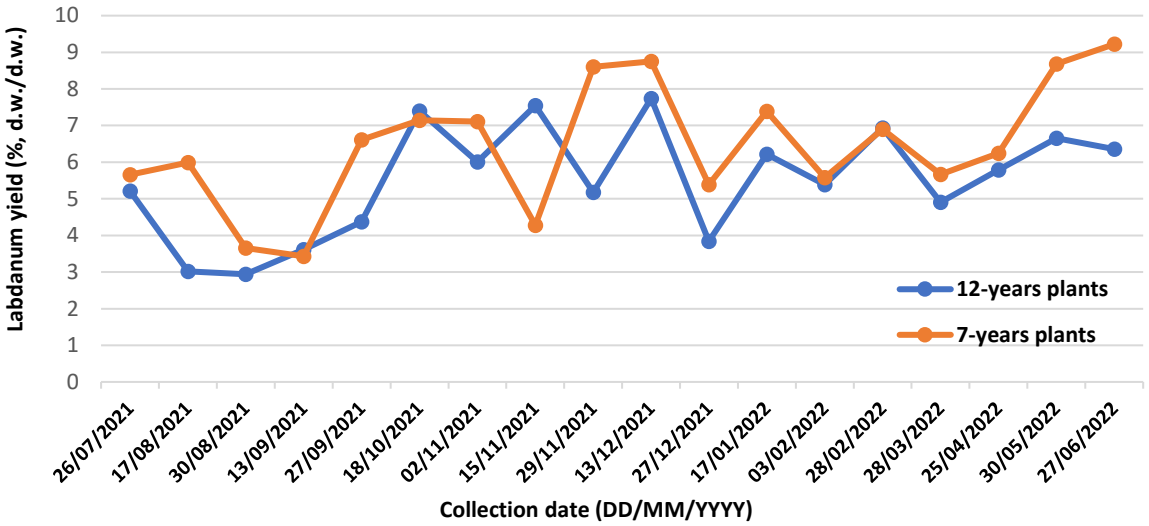

Figure 3. Annual yield of labdanum resin from differently aged *C. ladanifer* L. plants from central Spain

Discussion and Conclusions

Compared to other studies, Portuguese rockrose collected in August yielded 7.44±0.41 % (d.w./f.w.) [4] and 5.79 ± 0.52% (d.w./f.w.) [7]. In August, our plants yielded the lowest amount of the resin. Values exceeding the 7% yield was found from October onward. 7-years plants generally produced higher yields of labdanum resin. Plants from both locations exhibited similar yield variations over the seasonal changes, which could be caused by meteorological and environmental factors.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-Y-05

Extraction and bio-conversion of Limonene from waste sweet lime (*Citrus limetta*) peels by baker's yeast

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Keywords: Limonene, Waste peels, Baker's yeast, Terpineol, Bioconversion

Abstract

Citrus peels serve as a valuable reservoir of essential oils necessary in the realm of flavour and fragrance. Waste citrus peels, a by-product of the food processing industry, is a low-cost, easily available source. The inherent instability arising from the volatile nature, conjugated structure and very low content of terpene molecules in the source, makes conventional methods of steam distillation, cold-pressing relatively unviable. In the present work, waste sweet lime (*Citrus limetta)* peel from India, has been used to extract various classes of terpenes, majorly limonene (monocyclic monoterpene with several therapeutic benefits), present in it. A new extraction process has been developed using 'Baker's yeast' (*Saccharomyces cerevisae*), an inexpensive, whole-cell microbe to extract the essential oils. This uses mild conditions of temperature, pH, yeast concentration, etc. at elongated time to extract terpene mixture present in waste sweet lime peel. Process optimization resulted in a terpene yield of 0.93 ± 0.3 % (v/w) of the peels taken. It is comparable to the yield obtained of 1.1±0.1% by cold-pressing. After 60h of incubation extract obtained was characterised by TLC, GC-MS. Results confirmed presence of 97% limonene in the product extracted along with pinene, myrcene, carene, and terpineol. Extraction was carried out further to check any changes in composition. After 96h GC-MS confirmed presence of terpineol as the major terpene (67%). Thus a greener process was developed for extraction and in-situ bio-conversion of limonene by using baker's yeast accomplishing a sustainable approach.

1. Introduction

Citrus fruit residues like peels, seeds, skin, etc have become the preferred choices as raw material for extraction of various terpene molecules needed in sectors like perfumery and flavour industry due to their low cost and easy availability [1]. Apart from cosmetic uses, terpene molecules are presently widely used for therapeutic benefits [2]. The rising demand of natural terpenes for therapeutic applications have raised quality and stability concerns. Use of microorganisms for biosynthesis, bi-extraction and bio-conversion of terpene molecules are gaining popularity, owing to its sustainable and environment-friendly approach [3]. The present work makes use of citrus peels – a low-value raw material, to extract terpene mixture. Baker's yeast (freeze-dried *Saccharomyces cerevisae*) was chosen based in the market dynamics, easy availability and its GRAS status [4]. Furthermore, enzyme activities of this microbe has shown productivity trends related to the transition from oxidative to oxidoreductive growth [5]. All these factors led to selection of this whole-cell microbe for this work. Use of enzymes and whole cell organisms have been used till date for bio-conversion of various terpenes. However, extraction by using baker's yeast of solid-state extraction is not a popular method yet. Whole-cell microbe is used to rupture the cell walls for better permeation and diffusion of the terpene molecules into the matrix. The method was developed by optimization of process parameters. Further, the extracted terpenes were converted to a new class of terpenes by in-situ bio-conversion. The same experimental setup was used to extracted terpene to another class of terpene following the principles of in-situ bioconversion. Biotransformation of monoterpenes is difficult owing to the chemical instability, high volatility and high cytotoxicity of both precursors and products [6]. This results in low transformation rates. Keeping this in mind, in-situ bio-conversion of the limonene was focussed on.

2. Material and Methods

Waste sweet lime peels were collected from local juice stores from various parts of India. The peel condition was thoroughly checked such that it remained green and fresh while all rotten ones were screened out. Food grade active dry yeast (baker's yeast) was bought from the local grocery store. Distilled water was used throughout this work. Limonene, obtained from Sigma Aldrich USA was used as standard during various testing.

All other chemicals were obtained from Merck's India, Mumbai, India and were of analytical grade.

2.1 Process developed for extraction of limonene

The collected peels were washed thoroughly with running cold water 2-3times and commuted using a mixer grinder to reduced size. The prepared peels were taken in a 500ml Erlenmeyer flask and water was added to it. The solid to water ratio was varied from 2-7 times (w/v) keeping all other parameters constant. Activated baker's yeast was added with a varying amount of 2-10% (w/w) of the peels taken while keeping other parameters constant. pH of the medium was varied from 4 to7 using 0.1(M) HCl and 0.1(M) NaOH for obtaining best results. Inorganic salts of Mg^{2+} was added by 1% (w/w) of the yeast taken in order to facilitate the biochemical mechanism of yeast [7]. The whole set up was incubated at temperatures varying from 26-36□C for 12-72h maintaining all other requisite conditions and was shaken at 90-100rpm. After 60h, extracted terpene mixture was collected after removing the yeast cells and undigested peels by centrifugation (10,000 x g, 20min). The supernatant was extracted with di-ethyl ether (3×15ml). The terpene rich organic layer was separated using a separating funnel and passed through activated sodium sulfate to remove any trace of water. The obtained liquid was filtered through 0.5µm membrane (Agilent) to eliminate the presence of any solid residue. Finally, the solvent was lyophilized in order to avoid any thermal degradation of the extraction product. The terpene extract thus obtained was used for further analysis and characterization.

2.2 Process developed for in-situ bio-conversion of extracted limonene

The method involves in-situ bio-conversion of limonene to terpineol using baker's yeast right after extraction of limonene. After complete extraction the reaction was carried on for another 48h maintaining the same conditions in the same reaction medium. No other additional processing was done.

Parallely, a control experiment was performed. 0.5ml limonene was taken in 500-required amount of previously activated baker's yeast. This set up was run for 48-72h. The terpene obtained was characterised further.

Physical Characterization of the extraction product

The colour was checked visually. The odour of the extracted terpene mixture was checked by sensory characterization by expert panel. Solubility of the purified terpene was checked in 70% ethanol by addition of the product followed by vigorous shaking for 2min. The extracted terpene mixture was stored in a glass vial for 90 days at -20°C and the above mentioned sensory analysis was checked every 15 days in order to check the stability of the product.

Chemical Characterization Analysis of extracted terpene by thin layer chromatography (TLC)

Extracted product was analysed by TLC according to standard procedure with solvent system of n-hexane: Diethyl ether: 97:3 (v/v) [8].

Analysis of extracted terpene by Fourier transmission infrared (FTIR) analysis

Pure sample obtained by preparative-TLC and subsequent lyophilisation was subjected to FTIR Analysis (4000400cm-1, PERKIN ELMER universal ATR Sampling Accessory). The results are compared with standards.

Analysis of extracted terpene by GCMS analysis

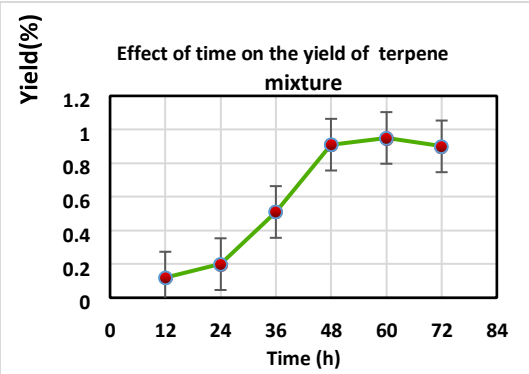
Chemical characterization of the essential oil was performed on a SHIMADZU gas Chromatograph-Mass spectrometer (ModelQP5050, Shimadzu Corporation, Japan) using a capillary column DB 5 MS (30meterX 0.

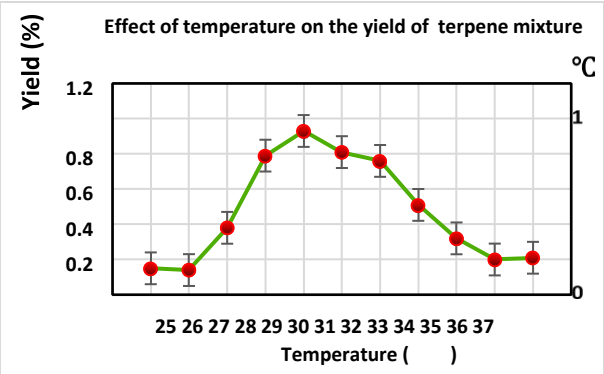
25mmid.X 0.25µmthickness). The injector temperature was 260°C and the interface ttemperature was maintained at 250°C.The oven temperature was programmed to increase from 40 to150°C at a rate of 5 °C/min and from 150 to 240°C at 30°C/min. The column inlet pressure was maintainedd at 48.9kPa. The carrier gas was helium at a flow rate of 1 ml/min with a split ratio 1: 10. Identification of compounds was based on comparisons with mass spectra from the literature (NIST, US National Institute of Standards and Technology).

3. Results and discussion

In this work, a wide array of results concerning the extraction of terpene from waste sweet lime peel by baker's yeast has been observed under varied process conditions. After incubation of the peels in presence of activated baker's yeast for 60h at 30±2℃ at suitable pH and 90-100 rpm the average yield of extracted terpene was 0.93±0.3 % (v/w) of the total peels taken. The yeast concentration for optimised extraction was nearly 8%. The peel: water ratio is an important factor as too much or too little water will lead to inefficient extraction. 1:5 peel: water ratio was found to be the best condition for obtaining maximum yield. The terpene mixture obtained was pale yellow in colour and had a lime odour. It was completely soluble in 70% ethanol. Chemical characterisation by GC-MS confirmed presence of limonene (97.16%) along with pinene, myrcene, carene, and terpineol (0.75%). It was further confirmed by FTIR.

Effect of process conditions of extraction of terpene mixture from sweet lime peels using baker's yeast





The impact of baker's yeast on the bioconversion of limonene was examined by prolonging the experimental procedure to 96h i.e. after obtaining optimized yield at 60h, it was further continued for another 48h in the same medium maintaining all other parameters. After 96h GC-MS analysis reported decline in percentage of limonene in the total terpene mixture. Concurrently, presence of an oxygenated terpene, terpineol was observed. Through optimization, it was noted that as the reaction kinetics advanced, there was an escalation in the quantity of terpineol alongside a reduction in the proportion of limonene. Moreover, a minor quantity of linalool was detected. Linalool is recognized as the precursor molecule of terpineol. Consequently, it can be inferred that with progress of reaction, limonene is being converted in-situ by baker's yeast to terpineol via linalool. This method demonstrated an approximately 70% in-situ conversion of limonene to terpineol, while the control experiment solely exhibited a 12% conversion rate.

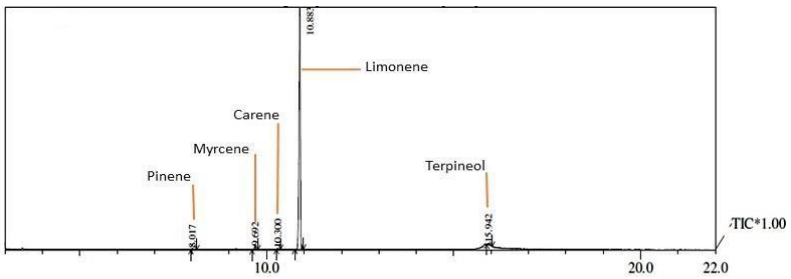


Fig1: GC-MS chromatogram of extracted terpene mixture

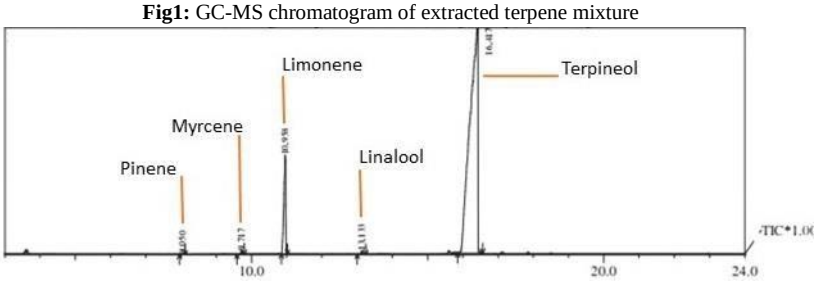


Fig 2: GC-MS chromatogram of in-situ bioconversion of limonene to terpin

4. Conclusion

The results clearly show that a novel method was developed for extraction and in-situ bio-conversion of limonene using baker's yeast with appreciable yield and conversion rate. Lowered temperature and considerably lesser use of solvent will enable application and use of the terpenes extracted in many a fields in terms of better quality and safety. The use of low-cost baker's yeast and citrus peels to obtain high value products aligns with the objectives of sustainable development.

Acknowledgement

Olivia Dhara acknowledges Late Dr. Mahua Ghosh for her intellectual inputs for carrying out this work.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-Y-06

Invitro and *Invivo* Antihyperglycemic effect of *Curcuma amada rhizome* essential oil in STZ induced diabetic rats

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Keywords: *Curcuma amada;* essential oil; in-vitro and in-vivo antidiabetic activity.

Abstract

The aim of this study is to investigate the antidiabetic potential of *Curcuma amada* rhizome essential oil by invitro and invivo methods. The extraction of rhizome essential oil (REO) is done by using the clevenger apparatus for eight hours. The obtained REO is stored in a vial covered with silver foil for further analysis. The phytochemical analysis of REO is done by using Gas Chromatography Mass Spectroscopy (GCMS). The analysis of functional group of essential oil is done by using Fourier Transform Infrared Spectroscopy (FTIR). Invitro and invivo antidiabetic studies are done for REO considering their antidiabetic potential, less toxicity and presence of various types of bioactive compounds like terpenoids, phenolic and alkaloids. The invitro antidiabetic activity of REO is done by the alpha amylase assay in which the acarbose is taken as the standard drug. The invivo antidiabetic activity of REO is conducted in the STZ (Streptozotocin) induced diabetic rats. The best result of inhibition % for REO is seen at a concentration of 100µl (77.5 %) which is near to acarbose (82.7%). The result of invivo antidiabetic study showed that our drug was able to lower the blood glucose level and there was a steady increase in the body weight of the animals after. The histopathological analysis also supported the fact that there is an improvement in the pancreatic cells of the animal. Thus from these results we can conclude that the REO has the antidiabetic potential and can be further investigated for drug formulation.

1. Introduction

The medicinal plants have been used by many local communities worldwide from thousands of years. Approximately 85 % of the world population is still using these medicinal plant as their primary healthcare agents and as a primary source of drug discovery. However, in the last few decades' variety of the drugs have been used in the treatment of various health problem but 80 % of these drugs are synthesized by using the medicinal plants [1]. From the past hundred years a prolific upsurge in the development, introduction and advancement in the analysis of herbal substance is seen. The invention of various analytical technique like chromatography, spectroscopy, extraction and isolation made it very easy in using these medicinal plant for the benefits of the local community. The genus, Curcuma belongs to the family Zingiberaceae and is composed up of almost 93 species [2]. The genus Curcuma has a great value for its medicinal and nutritional properties at global level [3]. In most of the Curcuma species the essential oil and curcumenoids are the two utmost pharmacological active constituents. Curcumenoids is basically a mixture of three compounds namely curcumin, desmethoxycurcumin and bisdemethoxycurcumin, all these compound have many health benefits and also prevent us from many diseases [4]. The essential oil of Curcuma species is composed more than 100 of compound which includes terpenoids, terpenes and sesquiterpenes [5]. The essential oils of different Curcuma species have been used in the cosmetic industry [6], in the food industry as colouring, flavouring and as a preservative agent [7] and also to deal with variety of health issue like digestive tract problems, wound healing, cancer, viral and diabetes [8]. Diabetes is a prevalent global condition characterized by insulin insufficiency, insulin resistance, and high blood sugar levels. Although, there are certain drug like metformin, repaglinide and thiazolidinedione are available in the market, but they do cause certain side effect like coronary arteries diseases. Efforts have been made to discover new kind of medicine for diabetes which is cheaper, less toxic and more effective. This prime objective of this study is to analyzed the antidiabetic potential of CAREO.

2. Material and Methods

The extraction of essential oil was done by using clevenger apparatus for 8hours at 60^{o}C. The extracted oil was subjected to GCMS for analysing the phytochemical composition. The invitro antidiabetic activity was carried out by using the alpha amylase assay. The invivo antidiabetic potential of oil was analyzed in streptozotocin induces

diabetic rats.

3. Results:

The GCMS analysis of CAREO showed the presense of 15 volatile compound. Out of thee 15 compound the compound found with major area (%) includes ar-curcumene (20 %), xanthorrhizol (18 %), β-curcumene (18 %), camphor (9 %), curzerenone (8 %) and were found representing about 74 % of total area. Similarly, some other minor compound found during GCMS analysis includes B-elemenone (7 %), curzerene (5 %), β -farnesene (1.3 %), eucalyptol (0.9 %), Isoborneol (0.7 %), B-elemene (0.7 %), camphene (0.6 %), borneol (0.4 %), camphene hydrate (0.3 %) and α -pinene (0.14 %). The chromatogram of CAREO is given in Fig a. The FTIR spectra of CAREO represent multiple peaks corresponding to various types of functional group present in essential oil which indicates the presence of variety of bioactive compound. The FTIR spectra of CAREO is presented in fig b

The invitro antidiabetic potential of CAREO is analyzed by using alpha amylase assay. The result showed that the inhibition % CAREO is 77.5 % and inhibition % of acarbose is 82.7 %. The inhibition % of CAREO lags only by 5.2 % in comparison with acarbose. The antidiabetic potential of CAREO is due to the presence of various types of bioactive compound present in the essential oil. In the literature it is reported that compound like camphor eucalyptol and curcumene have antidiabetic potential. Then, the invivo screening for antidiabetic potential of CAREO is also done in STZ induced diabetic rats. The female rats were used for this invivo screening. All the rats were divided into separate group. The toxicity test of CAREO is done with two different dose 200 mg/ kg and 400 mg/kg for 7 days. During the toxic study no abnormalities, death and panicness among rats is seen. After that all the rats were kept washing period of 7 days. During this all rats were treated with normal feed and water. Then all the rats were divided into five group. The detail of grouping is given in Fig C Then the blood glucose level of all the overnight fasting rats were measured and after that the STZ injection is given to them intraperitoneally for the induction of diabetes. After 3 hours the blood glucose level of all the rats is measured to confirm diabetes among them. After the confirmation of diabetes, a daily dose of CAREO is followed for 21 days. During the 21 day's analysis the blood glucose level of group 1 remained constant because this group has served as a normal group and only vehicle is given. The blood glucose level of group II increased from 120±4 to 340±4 because this group is treated with neither treated with CAREO nor with metformin. In group III metformin is given which is a market formulated drug to lower the level of glucose in blood. İn group IV there is a slight decrease in the blood glucose level. But in case of group V when the dose of CAREO is increased than the level of blood glucose is decreased more than the group IV and is similar to group III. The detail of blood glucose level is given in fig (d). Similarly, the body weight of group 1 remained unchanged because this group has served as a normal group and only vehicle is given. The body weight of group II decreased regularly because this group is neither treated with CAREO nor with metformin. In group III metformin is given, a market formulated drug due to which there is a recovery in the body weight can be seen. İn group IV there is a slight recovery in the body weight. But in case of group V when the dose of CAREO is increased than the recovery in the body weight is more than the group IV and is comparable to group III. The detail of body weight of all the five group is given in the fig (e). The histopathological analysis also supports the fact that the higher dose of CAREO has significantly prevented the cytoarchitectural symmetry of beta cells of pancreas. The separate images of histopathological analysis of each group is given in fig (f).

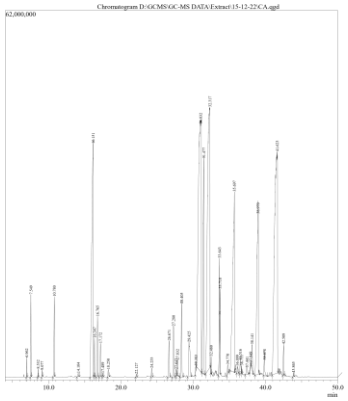

Fig (a) Chromatogram of CAREO

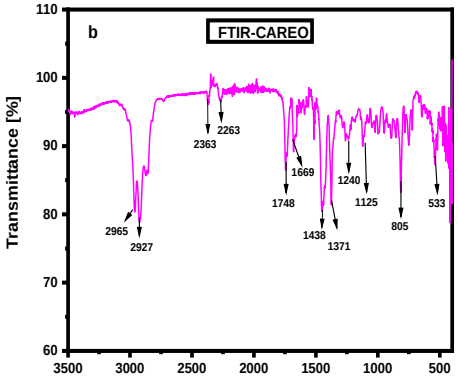

Fig (b) FTIR spectra of CAREO

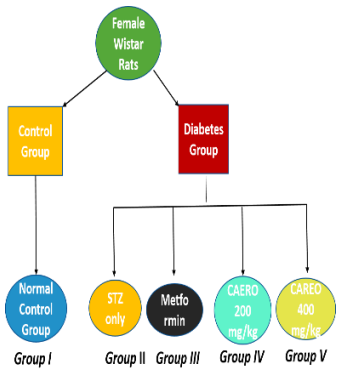

Fig (c) Grouping of rats

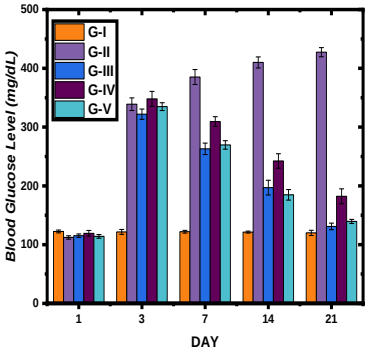

Fig (d) Blood Glucose Level

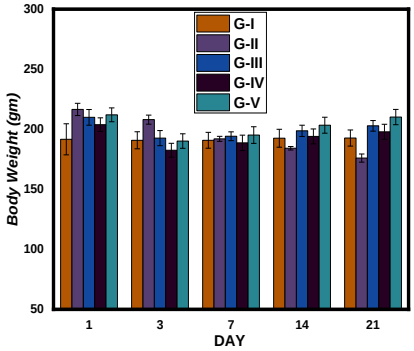

Fig (e) Body Weight

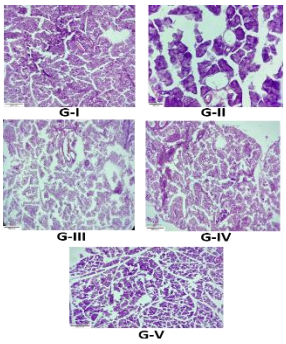

Fig (f) Histopathology

4. Discussion and Conclusions

In conclusion this study showcases the phytochemical composition of essential oil of Curcuma amada. The GCMS result showed the presense of 15 biologically active compound. Among these 15 compound five compound were identified as major compound which contribute more than 65 % of total area. Among these five compound curcumene and camphor have been documented for their antidiabetic potential. Then the invitro antidiabetic activity is done and the result showed that the CAREO has antidiabetic potential. Then the invivo antidiabetic analysis is done in which three parameter blood glucose level, body weight and histopathological analysis of pancreatic beta cells were taken into account. The invivo study also showed the antidiabetic nature of CAREO. So from this study we can conclude that the essential oil of Curcuma amada has antidiabetic potential and it can be turnout to be a natural antidiabetic agent.

Conflict of Interest

The authors declare no conflict of interest

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P-Y-07

In vitro antimicrobial evaluation of *Salvia sclarea* and *Origanum vulgare* essential oils in combination against sinusitis pathogens

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Keywords: Anti-sinusitis, essential oil combination, synergy

1. Introduction

Sinusitis is a common pathology that arises from a variety of causes including microbial infections within the upper respiratory tract. In this present study, commercial *Salvia sclarea* L. and *Origanum vulgare* L. essential oils, which are documented to have ethnobotanical were used. Various systematic essential oil combinations were prepared for targetted antimicrobial evaluation.

2. Material and Methods

The commercially available essential oils were analysed by GC-MS and GC-FID, for their quality. *In vitro* antimicrobial evaluation of the oils against methicillin-resistant *Staphylococcus aureus, Staphylococcus aureus* ATCC 23235, *Moraxella catarrhalis* ATCC 43617 and *Streptococcus pneumoniae* ATCC 6313, were performed using a microdilution assay. Combinations of the essential oils were evaluated using the checkerboard method. Active samples were then prepared for nasal spray formulations and the effects were challenged and compared also using the agar-well diffusion method. The in vitro safety of the essential oil nasal formulation was evaluated using the MTT method against L929 cell lines.

3. Results and Discussion

Analyses of *O. vulgare* essential oil resulted in 73% carvacrol as the major component, whereas the main component of *S. sclarea* oil was 67% linalyl acetate among other components determined, respectively. The calculated fractional inhibition concentration index (FICI) of combinations against *M. catarrhalis* resulted as synergistic (FICI value: 0.259), while the FICI value of the combinations against methicillin-resistant *S. aureus, S. aureus* ATCC 23235, and *S. pneumoniae* ATCC 6313, were found additive with 0.51, 0.509 and 0.508 FICI values, respectively. The tested two essential oils were relatively more effective in combination against all tested pathogens. No in vitro toxic effects of the formulation up to a concentration of 2 mg/mL was observed on healthy cell line evaluation. To the best of our knowledge, this is the first oregano-clary sage combination with the potential as nasal anti-sinusitis formulation.

4. Conclusions

The initial promising in vitro results of the sinusitis preparation needs to be further optimized and developed, confirmed by *in vivo* and clinical studies as well.

ACKNOWLEDGEMENTS

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P-Y-08

Chemical composition of essential oils from different parts of *Heracleum crenatifolium* Boiss. in Türkiye

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Keywords: *Heracleum*, Apiaceae, essential oil, GC, GC-MS

1. Introduction

The genus *Heracleum* L., or hogweed (Apiaceae family), contains 17 species in Türkiye. *Heracleum* species are traditionally used as food additives, spices, flavouring agents, and treatment of many disorders such as inflammation, stomachache, and wound healing. These plants have broad pharmacological activities: anti-inflammatory, antimicrobial, and antioxidant [1,2].

2. Material and Methods

In this study, the fruit, aerial parts, and root of *Heracleum crenatifolium* were collected in Konya, Türkiye. The plant material was identified by Dr. Ceyda Sibel Kılıç (Herbarium no: 31.001). The essential oils (EOs) of *H. crenatifolium* different parts were obtained by hydrodistillation using a Clevenger type apparatus for 3h. Essential oils were analysed both by GC-FID and GC-MS, simultaneously.

3. Results

The GC-FID and GC-MS analysis showed that major components of fruit, aerial parts and roots essential oils of *H. crenatifolium* were octyl acetate (95.4, 85.9, 9.5%) and myristicin (not detected, 10.6, 88%), respectively.

4. Discussion and Conclusions

To our knowledge, no chemical compositions of the essential oils of *H. crenatifolium* aerial parts and root have been reported in the literature.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-Y-09

Exploring the potential biological activities and chiral composition of *Lippia origanoides* Kunth essential oil: inhibition of enzymes linked to Alzheimer's disease and Type 2 diabetes

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Keywords: *Lippia origanoides* Kunth, essential oil, enzymatic inhibition, chiral analysis.

Abstract

Diabetes mellitus and Alzheimer's disease (AD) are increasingly common in ageing population. Type 2 diabetes is an important risk factor for AD due to shared phenotypes, including mitochondrial dysfunction [1]. The insulin resistance characteristic of diabetes alters the AD pathology by affecting amyloid precursor protein (APP) metabolism, contributing to plaque formation [2]. Impaired glucose and lipid metabolism, which is often associated with obesity, further exacerbate AD progression. In addition, there is new evidence of a link between the composition of the gut microbiota and the risk of obesity-related diseases [3]. Inhibition of enzymes such as acetylcholinesterase (AChE), butyrylcholinesterase (BChE), α-glucosidase and lipase offers potential therapeutic opportunities for both AD and diabetes. Essential oils (EOs), complex mixtures of volatile compounds obtained from individual plant species by hydro distillation and steam distillation or mechanical processes have diverse applications in perfumery, cosmetics, and pharmaceuticals [4], with recent focus on their potential in inhibiting disease-associated enzymes. This study therefore focused on a remarkable Brazilian native essential oil, *Lippia origanoides* Kunth EO, which has been investigated for some biological activities such as its antimicrobial, insecticidal, acaricidal and larvicidal properties [5,6,7], but not for its promising enzyme inhibitory activity with the exception of a paper dealing with AChE inhibition [6]. *Lippia origanoides* EO was chemically characterised, subjected to chiral analysis, and tested for its inhibitory effect on AChE, BChE, α-glucosidase and lipase. The results showed significant activity against all four enzymes, suggesting that the role of this EO as an adjuvant treatment with a broad spectrum of activity could be further investigated in addition to conventional therapies.

1.Introduction

Research interest in studying the biological properties of essential oils has increased due to their complex composition and widespread use in various fields such as cosmetics, food and pharmaceuticals. A particular focus is on investigating their influence on enzyme functions, especially those associated with critical human diseases like Alzheimer's and type 2 diabetes. In this framework, the present study centers on *Lippia origanoides* Kunth essential oil (family Verbenaceae), a slender, highly aromatic shrub or tree reaching heights of up to 3 meters. Indigenous to Central and Southern American countries like Mexico, Guatemala, Cuba, and Amazon region in countries such as Guiana, Venezuela, Brazil, and Colombia, it is known in northern Brazil as "Salva de Marajo" and "Alecrim d'Angola". In Brazil its leaves are traditionally used for culinary and medicinal purposes [5]. In this study, it was recognised as the only active essential oil showing remarkable activity against a spectrum of enzymes - AChE, BChE, α-glucosidase, and lipase - among numerous Brazilian indigenous plant essential oils investigated. To further explore the biological activity of this EO, preliminary tests of individual compounds from the essential oil are currently being carried out after determining its chemical composition and chiral characterisation by GC-MS and GC-FID to allow a more thorough investigation of the compounds responsible for the activity.

2. Material and Methods

Lippia origanoides Kunth samples were from Embrapa Western Amazon germplasm bank, in Manaus (AM), Brazil. The plant leaves were subjected to hydrodistillation in a Clevenger type apparatus. Access to biodiversity was authorized by CGEN under registry AC6AC63.

Lippia origanoides Kunth EO was subjected to an AChE and BChE *in vitro* colourimetric assay according to the Ellman method, the protocol used in this study was optimized by Pavarino et al. [8] by modifying that of Rhee et al. [9], a α-glucosidase *in vitro* colourimetric assay according to the protocol of Oboh et al. [10] and a lipase *in*

vitro colourimetric assay according to the protocol of Slanc et al. [11], all with slight modifications.

Qualitative and quantitative chemical characterisation of the essential oil was performed by GC-MS and GC-FID analyses, using both an apolar and a polar column, respectively a MEGA5 (95% polydimethylsiloxane, 5% phenyl, 30 m x 0.25 mm x 0.25 μm) and a MEGAWAX (polyethylene glycol, 60 m x 0.25 mm x 0.25 μm) to resolve coelutions.

For chiral analysis, two enantioselective capillary columns with stationary phases of 2,3-di-O-ethyl-6-O-tert-butyldimethylsilyl-β-cyclodextrin and 2,3-di-O-methyl-6-O-tert-butyldimethylsilyl-β-cyclodextrin were used.

3. Results

Lippia origanoides EO Kunth was obtained in 2.4% yield, and was chemically characterised by GC-MS and GC-FID using an apolar and a polar column, revealing carvacrol (41.8%), p-cymene (14.9%), γ-terpinene (8.5%), trans-β-caryophyllene (4.8%) and thymol (4.2%), as the main compound, percentages referring to the apolar column. In addition, enantioselective analysis revealed the presence of (1S,4R)-(-)-camphene, (S)-(+)-α-phellandrene, (1R,6S)-(-)-δ-3-carene, (1S,4S)-(-)-camphor, (1S,2R,4S)-(-)-borneol, (1Z,6Z,8S)-(-)-germacrene D and (1R,9R,E)-(-)-β-caryophyllene as enantiomerically pure compounds. On the other hand, α-thujene, α-pinene, β-pinene, sabinene, limonene, linalool and terpinen-4-ol were scalemic mixtures.

The EO of *L.origanoides* inhibits AChE by 61.9% ± 3.79 (at a concentration of 38.4 μg/ml in the final mixture), BChE by 21.3% ± 0.372 (at a concentration of 38.4 μg/mL in the final mixture), lipase by 84.8% ± 1.11 (at a concentration of 33.3 μg/mL in the final mixture) and α-glucosidase by 29.1% ± 2.27 (at a concentration of 50 μg/mL in the final mixture). To better compare the inhibitory activities of the *L.origanoides* EO over the four enzymes, the inhibitory concentration that halves the enzyme activity under the experimental conditions considered (IC_{50}) was determined, as shown in Table 1. The IC_{50} values confirmed the interesting inhibitory activity towards these enzymes. However, the IC_{50} value for BChE could not be calculated, even if the enzyme has an interesting inhibitory activity, since 50% inhibition was never achieved under the assumed experimental conditions. Preliminary tests of some compounds characterising the composition of the EO revealed that mainly oxygenated compounds of the EO such as carvacrol are responsible for the activity over AChE, with a contribution from minor compounds such as 1,8-cineole. Carvacrol is also active on BChE, while 1,8-cineole was inactive in this case. The compounds contributing to the biological activity against lipase and α-glucosidase were mainly hydrocarbon compounds. For the former, myrcene and limonene were active, for the latter limonene, α-pinene and β-pinene.

Table 1 IC_{50} values of *L.origanoides* EO towards AChE, BChE, α-glucosidase and lipase enzymes with their standard deviation value (n = 3).

Enzyme	**IC_{50} *L.origanoides* (μg/ml)**
AChE	22.9 ± 0.907
BChE*	Activity lower than 50%
α-glucosidase	14.6 ± 0.580
lipase	74.9 ± 3.84

*IC_{50} value of *L. origanoides* towards BChE was not calculated, because 50% inhibition was never achieved under the assumed experimental conditions.

4. Discussion and Conclusions

The aim of this study is to identify promising biological activities in plants traditionally used by the population. We specifically investigated the essential oil of *Lippia origanoides* Kunth due to its proven versatility, especially its inhibitory effect on enzymes such as AChE, BChE, lipase and α-glucosidase, which play a crucial role in diseases prevalent in the elderly. To summarise, this essential oil is a strong candidate for further research, which is currently being conducted, to investigate the compounds responsible for its inhibitory properties. Such exploration is promising for its potential use as a complementary treatment in these interrelated diseases.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-Y-10

Effect of wintergreen essential oil and black seed fatty oil in contact dermatitis *in vivo* model

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Keywords: *Gaultheria fragrantissima*, *Nigella sativa*, GC-MS, contact dermatitis, *in vivo* mouse model

Abstract

Introduction: Essential oils can be used externally in massage oil to reduce pain or inflammation associated with rheuma. *Nigella sativa* L., the fatty oil of black cumin seeds, has been used in Arab countries for centuries for food, cosmetic and medicinal purposes. Essential oils of *Gaultheria* species (*G. procumbens, G. fragrantissima*) are frequently used today as anti-inflammatory agents for joint diseases due to their high concentration of methyl-salicylate. In our study, we wanted to explore whether *Nigella* fatty oil or Gaultheria essential oil (wintergreen) reduce the symptoms of contact dermatitis. Contact dermatitis is a chronic, inflammatory skin disease, a late-type allergic reaction triggered, for example, by metals or certain plant substances.

Methods: The composition of the essential oil was determined by GC-MS. For our research, we chose the oxazolone-induced contact dermatitis mouse model. The first phase of the experiment was sensitization with oxazolone, followed by elicitation, then measurement of ear edema and blood flow between treatments, and finally the termination phase. Samples were collected for histopathological examination, and the measurement of myeloperoxidase (MPO) enzyme activity and cytokine levels.

Results: Methyl-salicylate has been identified as the main constituent of wintergreen. Based on literature data, the minor components proved that our essential oil was distilled from *G. fragrantissima*. In our experiments, oxazolone significantly increased ear edema, blood flow, MPO activity and the measured pro-inflammatory cytokine levels in the ear compared to controls. *Nigella* fatty oil and *Gaultheria* essential oil alone reduced some oxazolone-induced changes, while a mixture of them significantly reduced all parameters.

1. Introduction

Allergic contact dermatitis is an inflammatory skin disease that causes redness and itching. Today, 20% of children worldwide are affected [1]. This hypersensitivity reaction can be triggered by many factors, e.g. plant substances such as nettles, organic substances or metals. The biggest problem is that there is currently no cure. Medication can only alleviate the symptoms and inflammation. Treatments with steroids are often applied, which can have a number of side effects in case of long-term use. Therefore, it was important for us to develop a natural treatment that is safe and effective. The aim of our study was to examine the effect of *Nigella sativa* fatty oil and *Gaulteria* essential oil in contact dermatitis *in vivo* mouse model. We supposed that *Nigella* fatty oil or *Gaultheria* essential oil will show anti-inflammatory effect due to the thymoquinone [2] or methyl-salicylate [3] content, respectively.

2. Material and Methods

In our study, we used the fatty oil of *Nigella sativa* L. and the essential oil of *Gaultheria fragrantissima* L. individually and in combination. An *in vivo* mouse model was used for our study. First, inflammation was induced with oxazolone. Oxazolone was administered to only one ear of the mouse and once the characteristic symptoms of inflammation appeared, we continued the experiment with the treatment of the fatty oil or the essential oil. The mice were divided into 4 groups: The control group, the *Nigella*-treated group, the *Gaultheria*-paraffin oil combination group, *Nigella-Gaultheria* combination group. One ear of the mouse was treated with ethanol and the other ear (where we induced the inflammation with oxazolone) was with the given sample, so that we could compare not only the results of the groups but also the two ears to each other. The measurements were carried out for a total of 72 hours. During this time, we measured the blood flow in the ear with a laser Doppler and the ear thickness or edema with a micrometer. On the 10th day of the experiment, we terminated the animals, and ear samples were taken for further histological and immunological examinations.

Two parameters were measured during the immunological examinations: myeloperoxidase (MPO) enzyme activity and the concentration of some cytokines. MPO is expressed by cells involved in inflammation and its level is proportional to the degree of cellular components of inflammation. MPO measurement was performed from ear samples with spectrophotometer (at 620 nm). In the experiment, the concentrations of five types of cytokines (IL-1β, IL-10, IL-4, TNF-a and IFN-γ) were measured using the Luminex multiplex immunoassay. These samples were pipetted onto a 96-well plate containing beads coated with five types of cytokine-specific antibodies and colour-coded with fluorescent dye. Luminex MagPix was used to measure the fluorescence intensity of the immune complex-binding beads.

3. Results

Ear thickness measurement showed that the combination of *Gaultheria* essential oil and *Nigella* fatty oil significantly reduced edema from the 48th hour onwards. Laser Doppler perfusion measurements explored that oxazolone increased perfusion compared to the ethanol control group (Figure 1). *Gaultheria* and *Nigella* alone did not reduce blood perfusion, whereas in combination they significantly reduced perfusion by the 24th hour of the experiment.

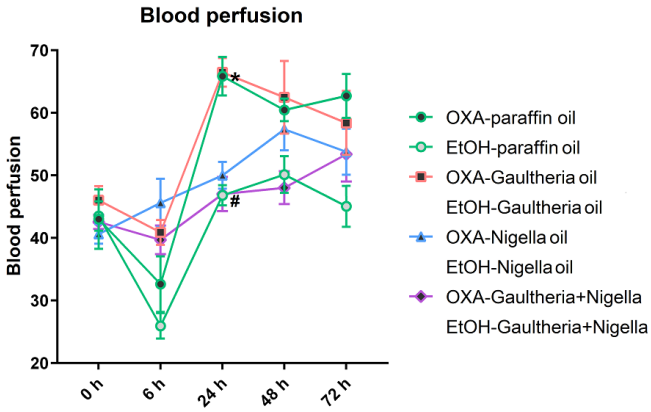

Figure 1. Blood perfusion in mouse ears measured with Laser Doppler. Two-way ANOVA + Tukey's test, n=5-6; *p<0,05 vs. respective EtOH-treated group; #p<0,05 vs. OXA-paraffin oil group

Oxazolone increased the activity of the MPO (Figure 2). In three groups, treatment with *Gaulteria* essential oil, and *Nigella* fatty oil and both of them, there was a significant decrease in MPO enzyme activity compared to our oxazolone control group.

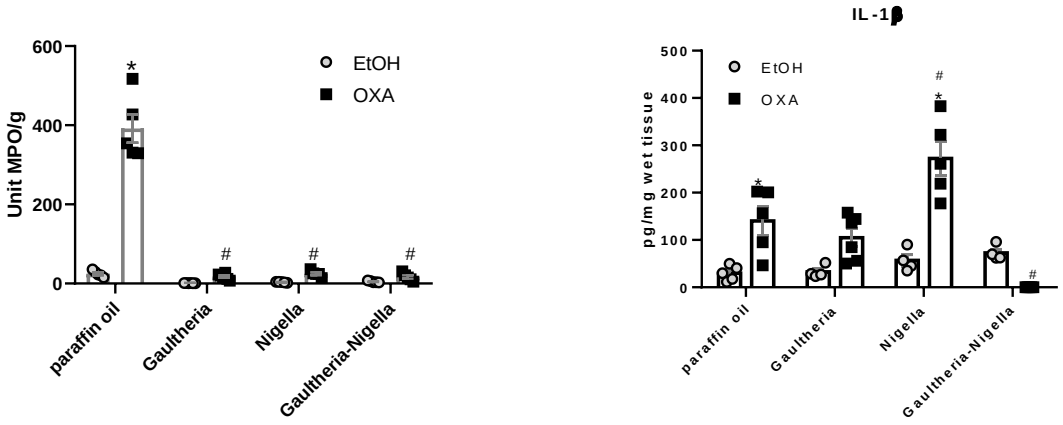

Figure 2. Myeloperoxidase activity. Two-way ANOVA + Tukey's test, n=5-6; *p<0,05 vs. respective EtOH-treated group; #p<0,05 vs. OXA-paraffin oil group and **Figure 3.** Cytokine level (IL-1β). Two-way ANOVA + Tukey's test, n=5-6; *p<0,05 vs. respective EtOH-treated group; #p<0,05 vs. OXA-paraffin oil group

The concentration of the pro-inflammatory cytokine interleukin-1β was measured. The concentration of IL-1β increased significantly in the *Nigella* treatment group compared to the oxazolone control group, while it reduced in the combination of *Nigella* and *Gaulteria* group (Figure 3).

Histopathological examination showed that the epidermis and dermis thickened with oxazolone compared to the ethanol control group. A comparison of the oxazolone-paraffin oil control group with the oxazolone group treated

with the combination showed that the combination caused fewer microabscesses and thus successfully reduced inflammation (Figure 4).

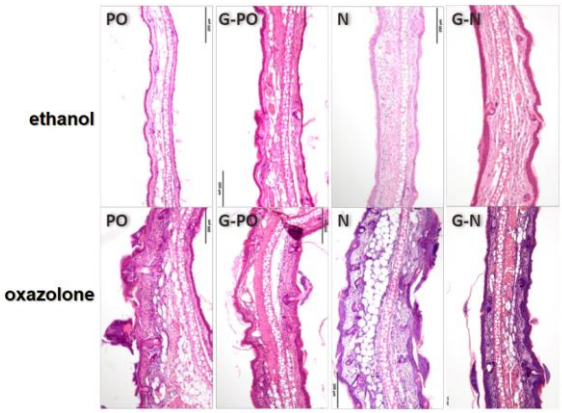

Figure 4. Histopathological examination. 100x magnification. From left to right: (PO) cross-sections of ears treated with paraffin oil, (G-PO) cross-sections of ears treated with *Gaultheria*-paraffin oil, (N) cross-sections of ears treated with *Nigella* fatty oil and (G-N) cross-sections of ears treated with *Gaultheria-Nigella*.

4. Discussion and Conclusions

In our experiment, the anti-inflammatory effect of *Nigella sativa* fatty oil and *Gaultheria fragrantissima* essential oil was measured in an *in vivo* contact dermatitis model. In summary, it should be mentioned that both plant extracts had an overall positive effect on the measured parameters of contact dermatitis. Furthermore, the combination of *Nigella sativa* fatty oil and *Gautheria fragrantissima* essential oil produced a significant reduction in most of the measured parameters (Table 1.) In contact dermatitis, the mixture of these two plant extract may be recommended to decrease the symptoms. A similar anti-inflammatory effect of *N. sativa* oil and thymoquinone was also reported by Aljambre (2015), although they did not investigate in contact dermatitis, but in psoriasis and acne vulgaris, among others [4] .

Table 1. Summary of the results

	blood perfusion	edema	MPO activity	IL-1β	histopathology
Nigella L.	↓	↓	↓#	↑#	↑
Gaultheria L.	↓	↓	↓#	↓	not obvious
combination	↓#	↓#	↓#	↓#	↓

means significance, p value: #p<0,05

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Conflict of Interest

The authors declare that there was no conflict of interest in this study.

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P-Y-11

Nanoencapsulated Coriandrum sativum essential oil as shelf-life enhancer of bananas

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Keywords: Essential oils, Nanoencapsulation, Antifungal, Antiaflatoxigenic, Preservative, Banana

Objective

The study aimed to synthesize *Coriandrum sativum* essential oil (CSEO) infused chitosan nanoemulsion (Ne-CSEO) and its practical applicability as novel green preservative to enhance the shelf-life of bananas from post-harvest fungal and aflatoxin B_1 contamination.

Methods

Coriandrum sativum seeds from Varanasi, India were subjected to hydrodistillation for essential oil extraction followed by GC-MS analysis. Nanoemulsion was prepared using chitosan via ionic gelation technique. Ne-CSEO was characterized using SEM, AFM, XRD, and FTIR. The antifungal and antiflatoxigenic assay was performed using food poisoning technique. *In silico* study was performed using Autodock Vina and the practical applicability of Ne-CSEO as edible coating on Banana was studied based on quality characteristics such as weight loss, total soluble solids, pH, titrable acidity, phenolic content, and sensorial attributes.

Results

Chemical characterization of CSEO revealed the presence of 35 components. Successful loading of CSEO into chitosan nanoemulsion was confirmed through SEM, AFM, XRD and FTIR. Ne-CSEO showed enhanced *in vitro* antifungal (0.6 µL/mL), and antiaflatoxigenic (0.5 µL/mL) activity over CSEO. The antifungal and antiaflatoxigenic mechanism of action of Ne-CSEO was studied in terms of leakage of cellular contents, inhibition of ergosterol biosynthesis, and impairment in cellular methylglyoxal biosynthesis. *In silico* studies validated interaction of linalool (major component) with Ver-1 and Omt-A proteins, confirming inhibition of AFB_1 production. Moreover, coating of Ne-CSEO on Banana significantly improved the shelf-life of stored Bananas.

Conclusions

Based on potential antifungal, and antiaflatoxigenic activity, Ne-CSEO may be recommended as, nano-smart, plant-based preservative to enhance the shelf life of stored Bananas.

P-Y-12

Bifora testiculata (L.) Spreng. essential oil and its principal constituent as anticancer agent

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Keywords: Apiaceae; *Bifora testiculata* (L.) Spreng.; aldehydes; *trans*-2-dodecenal; essential oil; anticancer activity

Abstract

Bifora testiculata (L.) Spreng., belonging to the Apiaceae family, is a species that grows in Europe, mainly in the Mediterranean regions and in Saudi Arabia. The history of *Bifora* genera application in traditional medicine highlights its various biological properties. Trying to explore the phytochemistry and pharmacological aspects of this species, the essential oil (EO) extracted from aerial parts (flowers, leaves and stems) of a locally wild accession, never previously investigated, growing in Sicily, Italy, was investigated. The chemical composition of EO, obtained by the hydrodistillation method, was evaluated by GC-MS. Aldehydes represent the principal class (86.1%) with *trans*-2-dodecenal (67.49%) as major metabolite. This EO, and its main component, were tested for their possible anticancer activity against human breast adenocarcinoma cell line (MDA-MB 231), human malignant melanoma cell line (A375) and human colon adenocarcinoma cell line (CaCo2). After 72h of incubation, the EO showed quiet good results exerting a remarkable cytotoxic effect, accompanied by morphological changes represented by cell shrinkage, but the best results were obtained by *trans*-2-dodecenal, and in particular against human breast cancer cell lines MDA-MB 231, showing an IC_{50} value of 2.66 μg/ml. The high percentage of this active compound in *Bifora testiculata* EO suggests it as a potential source of anticancer drugs.

1. Introduction

Bifora is a cosmopolitan genus of flowering plants in the Apiaceae family, belonging to the Tribe Coriandreae [1], characterized by a disjoint distribution and comprising 3 species: two Eurasian (*Bifora radians* M. Bieb. and *Bifora testiculata* (L.) Spreng.) and one American (*Bifora americana* (DC.) A. Gray) [2].

Bifora radians, the first species, has been tradionally used, as spice added to meal since ancient time [3], and is now recognized for its broad spectrum of biological activities. Extacts from various aerial parts have been investigated for their antioxidant activity, total phenolic content [4], as well as their antimicrobial properties against *Escherichia coli, Pseudomonas aeruginosa, bacillus subtilis Staphylococcus auerus, Candida albicans* [5]. Leaf extract of *B. radians* are also noted for their high insectidicial activity against pests such as the milkweed bug, mosquitoes, Colorado potato beetle, and grape berry moths [6,7,8].

Regarding the essential oils (EO) of *B. radians*, known for their high aldehyde content [9,10], studies have demonstrated their antibacterial effects against *Paenibacillus* larvae and insecticidal properties against adult turnip aphids [11,12].

No informations are reported about the chemical composition and biological acticity of the other two species within the genus (*B. americana* and *B. testiculata*), except for a paper by Evergetis [13], which reported the EO chemical composition of greek accession of *B. testiculata,* suggesting it as a potential resources of Fine Chemicals (FC), in particular aldehydes.

Given the extensive biological activity of *Bifora radians* and the dearth of knowledge regarding the other two species, we undertook an investigation into the essential oil chemical composition and antitumor activity of the Sicilian (Italy) accession of *Bifora testiculata* against three different cancer cell lines, along with an analysis of its main compound.

2. Material and Methods

2.1 Plant material

The flowering aerial parts (flowers, stems, leaves) of *B. testiculata* were collected on sandy substrates in Villalba, Caltanissetta, Italy, (37°38'41.6"N 13°50'16.7"E 700 m s/l) in May 2023.

2.2 Essential Oil Extraction

The fresh sample was ground in a Waring blender and then subjected to hydrodistillation for 3 h. The essential oil dried over anhydrous sodium sulphate, was stored in a sealed vial under N_2 at -20°C, ready for GC-MS analyses. The essential oil yielded 0.20% (w/w).

2.3 GC-MS analysis

GC-MS analysis of essential oil was performed according to the procedure reported by Porrello et al. [14]. Linear retention indices (LRIs) were calculated using a mixture of pure *n*-alkanes (C8–C40), and all the peaks' compounds were identified by comparison with MS and by comparison of their relative retention indices with WILEY275, NIST 17, ADAMS, and FFNSC2 libraries.

2.4 Pure Compound

trans-2-Dodecenal was purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Eschenstr. 5, 82024 Taufkirchen, Germany).

3. Results

Bifora testiculata EO extracted from aerial parts (flowers, stems, leaves) had a yellow straw colour. Overall, twenty-six compounds were identified, representing the 91.81% of the total composition.

The main class, representing almost all the EO, turned out to be that of aldehydes (86.10%) with *trans*-2-dodecenal (67.49%) as major compound. Other aldehydes in a large amount were found to be *cis*-2-decenal (3.25%), *trans*4-undecenal (3.38%), dodecanal (4.50%), and *cis*-2-dodecenal (2.91%).

The EO and its main compound *trans*-2-dodecenal have been tested against human breast adenocarcinoma cell line (MDA-MB 231), human malignant melanoma cell line (A375) and human colon adenocarcinoma cell line (CaCo2). IC_{50} values are listed in the Table below.

Table 1. *In vitro cytotoxic activity of B. testiculata essential oil and trans-2-dodecenal.*

	Cell line (IC_{50} µg/ml)[a]		
	MDA-MB 231[b]	**A375[c]**	**CaCo2[d]**
***B. testiculata* EO**	10.46	7.93	14.41
***trans*-2-Dodecenal**	2.66	5.29	4.74
Cisplatin	3.45	0.55	3.28

[a] IC_{50} = The concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation).
[b] Human breast adenocarcinoma cell line. [c] Human malignant melanoma cell line. [d] Human colon adenocarcinoma cell line.

Discussion and Conclusions

The chemical composition of the essential oil (EO) from the Sicilian accession of *Bifora testiculata* is reported here for the first time. Its primary compound, *trans*-2-dodecenal constitutes 67.49% of the oil, distinguishing it from the EOs of *B. radians*, predominantly rich in *trans*-2-tridecenal (ranging from 47-66%) and *trans*-2tetradecenal (14-23%) [9,10].

Interestingly, the EO from the Greek accession of *B. testiculata*, while also featuring longer-chain aldehydes, shares similarities with ours, with *trans*-2-dodecenal as the main compound, albeit in a lower concentration (56.3%). This observation suggests the superiority of the Sicilian accession over the Greek one in terms of fine chemical production potential.

trans-2-Dodecenal, the primary compound in our EO, is also found in other Apiaceae EOs, such as *Eryngium foetidum* [15], and has previously been investigated with their EOs, for its antitumoral properties, showing

promising results.

Motivated by this, we decided to assess both the EO and *trans*-2-dodecenal, against three different cancer cell lines. While the EO exhibited promising results, *trans*-2-dodecenal outperformed it, particularly against the human breast adenocarcinoma cell line (MDA-MB 231), with an IC_{50} value of 2.66 µg/ml, surpassing the positive control with cisplatin.

These findings highlight the potential of Sicilian *Bifora testiculata* EO as a source of novel anticancer agents.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-Y-13

Valorization of ginger leaf and peel waste into essential oils as natural grain protectants against major stored product insects

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Keywords: essential oils, contact toxicity, *Zingiber officinale*, rice weevil, red flour beetle

Objective

Post-harvest losses from insect infestation during storage prompt the emergence of essential oils (EOs) as viable alternatives. Ginger (*Zingiber officinale* Roscoe), a rhizomatous medicinal herb, holds untapped potential in its often-discarded leaves and peel. The study aimed to determine the chemical composition of ginger EOs extracted from leaf and peel waste from India and to evaluate its bioefficacy against the major stored grain pests, *Tribolium castaneum* (Herbst), and *Sitophilus oryzae* (L.).

Methods

Ginger leaves and peels EO were extracted through hydrodistillation. The EOs were then analyzed using gas chromatography-mass spectrometry (GC-MS). Contact, fumigant, repellent and phytotoxicity assays were conducted to assess the insecticidal potential.

Results

The ginger leaves and peel waste yielded 0.13 ± 0.18% and 0.59 ± 0.31% (v/w) oil, respectively. GC-MS analysis revealed prominent compounds such as caryophyllene oxide and caryophyllene in ginger leaf EO, and zingiberene and β-sesquiphellandrene in ginger peel EO. The bio-efficacy assessment demonstrated significant repellent, contact (at 24h, for *T. castaneum*: LC_{50}= 20.26 (leaf) and 14.93 mg/cm^2 (peel); for *S. oryzae*: LC_{50}= 11.8 (leaf) and 4.14 mg/cm^2 (peel)) and fumigant (at 24h, *T. castaneum*: LC_{50}= 17.4 (leaf) and 5.32 mg/L air (peel); *S. oryzae*: LC_{50}= 9.34 (leaf) and 7.23 mg/L air (peel)) toxicities of ginger leaf and peel EOs against the test insects. Phytotoxicity testing on paddy seeds revealed no adverse effects on germination or seedling growth, affirming the safety of these EOs.

Conclusion

This investigation underscores the potential valorization of ginger leaf and peel waste EOs as biopesticides against stored grain pests.

P-Y-14

Phytochemical profiling and bioactivity assessment of volatile components of *Nectaroscordum tripedale* Trautv. from Iran: Antioxidant, antimicrobial, and cytotoxic investigations

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Keywords: Essential oil, *Allium tripedale, Nectaroscordum tripedale*, Antimicrobial, Cytotoxic evaluation, Antioxidant

Abstract

Objective: This study aims to investigate the chemical composition of volatile constituents in the aerial parts of *Nectaroscordum tripedale* (Trautv.) Grossh., a plant belonging to the Liliaceae family, mainly found in semi-arid regions in Europe, North America, North Africa and Asia. In addition, it evaluates the antioxidant, antimicrobial, and cytotoxic activities of these constituents to explore their potential for modern medical applications. Methods: The volatile components were isolated using simultaneous distillation extraction (SDE) and analyzed through GC-Mass-FID for identification and quantification. The study included DPPH radical scavenging, hydrogen peroxide scavenging, reducing power determination, and β-carotene-linoleic acid bleaching assays to assess antioxidant activity. Antimicrobial activity against *E. coli*, *S. aureus*, *S. epidermidis*, and *S. dysenteriae was evaluated.* Cytotoxicity was determined using the brine shrimp lethality assay. Results: The analysis identified 44 volatile constituents, with trans-Anethol (22.06%), 5-butyl-2,3-dimethyl-Thiophene (15.55%), 1-Butyl thienothiine (10.56%), dimethyl trisulfide (6.20%), 6-hydroxy-2-methyl-4H-1-Benzopyran-4-one (4.39%), and dibutyl phthalate (4.07%) as the main components. The antioxidant assays demonstrated variable activities. The essence showed significant antimicrobial activity and the plant showed low cytotoxicity. Conclusion: *N. tripedale* exhibits a rich composition of volatile constituents with significant antimicrobial, limited antioxidant capacity and low cytotoxic activities, suggesting its potential for further research in pharmaceutical applications. The study underscores the importance of exploring the flora of the semi-arid region for beneficial bioactive compounds in modern medicine.

1. Introduction

The study of essential oils of plants has long been a cornerstone of pharmaceutical and nutritional science, offering insight into the potential of natural products to contribute to human health [1]. *Nectaroscordum tripedale* (Trautv.), also known as *Allium tripedale* Trautv., belonging to the Liliaceae family, is characterized by its robust growth, featuring thick stems ranging from 50 to 150 cm in length, crowned with umbel inflorescences. This plant species is part of the *Allium* genus, which encompasses more than 700 species distributed in semi-arid regions of Asia, North Africa, Europe, and North America. In particular, the genus includes well-recognized medicinal crops such as garlic (*Allium sativum* L.) and onion (*Allium cepa* L.), known for their antibacterial and antimicrobial properties [2]. Within this genus, the subgenus *Nectaroscordum*, considered a distinct genus, includes species mainly appreciated for their decorative appeal in western cultures due to their distinctive inflorescences. These plants, particularly prevalent in the mountainous terrains of northwest Iran and surrounding places, are harvested in spring. *N. tripedale* emits a potent aroma upon crushing, noted for its disagreeable smell and mild eye irritation, with a distinctly spicy taste[3], [4].

This study aims to examine the chemical profile of volatile compounds present in *N. tripedale*, assessing their antioxidant, antimicrobial and cytotoxic potentials. By employing advanced analytical techniques, this research not only identifies key volatile components but also evaluates the antioxidant, antimicrobial, and cytotoxic activities of the plant. Through this investigation, we hope to shed light on the scientific basis behind the traditional uses of this plant and explore its potential as a source of novel bioactive compounds.

2. Materials and Methods

Chemicals such as DPPH, β-carotene, linoleic acid, BHT, and gallic acid were sourced from Sigma-Aldrich, Germany, with other chemicals and culture media from Merck, Germany. The microbial strains were acquired from the Iranian Research Organization for Science and Technology. The brine shrimp larvae (*Artemia salina*) were obtained from Advanced Hatchery Technology, USA. Double-distilled water was utilized throughout the experiments. The aerial parts and bulbs of *N. tripedale* were harvested from the Kabirkooh mountain, Zagros Mountains, Iran, during spring, identified by Dr. Hossein Batooli. They were dried, ground, and stored refrigerated. Volatile components were isolated using Simultaneous Distillation-Extraction (SDE) with n-Pentane. The methanol extracts of the plant parts were prepared by Soxhlet extraction.

Volatile compounds were analyzed using GC-Mass-FID on an Agilent HP-6890 gas chromatograph. Antioxidant activities were evaluated by DPPH radical scavenging, reduction power, and β-carotene/linoleic acid assays. Cytotoxicity was evaluated using a brine shrimp lethality assay. Antimicrobial activity against a panel of 12 microorganisms was tested using disc diffusion and microwell dilution assays to determine sensitivity and minimal inhibitory concentrations (MIC) [5], [6].

3. Results

The chemical analysis of the volatile *N. tripedale* revealed 44 constituents, which comprise 98.09% of the samples, with trans-Anethol (22.06%), 5-butyl-2,3-dimethyl-Thiophene (15.55%), 1-Butylthienothiine (10.56%) and dimethyl trisulfide (6.20%) as the primary components. Antioxidant activity evaluations demonstrated weak action in β-carotene/linoleic acid bleaching assays, with less than 40% inhibition, and negligible activity in other assays, suggesting a limited antioxidant capacity possibly due to low phenolic content. Cytotoxic activity against brine shrimp highlighted LC_{50} values greater than 400 μg/ml for aerial parts and greater than 105 μg/ml for bulbs, indicating low cytotoxicity. Antimicrobial evaluations showed aerial volatile components exhibiting effective activity against strains such as *E. coli*, *S. aureus*, *S. epidermidis*, and *S. dysenteriae* (Table 1).

Table 1. Antimicrobial activity of the aerial parts of N. tripedale

			Antibiotics					
	Aerial part		Rifampin		Gentamicin		Nystatin	
Microorganisms	DD[a]	MIC[b]	DD	MIC	DD	MIC	DD	MIC
P. aeruginosa ATCC 27853	-	-	-	-	8	0.50	NA	NA
B. subtilis ATCC 6633	-	-	13	0.125	21	0.50	NA	NA
E. coli ATCC 10536	9	2000	11	0.50	21	0.50	NA	NA
S. aureus ATCC 29737	10	2000<	10	0.250	21	0.50	NA	NA
K. pneumoniae ATCC 10031	-	-	7	0.250	22	0.25	NA	NA
S. epidermidis ATCC 12228	10	500	40	0.250	35	0.50	NA	NA
S. dysenteriae PTCC 1188	9	1000	8	0.250	18	0.50	NA	NA
P. vulgaris PTCC 1182	-	-	10	0.125	23	0.50	NA	NA
S. paratyphi-A serotype ATCC 5702	-	-	-	-	21	0.50	NA	NA
C. albicans ATCC 10231	-	-	NA	NA	NA	NA	33	0.125
A. niger ATCC 16404	-	-	NA	NA	NA	NA	27	31.2
A. brasiliensis PTCC 5011	-	-	NA	NA	NA	NA	30	31.2

(-) indicate no antimicrobial activity.

[a] Inhibition zone in diameter (mm) around the impregnated discs.

[b] Minimal inhibition concentrations (as mg/ml).

[c] NA (not applicable).

4. Discussion and Conclusions

The chemical profile of *N. tripedale* revealed a diverse composition, but the plant demonstrated limited antioxidant and cytotoxic activities. These findings are intriguing, considering that some reports suggest potential anticancer benefits, which needs further exploration. The weak antioxidant activity could be attributed to the low phenolic content, which is often linked to such biological effects. However, the significant antimicrobial activity of the aerial parts indicates the presence of compounds that may be useful against certain bacterial strains.

This study serves as a preliminary investigation into the chemical and biological properties of *N. tripedale*, a plant with scarcely reported potentials [7], [8]. Despite the limited antioxidant and cytotoxic activities observed, the antimicrobial efficacy suggests promising results for further research, particularly in exploring the plant's use against specific microbial infections. The discovery of significant antimicrobial agents highlights the potential utility of the plant in medical applications. More detailed studies are necessary to fully understand the scope of *N. tripedale's* bioactive compounds and their mechanisms, which could provide valuable insights into their pharmacological potential and support their use in modern medicine. This work lays the groundwork for future investigations aimed at uncovering the full spectrum of *the* biological activities of N. tripedale and potential

therapeutic applications.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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POSTER ABSTRACTS

P-15

Aroma profile of fresh *R.alba* L. blossom by solid-phase microextraction and head space gas chromatography

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Keywords*: Rosa alba* L., solid-phase microextraction, GC/MS, aroma profile, volatile organic compounds

Introduction

The white oil-bearing rose (*R. alba* L.) is the second most important plant for Bulgarian rose cultivation and essential oil production. In recent year, there is a revival in the interest in white oil-bearing rose, in line with with the worldwide tendencies for searching new aromatic alternatives [1].
The purpose of the current research is to evaluate the aroma profile of fresh *R .alba* L. flowers using head space solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS).

Materials and methods

Fresh flowers of *R. alba* L., harvested early in the morning during the most suitable phases of development - semi - to full open was used as raw material. The population consists of four clones, which differ mainly in the number of petals and the color tint, fig. 1. In current study two clones were used.

The analysis was performed be means of HS-SPME-GC/MS, which allows efficiently to extract and concentrate aromatic compounds. Semi-polar divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibres were used for extracting the volatiles.

Figure 1. Variations of colour tint and number of petals in the *R. alba* population.

Results

More than 75 individual compounds were identified and quantified in the fresh *R.alba* L. flowers using HS-SPME-GC/MS. The study revealed that the aroma-bearing fraction of rose volatiles consists mainly of monoterpene alcohols: geraniol (17.5-35.9%) and citronellol+nerol (18.4-27.2%), followed by 2-phenylethanol (11.5÷27.1%). Linalool, α-pinene, β-myrcene, rose oxides were also observed in low concentrations. The stearopten fraction in the HS phase logically was observed in low concentration, with main representatives nonadecane+nonadecene, heptadecane, heneicosane and tricosane.

Conclusion

HS-SPME-GC/MS offers quick and easy approach for flavor analysis without using solvents and expensive chemicals The HS-GC profile of the *R. alba* fresh flowers shows distinct differences between two studied clones of the population, as well as between volatiles in petals and in the whole flower.

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P-16

Volatile organic compounds in traditional Bulgarian red wines - development of fast and easy analytical method using solid-phase microextraction and head space gas chromatography

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Keywords*: red wine*, solid-phase microextraction, GC/MS, volatile organic compounds

Objective

Wine is a complex mixture of several hundred compounds, many of them found at very low concentrations, however, they play an important role in its quality and sensorial properties. The average concentration of volatile organic compounds (VOCs) is 0.5%, which makes their analysis a challenging task. The content of VOCs in wine depends on the varietal characteristics, viticulturally practices and wine-making technology.

The aim of this study was to develop sensitive and reproducible procedure using headspace solid-phase micro extraction (HS-SPME) combined with gas chromatography/mass spectrometry (GC/MS) for fast profiling of the free volatile organic compounds (VOCs) in Bulgarian red wines.

Materials and methods

A semi-quantitative SPME-HS-GC/MS method has been developed for the red wine aroma profile analysis. Semi-polar divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibres were used for extracting the free wine VOCs.

More than 100 commercial wine samples from traditional Bulgarian grape varieties (*Mavrud, Shiroka Melnishka Loza, Gamza, Misket Cherven, Dimyat, etc.)* were collected ad studied by means of SPME-HS-GC/MS.

Results

More than 50 individual free volatile and semi-volatile compounds were identified, with the representatives of terpenes, esters, alcohols, fatty acids, sulphur compounds, etc. The study revealed that 3-methyl, 1-butanol was the most abundant component, ranging from 55.23% - 28.76%, followed by octanoic acid, ethyl ester (26.34% - 7.52%), phenylethyl alcohol (18,61% - 8,04%) and butanedioic acid, diethyl ester (7.34% - 4.71%). The best HS-SPME conditions were as follows: conditioning time 15 min, conditioning temperature 45ºC and extraction time 45 min, with addition of 4 grams NaCl. All the samples were analysed in triplicates, with St Dev <5%.

Conclusion

The developed easy and solvent-less HS-SPME-GC/MS method is suitable for fast, sensitive and reproducible analysis of VOCs and can be used for comparative aroma profiling of Bulgarian red wines as a part of the BG Wine database.

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P-17

Quality of the essential oil from two hop varieties introduced in Rio de Janeiro, Brazil

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Keywords: Comet, Triumph, myrcene, gas chromatography, *Humulus lupulus*.

Abstract

The objective of this work was to compare the essential oil yield and quality from two hops (*Humulus lupulus* L., Cannabaceae) varieties, namely Comet and Triumph cultivated in the mountain area of the state of Rio de Janeiro. The samples consisted of dry cones, obtained with local producers. The essential oils were obtained by hydrodistillation in a Clevenger apparatus. Residual humidity content was determined by azeotropic distillation with a Dean-Stark apparatus. Oil composition was evaluated by gas chromatography with flame ionization and mass spectrometry detectors. For quantitation, four replicates were distilled and analyzed after the addition of an internal standards. Identification was based in mass spectra comparison with commercial libraries and calculation of linear retention indices. For the samples tested, the Comet variety yielded 2 times more essential oil, with 4.1%, while the Triumph yield was only 2.0% (dried basis). Comet oil was rich in myrcene (64.9%) and β-caryophyllene (8.3%). The major compounds in Triumph oil were α-humulene (41.7%), myrcene (28.5%) and β-caryophyllene (11.5%). While the data from Triumph are in good agreement with literature, a higher yield for the Comet variety was recorded. For both varieties, it was verified a good adaptation of the hops to the mountain area near Rio de Janeiro.

1. Introduction

One of the 4 obligatory ingredients in beer, hop (*Humulus lupulus* L., family Cannabaceae) is a species originary from the northern hemisphere, with occurrence in Europe, North America, and Asia [1]. Although some introduction attempts have been made in Brazil during the last quarter of the 19th century, only recently this culture has reached commercial success. Several varieties have been introduced in the South of the country, as well as in the mountain areas of the states of São Paulo and Rio de Janeiro. Following the boom in the market of artisanal breweries, many local producers started cultivation of new varieties. Herein, we report the results on the quality assessment of the essential oil from two hop varieties, Comet and Triumph, cultivated in the city of Petrópolis, a mountain area near Rio de Janeiro.

2. Material and Methods

All solvents used were from HPLC grade (Tedia Brazil). The dried hop samples (*Humulus lupulus* L. var. Comet and *H. lupulus* L. var. Triumph), crop 2023, were kindly provided by a producer from the city of Petrópolis, RJ (S 22°25' 36.5", W 43°17'2.5"). Each sample (100 g) was distilled in a Clevenger-type apparatus according to the Brazilian Pharmacopoeia [2] (the same as in the European Pharmacopoeia). Oil yield was expressed in volume by weight and are expressed in dried basis. A 0.1 % solution of the oil in hexane and methyl octanoate as added as internal standard, and 1.0 µL was injected in split mode (1:50). Oils were analyzed in an Agilent 7890B gas chromatograph (GC) fitted with flame ionization detector (FID) and using a DB-5 column (30 m x 0.25 mm x 0.25 µm), with hydrogen as carrier gas (1.5 mL/min). Oven temperature was programmed from 60 to 240 °C, at 3 °C/min. The injector was operated at 250°C and the detector at 280 °C. For quantitation, predictive response factors were used. All calculations were performed using a series of Excel® pre-programmed electronic sheets [3]. The samples were also injected in an Agilent 5975C mass selective detector system, using the same column and conditions as stated for GC-FID. Helium was used as carrier gas (1.0 ml/min). Ionization energy was 70 eV, at 3.15 scans/s, from 40 to 350 u. Ion source was kept at 200 °C, mass analyzer at 150 °C and transfer line at 260 °C. For compound identification, mass spectra were compared to data from commercial libraries, and linear retention indices were calculated [4].

3. Results

The oil yield (dried basis) was 4.1 for the Comet and 2.0% for the Triumph varieties. Main constituents for the

Comet oil were myrcene (64.9%), β-caryophyllene (8.3%), α-selinene (3.7%), β-selinene (3.5%), and (*E*)-β-ocimene (1.8%). For the oil from the Triumph variety, α-humulene was the main constituent (41.7%), followed by myrcene (28.5%), β-caryophyllene (11.5%), δ-cadinene (2.1%), and humulene epoxide II (1.5%).

4. Discussion and Conclusions

The oil yield for the Triumph is slightly above the average, but its chemical profile is within the reported data for this variety [5]. The composition of the Comet variety was characteristic, with a high content of myrcene and low content of α-humulene. The yield, however, was twice (4.1%) the average for the variety. These results are quite interesting and stimulating for the hop producers in the mountain areas from the state of Rio de Janeiro, as they point out to a good adaptation of both varieties to this production area.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-18

Combination of thyme, tea tree essential oils and antibiotics against MRSA and *Pseudomonas aeruginosa*

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Keywords: Essential oil, checkerboard titration, MRSA, *Pseudomonas aeruginosa*, antibiotics, synergy

Abstract

Essential oils (EOs) are complex plant extracts that have antimicrobial activity, which makes them a good alternative to antibiotic treatment. Previous researches have demonstrated synergistic effect of certain antibiotic-EO combinations. Thyme and tea tree EOs are important antimicrobial oils and biofilm inhibitors. In our research, thyme (*Thymus vulgaris* L.) and tea tree (*Melaleuca alternifolia* [(Maiden & Betche) Cheel] EOs were combined with antibiotics (gentamicin, vancomycin) and tested on nosocomial bacteria such as *Pseudomonas aeruginosa* (ATCC 27853) and methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 700698). The composition of the EOs was determined by GC-MS, and the minimum inhibitory concentrations (MICs) of the EOs and antibiotics were determined by microdilution method. The antibacterial activity of the EO-EO and EO-antibiotic combination was measured by *in vitro* checkerboard method. The main component of thyme EO was thymol (53.97%), while that of tea tree EO was terpinen-4-ol (38.40%). In the combination study, the fractional inhibitory concentration index (FICI) values showed that thyme EO has a synergistic effect with gentamicin, whereas the combination of tea tree EO and gentamicin and the two EOs with each other showed an additive effect on *P. aeruginosa*. In case of MRSA, the combination of thyme EO and vancomycin showed an additive effect, while the combination of tea tree EO and vancomycin and the two EOs with each other showed synergistic effects. Our results confirm the effectiveness of the combination of the tested EOs and antibiotics against MRSA and *P. aeruginosa*.

1. Introduction

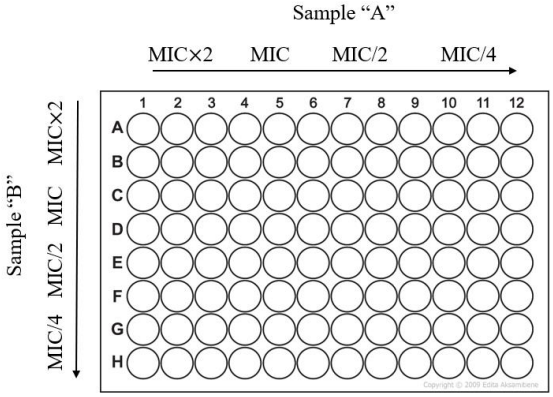
Antibiotic resistance is one of the major public health challenges of the 21st century [1]. Several multi-resistant pathogens have been observed among the strains causing nosocomial infections. The number of effective antibiotics is limited and it is important to explore new alternatives. EOs have antibacterial activity. Some of them are able to inhibit bacterial proliferation (bacteriostatic effect) or to kill the microorganism (bactericidal effect) [2]. The antimicrobial effect of EOs is mainly due to their volatile components (phenols, alcohols, aldehydes, ketones, esters, etc.). These components can have antibacterial activity on their own, but recent researches have shown that their activity is the result of complex interactions between their individual components [2,3]. Some EOs and antibiotic combinations have synergistic effects as well [4]. The aim of our study was to investigate the combination of thyme, tea tree EOs and gantibiotics (gentamicin, vancomycin) against MRSA and *Pseudomonas aeruginosa*.

2. Material and Methods

In our research, the EO of thyme (*Thymus vulgaris* L.) and tea tree (*Melaleuca alternifolia* [(Maiden & Betche) Cheel] was involved. First, gas chromatography-mass spectrometry (GC-MS) analysis was performed. Subsequently, the minimum inhibitory concentration (MIC) of the EOs was determined by microdilution method. Afterwards, the combination effect of EOs and antibiotics was determined by a checkerboard titration method. 96-well microtiter plates were used (Figure 1), and 6 parallel measurements were performed. All assays were made in two configurations: in the case of *Pseudomonas aeruginosa* (ATCC 27853), the EOs were combined with gentamicin, and in the case of methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 700698), the EO samples were combined with vancomycin. The EOs were also combined with each other in a similar experimental setup. The bacteria were cultured in Brain Heart Infusion (BHI) medium with a bacterial count of 10^5 CFU/ml. Because of EOs hydrophobic properties, emulsions were prepared using 1% Tween40 and BHI medium. Both positive and negative controls were used in the assay. The treated samples served as positive controls. BHI solution

was the negative control. In order to exclude the effect of the emulsifier, a Tween40 control was also applied. The 96-well microtiter plates were incubated at 37°C for 24 hours and then absorbance was measured at 600 nm. From the absorbance values, MIC in combination values were calculated. FICI was calculated based on the formula of Figure 2.

Figure 1. The combination of EOs and antibiotics. Sample "A": thyme or tea tree EO (in EO combination: thyme EO); Sample "B": gentamicin or vancomycin (in EO combination: tea tree EO) [8].



$$FICI = \frac{MIC_A \text{ in combination}}{MIC_A \text{ alone}} + \frac{MIC_B \text{ in combination}}{MIC_B \text{ alone}}$$

Figure 2. Calculation of FICI value [8].

3. Results

The main component of thyme EO was thymol (53.97%) and that of tea tree EO was terpinen-4-ol (38.40%). The combination results are shown in Table 1.

Table 1 FICI values of the samples and the results of the combinations.

Pathogens	Samples	FICI	Result of Combination
P. aeruginosa	Thyme EO / Gentamicin	0.372	Synergy
	Tea tree EO / Gentamicin	0.75	Additive
	Thyme EO / Tea tree EO	0.62	Additive
MRSA	Thyme EO / Vancomycin	0.75	Additive
	Tea tree EO / Vancomycin	0.5	Synergy
	Thyme EO / Tea tree EO	0.47	Synergy

FICI≤0.5: synergy; FICI: 0.5-4: additive; FICI<4: antagonist [5].

Our results have revealed that the combination of EOs used in our experiment showed synergistic or additive effect. There was synergy based on FICI in the case of thyme and tea tree EOs combination. The FICI values showed synergistic effect in three cases: *P. aeruginosa*: thyme EO-gentamicin, MRSA: tea tree EO-vancomycin, and EOs together.

4. Discussion and Conclusions

In our experiment, the combinations of thyme and tea tree EOs and antibiotics were examined. The studies of the

last two decades also detected synergistic effects betwen EOs and antibiotics [4,6]. A previous study found an additive/antagonistic (FICI>0.5) effect between tea tree EO and vancomycin against *S. aureus* [7], but our results, in contrast, confirmed synergistic effect (FICI=0.5). Our study explored synergistic or additive effects when thyme or tea tree oils were combined with gentamicin or vancomycin. This result may support the reduction of antibiotic concentrations to prevent the spread of antibiotic-resistance. In the future experiments, our results will be used for products development, when patients' wound care is in the focus.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-19

Composition of the essential oil from the needles and twigs of organic dwarf pine (*Pinus mugo* Turra) from Tyrol

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Keywords: Pine, Pinus mugo, Pinaceae, Tyrol, Essential oil composition

Objective

The aim of this study was to assess the chemical composition of the essential oil of organic dwarf pine (*Pinus mugo* Turra) from Tyrol, a region encompassing South-West Austria and Northern Italy. *Pinus mugo*, also known as Mountain Pine, is a shrub with a height of up to 3.5 meters that grows spontaneously in the mountainous regions of Central Europe and the Carpathians at altitudes ranging from 1300 to 2200 meters. The essential oil is obtained through steam distillation of the crushed needles and branches and has a very pleasant pine-type odour, with balsamic-sweet, slightly woody and spicy nuances and an undertone of great tenacity.

In this study, 33 industrial batches of *Pinus mugo* essential oil have been analysed, spanning the years from 2020 to 2024.

Methods

The batches of *Pinus mugo* essential oil were produced by steam distillation for 6 hours (using a 5 m^3 still) to 10 hours (using a 10 m^3 still). The yield was 0.2 to 0.35% of a colourless to pale yellow essential oil. The composition was determined by GC-MS and dual channel GC-FID. Additionally, the enantiomeric distribution of selected constituents was evaluated using enantio-GC with a chiral cyclodextrin-based stationary phase.

Results

The essential oil of *Pinus mugo* mainly comprised monoterpene hydrocarbons, with α-pinene (11–20%), β-pinene (4.5–8.4%), myrcene (5.8–12.8%), δ-3-carene (19–39%), limonene (3.6–9.6%), and β-phellandrene (11–17%) as the major constituents. Chiral analysis indicated that the levorotatory enantiomer predominated in each case.

Conclusions

The primary constituent of *Pinus mugo* essential oil is δ-3-carene, a compound typically found in minor concentrations or entirely absent in essential oils from other *Pinus* species. This also applies to bornyl acetate (up to 1.9%). Another characteristic constituent is ß-phellandrene, which has been found in similarly high concentrations only in the essential oil from *Pinus cembra*.

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P-20

Studies on the composition of floral scent and essential oils of soapwort (*Saponaria officinalis*), growing in the wild in Lithuania, and the antioxidant activity of these oils and alcoholic extracts.

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Keywords: *Saponaria officinalis* L., essential oil, chemical composition, GC-MS, antioxidant activity

Abstract

The analysis of volatile organic compounds (VOCs) emitted by the flowers of soapwort (*Saponaria officinalis*) and the components of their essential oils, extracted by hydrodistillation, was performed using gas chromatography-mass spectrometry (GC-MS). The smell of the flowers was largely composed of compounds belonging to the alcohol group. 2-heptanol was the predominant compound not only in the alcohols group, with a content of 20,46 ± 1,03 %, but also among all the compounds identified in the sample. The second most abundant compound identified was phenylethyl alcohol (9.16 ± 0.98 %). Hydrocarbons and ketones were the most abundant groups of compounds in *S. officinalis* essential oil (about 34 and 24 %, respectively). One of the main compounds, 2-heptadecanone (11.38 ± 0.14 %), belonged to the ketone group. The second most abundant compound was the only diterpene identified, caur-16-ene (11.82 ± 2.37 %). Aldehydes (9.0 %)), esters (7.8 %), sesquiterpenes (4.5 %) and alcohols (3.4 %) and a few unidentified compounds accounted for the remaining part of the essential oil. To the best of our knowledge, this is the first time that the results of studies on the antioxidant activity of *S. officinalis* flower essential oil are presented. It proved to better scavenge free radicals than methanol-water extracts from the same plant. The aim of this work was to characterise the volatile organic compounds of S. officinalis, growing in the wild in Lithuania, and to evaluate their antioxidant activity for the first time.

1. Introduction

Saponaria officinalis L., a perennial, flowering plant, commonly named: “soapwort”, “wild sweet William” or “soapweed”, a prominent member of the Caryophyllaceae family, is indigenous to Europe and Asia but is cultivated worldwide for its medicinal benefits [1]. This plant contains valuable compounds such as saponins, for witch it is famous mostly, flavonoids, and polyphenols, which are highly regarded in pharmaceutical applications. *S. officinalis* exhibits antioxidant, antibacterial, and mucoactive properties and is traditionally used as a bile laxative, blood purifier, sudorific, diuretic, anti-congestion remedy, laxative, and for treating skin itching [2].

Research on the volatile compounds of *Saponaria officinalis* is limited compared to the extensive studies on its non-volatile compounds. In an investigation of the floral scent by Jurgens and his collegues [3], they found that the primary compound in the fragrance composition of this species is methylbenzoate, which constitutes more than half of the scent profile. Until 2018, there have been no studies published in the international scientific press related to the extraction and chemical composition of the essential oils of soapwort. The predominant class of compounds of shoot essential oil was nonterpenoids (53.7%), with tricosane-6,8-dione (13.4%) and tricosane (7.2%) being the most abundant. Among the sesquiterpenoids, the second acountable class, patchouli alcohol (7.9%) was a major component. However, the most abundant component overall was the phytol (14.1%). Patchouli alcohol (20.0%) and heneicosane (11.5%) were the main representatives of the oxygenated sesquiterpenes and nonterpenoid classes in the essential oil of the flower.

There is a strong correlation between total phenolic content and antioxidant activity in certain plants. Methanolic extracts of *S. officinalis* underground parts and roots were significantly rich in phenolic compounds and showd hight free radical scavenging ability [2,4].
The primary aim of this study was to compare the composition of volatile compounds emitted by *Saponaria officinalis* flowers growing in the wild in Lithuania with the essential oils obtained through hydrodistillation. Additionally, for the first time, the free radical scavenging ability of the essential oil was analyzed and compared to the antioxidant activity of the alcoholic extract.

2. Material and Methods

Saponaria officinalis plants (up to 0.7 kg) were collected at full flowering stage (in July 2023) in the meadow near Kernave, Sirvintai district.

Sampling of VOC emitted by *S. officinalis raw* flowers was performed in vitro through exposure of PDMS/DVB 65 um Solid Phase Micro Extraction (SPME) fibres, (Supelco, USA) for 2 hr from the headspace of Erlenmejer flask with 10 g of plant. As soon as VOCs collection was completed the SPME fibre was removed and introdused for analyses using GC-MS system (Shimadzu GC-2010 gas chromatograph (GC) coupled with Shimadzu MS-QP 2010 Plus mass selective detector (MS) (Shimadzu, Kyoto, Japan) and equipped with a non-polar Rxi-5SilMS capillary column (30 m × 0.25 mm × 0.25 μm; Restek, USA)). The essential oil was isolated by hydro-distillation of dried material (approximatly 100 g) in a Clevenger-type apparatus for 2 h, as per the European Pharmacopoeia. The ratio of plant material to water was 1:20. The conditions of chromatographic separation for bouth sample types were as follows: GC oven's temperature increased from 50 °C (isothermal for 1 min) to 160 °C (isothermal for 2 min) at a rate of 5 °C/min, then increased to 250 °C at a rate of 10 °C/min; the final temperature was kept for 4 min. The temperature of the injector and detector was 250 °C and 220 °C for ion source. The flow rate of carrier gas (helium) was 1 mL/min, analysis was performed in split mode (1:20). At least 2 repetitions ($n \geq 2$) per analysis were performed. Two and a half grams of dried, crushed plant material (flowers and leaves) were soaked in 25 mL of 50% methanol and extracted using an ultrasonic bath for 30 minutes. The mixture was then filtered and analyzed spectrophotometrically to determine total phenolic content (TPC) according Folin-Ciocalteu method and $DPPH^{\cdot}$ assay was employed to reveal radical scavenging abilities of investigated samples.

3. Results

Despite the fact that the scent of soapwort is very strong, the average yield of the obtained essential oils of three repetitions was only 0.12 ± 0.04 % v/w.

The essential oil of *Saponaria officinalis* primarily consisted of simple hydrocarbons (33.85%), with over half of these having chains of more than 20 carbon atoms. Ketones were the second most abundant group (24.05%). The diterpene kaur-16-ene, the only diterpene identified in the oil, was the dominant compound (11.82%), along with the ketone 2-heptadecanone (11.38%). The latter two compounds were also found among the volatile constituents collected from the headspace of the flowers, but in very small quantities. The odour composition of the blossoms was composed of almost 50 % compounds classified as alcohols, of which 2-heptanol was the predominant one among those identified in the sample. Aldehydes were the second most abundant group of compounds (23.9 %). Compounds belonging to the groups of ketones, hydrocarbons, esters, mono-, sesqui- and diterpenes accounted for a very small proportion of the floral odour. Aldehydes, esters and sesquiterpenes were significantly more abundant in the essential oil compared to the floral scent. The compounds with a content greater than 5 % among the VOCs identified in *S. officinalis* floral scent and in the essential oil are presented in Table 1.

Table 1 Most abundant VOCs of *S. officinalis* floral scent and EO.
(Amounts are presented as mean values (n=3) ± SD)

Compound	RI $_{exp.}$	Amount in EO, %	Amount in headspace, %
Hexanal	801		7.87 ± 0.82
2-Heptanol	**894**		**20.46 ± 1.03**
Benzaldehyde	960		5.26 ± 0.08
Benzyl alcohol	1026		7.85 ± 1.17
Phenylethyl alcohol	**1108**		**9.16 ± 0.98**
2-Heptadecanone	**1906**	**11.38 ± 0.14**	
Kaur-16-ene	**2024**	**11.82 ± 2.37**	
Heneicosane	2100	5.76 ± 0.05	
Tricosane	2300	7.05 ± 0.66	
Tetracosane	2400	6.43 ± 1.21	
Tricosane-6,8-dione	2576	7.32 ± 1.09	

$RI_{exp.}$ - Kovat's indices determined experimentally on the non-polar column Rxi-5 Sil MS integra guard

Even though the alcoholic extract of *S. officinalis* flowers was richer in phenolic compounds than the essential oil (36.98 ± 0.13 and 9.22 ± 0.96, g/L GAE, respectively), the latter showed almost 1.5-fold better free radical scavenging ability.

4. Discussion and Conclusions

In the only study on the composition of the essential oil of *S. officinalis* flowers published so far, the main compounds identified were patchouli alcohol, heneicosane and tricosane [1]. In our study, it was found that the wild-growing soapwort in Lithuania contains the diterpene kaur-16 ene and the ketone 2-heptadecanone as the main compounds in its essential oil composition. Patchouli alcohol was not found nor in the essential oil we studied, nor in the odour composition collected from the headspace of the flowers. Despite the fact that several compounds were common to the essential oils of the same species and part of the plant studied by us and the Serbian authors, it must be stated that the results obtained may differ considerably. The same can be said when comparing the floral scent composition studied by Jurgens with our results for the VOCs emitted by the flowers. Since, to our knowledge, the antioxidant activity of *S. officinalis* essential oils has not been studied, the obtained result can only be compared with the free radical scavenging ability of essential oils of other plants [5]. Such a comparison allows us to say that the essential oil of the flowers of soapwort is characterized by a rather high antioxidant activity.

The composition of the volatile compounds emitted by the flowers of *S. officinalis*, which is largely composed of alcohols, is very different from that of essential oils obtained by hydrodistillation. In addition, essential oils have the potential to become components of products used to reduce antioxidant stress.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-21

Chemical composition and biological activity of *Juniperus sabina*, *Juniperus foetidissima*, and *Juniperus phoenicea* essential oils

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Keywords: juniper, essential oils, antibacterial agents, multivariate analysis

Abstract

In this study, chemical composition and antimicrobial activity of leaf and cone essential oils (EO) of three juniper species were analyzed. Branchlets with leaves and berries of *J. feotidissima* and *J. phoenicea* were collected from one population (North Macedonia and Greece, respectively), while leaves of *J. sabina* were collected from two (Albania and Montenegro). For the antibacterial analysis, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against three gram (+) and three gram (–) bacteria were tested. In EOs, 108 compounds were detected and identified. *J. foetidissima* leaf EO had the highest chemical diversity, while the lowest was found in cones. In leaf EOs, monoterpenes were the most abundant compounds: sabinene and *trans*-sabinyl acetate in *J. sabina*, and *α*-pinene, and limonene in *J. phoenicea*. Only in leaf EO of *J. foetidissima* a sesquiterpene allo-cedrol was the dominant compound. EOs of cones differed from leaf EOs in their simplicity and chemical profile, with *α*-pinene being in high concentration. All EOs showed antibacterial activity, with *J. sabina* EO being the most active. Statistical analyses showed compounds that had a positive correlation with antimicrobial activity: terpinen-4-ol and *δ*-cadinene against *S. aureus, P. aeruginosa*, and *S. enteritidis, α*-thujene, sabinene, and methyl citronellate against *E. coli*, *P. aeruginosa*, and *S. enteritidis*, and *γ*-terpinene and *trans*-sabinyl acetate against *P. aeruginosa* and *S. enteritidis*. Additionally, some compounds present in lower concentrations, which may exert synergistic effects on antimicrobial activity, also showed positive correlations.

1. Introduction

The genus *Juniperus* is the second most diverse genus of conifers and is divided into three sections: *Caryocedrus* (1 species), *Juniperus* (= *Oxycedrus*) (10 species), and *Sabina* (56 species). Species of the genus *Juniperus* can be evergreen trees, pyramidal, or prostrate shrubs. The leaves can be needle-like (sections *Caryocedrus* and *Juniperus*) or scale-like (section *Sabina*), sometimes with a visible gland on the leaf surface, arranged spirally or opposite [1-3]. In this study, three species of juniper from the *Sabina* section were analyzed: *Juniperus sabina* L., *Juniperus foetidissima* Willd., and *Juniperus phoenicea* L.

2. Material and Methods

Essential oils were analyzed using a gas chromatography–mass spectrometry (GC-MS/FID). For the antibacterial analysis, the following gram (+) bacteria were selected: *Bacillus cereus* (food isolate), *Listeria monocytogenes* (ATCC 13922), and *Staphylococcus aureus* (ATCC 6538); and the gram (-) strains: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), and *Salmonella enteritidis* (ATCC 13076). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the EOs were determined using microdilution method.

3. Results

In the leaves of *J. sabina* 69 compounds were identified, 71 in *J. foetidissima* leaves, while the lowest components were detected in the cones EO of *J. phoenicea* (45 and 41). In the EO of *J. sabina* 69 and 55 compounds were identified (in JS1 and JS2, respectively). Monoterpene hydrocarbon sabinene dominated in this EO (44.2% in JS1 and 37.1% in JS2) along with its oxidized derivative, *trans*-sabinyl acetate (9.3% in *J. sabina* from Albania (JS1) and 20.8% in *J. sabina* from North Macedonia (JS2). Furthermore, JS1 exhibited lower total amount of monoterpenes compared to JS2 (74.7% versus 90.3%) and higher sesquiterpenes (17.7% versus 4.3%). The oils *of J. sabina* had 5-15 times more oxidized monoterpenes compared to other analyzed oils (15.1% and 27.8%). *α*-pinene, dominant in the oils of *J. foetidissiama* and *J. phoenicea*, was found in low concentrations (below 5 %) in *J. sabina*. Monoterpenes (46.0%) were the dominant group in *J. foetidissima* leaf EO, with α-pinene (19.7%) and limonene (18.5%) being prominent, followed by sesquiterpene allo-cedrol (33.1%). Conversely, fewer compounds (55) were found in the cone EO, dominated by monoterpenes (88.0%), particularly α-pinene (67.8%) and limonene

(12.8%). Sixty-seven compounds were identified in the leaf EO of *J. phoenicea*, with α-pinene (63.3%) and limonene (6.0%) being the most abundant. In the cone EO, 45 compounds were found in immature cones and 41 in mature cones. The antimicrobial activity against *S. aureus* showed a strong positive correlation with the *P. aeruginosa*, *S. aureus*, and *S. enteritidis*. The antibacterial acitivity against these bacteria had a positive correlation with terpinen-4-ol and δ-cadinene. On the other hand antibacterial acitivity against the gram (-) bacteria, *E. coli*, *P. aeruginosa*, and *S. enteritidis*, was correlated with α-thujene, sabinene, and methyl citronellate, while antibacterial acitivity against *P. aeruginosa* and *S. enteritidis* also exhibited a positive correlation with γ-terpinene and *trans*-sabinyl acetate. Additionally, some compounds present in lower concentrations, which may exert synergistic effects on antimicrobial activity, also showed positive correlations.

nMDS analysis examined the correlation between antimicrobial activities in relation to the composition of EO (Figure 1). The graph shows that EOs from *J. sabina* (JS) exhibited the strongest antimicrobial activity against Gram (+) bacterial strains: *B. cereus* and *S. aureus*, and Gram (-) *P. aeruginosa* and *E. coli*. The EO from *J. foetidissima* (JF) leaves showed the best antimicrobial activity against *B. cereus* and *L. monocytogenes*.

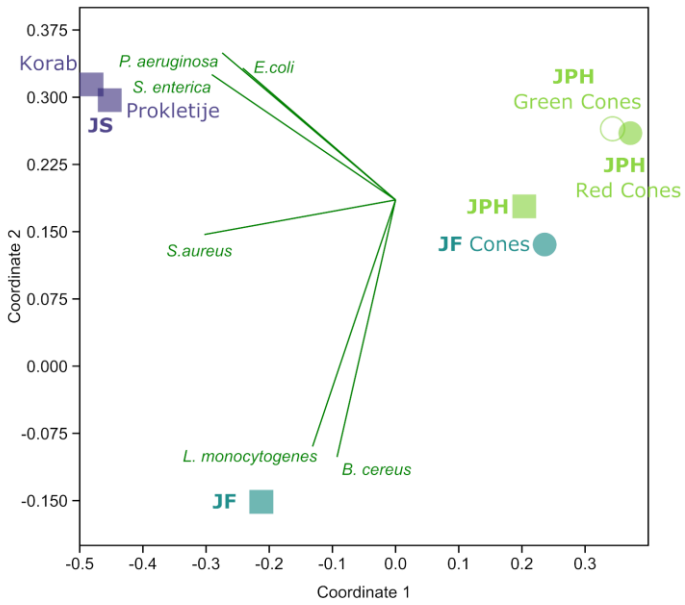

Figure 1. nMDS - MIK values in correlation with oil composition

4. Discussion and Conclusions

EO composition showed species-specific composition. The cone EO had much simpler composition, with one or two components having a high concentration, though the general profile of cone EO matched the leaf EO, with some minor components missing. In this study, leaf EOs generally exhibited better antimicrobial potential compared to cone EOs, probably due to a lower percentage of α-pinene and higher diversity of compounds present. The leaf and cone oils of *J. phoenicea* showed the highest antibacterial activity against *S. enteritidis* and *B. cereus*, with leaf EO exhibiting better activity against *S. enteritidis* and cone EO against *B. cereus*. However, they showed no significant activity against *S. aureus*, *E. coli*, *P. aeruginosa*, and *L. monocytogenes*. Similarly, Angioni et al. (2003) found that leaf oil exhibited activity against *S. aureus* (MIC 0.90 mg/mL) and weak activity against *E. coli* and *P. aeruginosa* (MIC >0.90 mg/mL). Ait-Ouazzou et al. (2012) found that leaf oil had good activity against *S. aureus* (MIC <0.5 µl/mL) but also towards *L. monocytogenes* (MIC 1 µl/mL) and didn't exhibit activity against *E. coli* and *P. aeruginosa* (MIC >30 µl/mL). *E. coli* appeared as the most resistant bacterium among the tested strains; however, the cone EOs, which contain the highest percentage of terpinen-4-ol, exhibited slightly stronger activity against it. Ait-Ouazzou et al. (2012) also found that terpinen-4-ol had a strong impact on *E. coli*. The most sensitive bacterium was *B. cereus*, and its antimicrobial activity is positively correlated with *L. monocytogenes*. These bacteria were most sensitive to the leaf EO of *J. foetidissima*. The antimicrobial activity against *S. aureus* was positively correlated with the gram (-) bacteria *P. aeruginosa*, *S. aureus*, and *S. enteritidis*, which were most sensitive to the leaf oil of *J. sabina*.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-21

In vitro wound healing evaluation of *Mentha spicata, Matricaria chamomilla* essential oils with *Cocos nucifera* oil nanoemulgel formulations

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Keywords: essential oil, wound healing, nanoemulgel, *Mentha spicata, Matricaria chamomilla*

Objective
Wound treatments require novel preparations. Nanoemulsion systems are effective, have low side effect profiles and enhance the absorption in wound treatment. For this purpose, *Mentha spicata* L., *Matricaria chamomilla* L. and *Cocos nucifera* L. oils were selected based on their traditional usages for wound treatment.

Methods
The commercially available essential oils were analysed by GC-MS and GC-FID, for their quality. Nanoemulgel formulations were designed and *in vitro* wound healing activity of the commercial oils and formulations were performed using a in a cell culture wound model using the scratch assay. Finished formulations were assessed for toxicity in L929 healthy cell line using the MTT assay.

Results
According to standardization analysis, the main components were determined as %62.3 carvone, %23.1 limonen for *M. spicata*, %46.3 (E)-β-farnesene, %9.1 α-bisabolol oxide B for *M. chamomilla* and %40.8 lauric acid, %20.51 myristic acid for *C. nucifera*. Their effective concentrations were found 12.5 µg/mL; 25 µg/mL; 12.5 µg/mL, respectively. Stable formulation series that are thermodynamically stable and have droplet size and distribution within normal limit. Prepared loaded gels showed wound healing activity on cell lines without exhibiting toxic effects.

Conclusions
To the best of our knowledge, this triple combination was tested for the first time in this study in terms of wound healing activity and showed a higher effect than the positive control substances in terms of both activity and toxicity. However, it is planned to confirm the results with animal experiments in future studies.

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P-23

Chemical and biological evaluation of three Cineol rich herbal drug and infusion volatiles

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Keywords: herbal volatiles, antimicrobial activity.

Abstract

This study aimed to determine the volatile components of essential oils and their infusions obtained from *Eucalyptus globulus* L. leaves, *Salvia officinalis* L. and *Rosmarinus officinalis* L. herbal parts to evaluate their antimicrobial activity against selected human respiratory tract pathogens.

1. Introduction

The herbal drugs are among the most commonly used herbal teas for the treatment due to their antimicrobial, antifungal and antioxidant effects [1].

2. Material and Methods

The herbal drugs were obtained from a pharmacy in Munich, Germany. Herbal tea of each material was freshly prepared as 5% infusions of which volatile components were determined by HS-SPME-GC/MS methods. The infusions were lyophilized for biological activity studies. The essential oils were obtained by hydrodistillation subsequently analysed by GC-FID and GC/MS systems [2]. The antimicrobial activity of the samples were determined by broth microdilution against *Escherichia coli* NRRL B-3008, *Bacillus cereus* NRRL B-3711, *Bacillus subtilis* NRRL B-4378, *Staphylococcus aureus* ATCC 6538, *Streptococcus mutans* ATCC 25175, and *Salmonella typhimurium* ATCC 13311 [2, 3].

3. Results

1,8-Cineol was determined as major components for all tested samples (8.0-63.4%), followed by globulol (11.1 and 1.8%) as major component of *E. globulus* essential oil and its infusion, respectively. Camphor (18.1 and 8.5%), α-terpineol (5.1 and 4.9%) and borneol (5.8 and 2.7%) were found as main constituents of *R. officinalis* essential oil and its infusion, respectively. α-Thujone (27.4 and 23.9%), camphor (23.4 and 19.9%) and β-thujone (7.1 and 9.9%) was determined as major components for *S. officinalis* oil and its infusion, respectively. Minimum inhibition concentration (MIC) of samples were in the range of 0.6-12.5 mg/mL, compared with standard antimicrobial agents. When compared with current literature the volatile constituents were in line, and the antimicrobial activity were relatively moderate. The infusions were more susceptible to the tested pathogens compared to the tested oils

4. Discussion and Conclusions

The biological activity of thujones need to be evaluated in more detail along with toxicological data before testing the herbal tea combinations.

ACKNOWLEDGEMENTS

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-24

Wound care formulation using chitosan and *Hamamelis*p preparations

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Keywords: combination, formulation, antimicrobial, anti-inflammatory, toxicity

Objective

Wound healing is a major issue in skin injuries as it is well-known. Natural products have been utilized since early times of humanity. Chitosan is a biopolymer, which is used internally and externally for its antimicrobial as well its anti-inflammatory properties along with is biofilm forming features. *Hamamelis virginiana* L., native to N. America, is known to have medicinally valuable products, which contains various bioactive compounds such as volatiles. The aim of the study is to optimize the chitosan – *Hamamelis* combination according to its bioactivity.

Methods

The commercially available chitosan, *Hamamelis* distillate and pluronic constituents were formulated for potential external use. *In vitro* antimicrobial evaluation against human skin pathogens were performed initially using a microdilution assay, where ingredients and formulations were evaluated using a checkerboard assay. In addition, in vitro lipoxygenase enzyme inhibition experiments were performed for the anti-inflammatory potential and a cell line MTT assay for toxicity evaluation.

Results

According to the first formulation results the human pathogens *Escherichia coli* NRRL B-3008, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Corynebacterium striatum* ATCC BAA 1293, *Candida albicans* ATCC 90028 were susceptible with a MIC >1000 mg/mL concentration. Various combinations towards bioactive formulations are ongoing, also for anti-inflammatory, and toxicity evaluations. To the best of our knowledge, the combination of chitosan with *Hamamelis* preparations is for the first time.

Conclusions

This type formulations have potential acute and chronic application in various skin conditions, however, more detailed bioactivity tests are needed.

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P-25

Essential oil profile of pellets from hop cultivars produced ın South Brazıl

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Keywords: *Humulus lupulus*; hop quality; essential oil; myrcene.

Abstract

Hops produce essential oil to impart the odor and aroma characteristics to beer. Since the hop production started in Brazyl, the quality of the raw material have been investigated. The objective of this work was to evaluate the essential oil yield and content of pellets from hop cultivars produced in Brazil as to compare with imported pellets. The experimental design was completely randomized comparing the pellets of the cultivars Comet, Cascade, Chinook and Zeus, produced in Brazil e imported, with three replications. The essential oil samples were isolated using a Clevenger apparatus and its profile was determined by GC/MS. A total of 43 compounds were identified in the essential oil samples and the main compounds (myrcene, (E)-cariophyllene, α-humulene, β-selinene, α-selinene, β-bisabolene, γ-cadinene and δ-cadinene) were present in all cultivars. The essential oil yield was higher in pellets of Comet cultivar compared to the other cultivars produced in Brazil and imported. Myrcene was the main compound in the essential oil of all hop pellets, and was higher in national pellets of both Comet and Cascade cultivars. The other essential oil constituents (E)-caryophyllene, α-humulene, β-selinene, α-selinene, β-bisabolene, γ-cadinene and δ-cadinene showed higher percentage in imported pellets, according to the cultivar. The imported pellets of the Comet cultivar showed superior percentage of (E)-caryophyllene, β-selinene and α-selinene, similar to α-humulene and β-bisabolene in imported pellets of Chinook and Zeus cultivars. Although the higher essential oil yield was observed in national pellets, the constituents showed higher percentage in the imported samples, except for myrcene.

1. Introduction

Hops (*Humulus lupulus* L.) are perennial herbaceous climbing plants belonging to the Cannabaceae family known worldwide as one of the most important ingredients for the brewing industry, providing bitterness and aroma. With the increase of hop cultivation increased in new regions, including Brazil, the quality of the raw material need to be evaluated in order to provide good quality for the brewing industry.

2. Material and Methods

Samples of dried pellets of the hop cultivars Comet, Cascade, Chinook and Zeus, produced in Brazil e imported,were used in the experiment. For each treatment, three replications of 50 grams of dry pellets were used for essential oil isolation by hydrodistillation in a Clevenger apparatus during 3 hours. The essential oil content was determined on a dry mass basis, expressed as a percentage. To identify and quantify the essential oil constituents, the samples were diluted to 1% concentration using hexane and 1 µl of this solution was injected into a gas chromatograph coupled to a mass spectrometer (GC/MS) Shimadzu (2010 Plus). The injector was kept at 250°C and the constituents were separated using a HP-5MS capillary column (30m x 0.25mm x 0.25µm) with helium gas as a carrier (1 ml min^{-1}). The oven temperature was programmed incrementally (60 to 240°C / 3ºC min^{-1}). The chemical constituents were identified by their linear retention indices, calculated from the injection of a homologous series of n-alkanes (VAN DEN DOOL; DEC. KRATZ, 1963) and mass spectra, both compared with the literature data (ADAMS, 2017). The compounds were quantified using a GC with a flame ionization detector (DIC) under the same conditions as the GC/MS, now using hydrogen (1.5 ml min^{-1}) as the carrier gas. Their percentage composition was obtained via electronic integration of the DIC signal by dividing the area of each component by the total area (%).

3. Results

Table 1. Essential oil yield and composition of pellets from hop cultivars produced in South Brazil and imported.

			Constituent (%)							
Origem	Cultivar	Yield (%)	mircene	(E)-cariophyllene	α-humulene	β-selinene	α-selinene	β-bisabolene	γ-cadinene	δ-cadinene
National	Comet 1	**1.92 a**	**56.78 a**	10.14 c	0.32 c	4.52 d	4.85 d	1.38 de	2.04 d	0.48 e
	Comet 2	0.98 c	43.88 bc	15.01 b	0.49 c	6.70 b	7.10 b	1.87 bc	1.72 ed	0.70 de
	Comet 3	**1.93 a**	**54.49 a**	10.03 c	0.38 c	4.54 d	4.93 d	1.48 de	2.63 c	0.52 e
	Comet 4	1.29 b	**50.26 ab**	11.34 c	0.43 c	5.22 c	5.55 c	1.60 cd	2.59 c	0.57 de
	Cascade	0.60 ef	**52.02 ab**	5.25 e	12.12 b	1.38 fg	1.57 f	0.93 f	1.75 ed	0.95 d
	Chinook	0.48 fg	27.43 d	8.10 d	16.73 b	1.58 fg	2.25 e	**3.34 a**	1.73 ed	3.39 b
	Zeus	0.32 g	28.34 d	11.03 c	14.92 b	1.55 fg	1.95 ef	1.95 b	**4.05 a**	3.10 b
Imported	Comet	0.76 de	27.64 d	**17.06 a**	1.19 c	**9.58 a**	**9.12 a**	1.63 bcd	3.19 b	0.76 de
	Cascade	0.45 fg	39.28 c	7.13 d	13.68 b	2.16 e	2.24 e	1.15 ef	1.50 e	1.37 c
	Chinook	0.92 cd	26.38 d	9.89 c	**21.2 ab**	1.64 f	2.46 e	**3.12 a**	0.96 f	**4.69 a**
	Zeus	0.55 f	20.92 d	13.79 b	**29.48 a**	1.08 g	2.47 e	**3.09 a**	0.88 f	3.35 b
CV(%)		7.74	8.21	5.42	33.47	4.87	4.63	5.81	7.48	7.48

*Means followed by the same letter in the column are not significantly different by Tukey's test at p≤0.05.

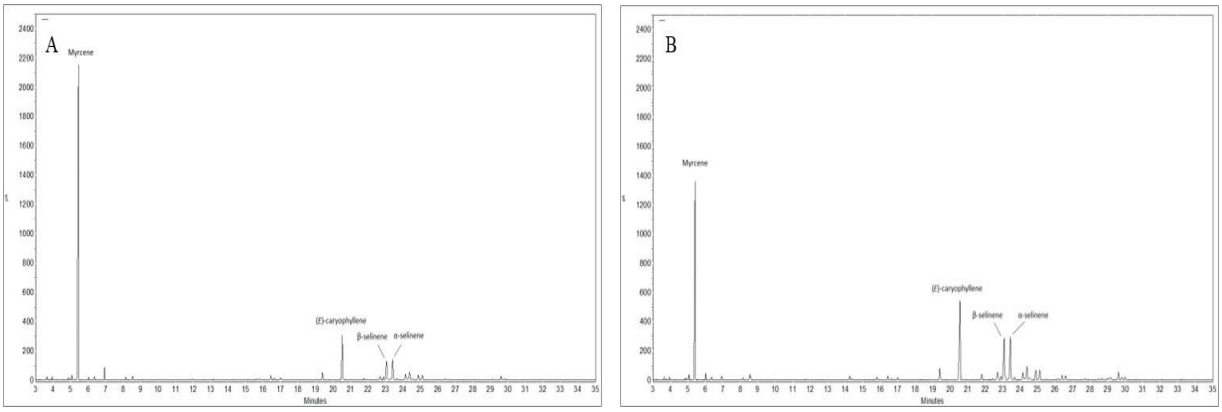

Figure 1. Essential oil constituents of pellets from the hop cultivar Comet produced in South Brazil (A) and imported (B).

4. Discussion and Conclusions

This study represents a new investigation of pellets hop cultivars produced in Brazil compared to the imported ones. The main result is that some cultivars produced in Brazil present higher essential oil yield and mircene content. The other essential oil constituents showed higher percentage in imported hop pellets. These results can be related with the climatic conditions in Brazil as with the post-harvest techniques, including the pellet production. We conclude that the hop cultivars produced in Brazil should be evaluated before cultivation in large areas.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-26

Effects of solvent free-microwave green extraction of essential oil from orange peel (*Citrus sinensis* L.) on shelf life of flavoured liquid whole eggs during storage under commercial retail conditions

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Keywords: Orange peel essential oil, Green extraction, Scanning electron microscopy, Liquid whole eggs , Shelf-life

Abstract

This study compares the extraction of essential oil (EO) from orange peel (Citrus sinensis L.) by three diferent methods, namely solvent-free microwave assisted extraction (SFME), traditional hydrodistillation (HD) and cold-pressing (CP), in terms of efciency and chemical composition using gas chromatography coupled to mass spectrometry (GC–MS). Microstructure analysis of the behaviour of the epithelial cells of the orange peel bark was carried out by scanning electron microscopy (SEM). Results showed that the traditional HD extraction method caused greater modifcations of the cellular structure than the SFME method. The comparison between SFME and HD indicated that SFME showed advantages such as faster kinetics and higher efciency with similar yields (0.40% dry basis in 30 min by SFME versus 3 h by HD). The antioxidant activity of EO was evaluated in vitro by the DPPH assay, resulting in high radical scavenging activity exceeding 80%. The EO was added at three levels (0.1, 0.3, and 0.5%, v/v) to liquid whole egg in order to evaluate its efect on oxidative stability and organoleptic attributes (colour and odour) during simulated cold commercial retail conditions. The thiobarbituric acid reactive substances assay showed that the EO addition signifcantly reduced the lipid oxidation. The results obtained confrm orange peel EO as a promising functional food ingredient.

1. Introduction

Citrus essential oils are widely used in food industry as natural favouring agents for foods [1]. Eggs play an important role in the human diet as a source of highly digestible proteins, lipids, minerals, and vitamins. Eggs are processed into various egg products for uses in the food service industry or as ingredients in various foods. However, the quality of liquid eggs during cold storage is negatively afected by the oxidation of polyunsaturated fatty acids and cholesterol, change colour, and destruction of carotenoids. In this scenario, the search for new strategies and new bioactive agents for stabilization of liquid eggs has become a central goal for food industry. EOs can be an interesting alternative to preserve oxidative stability as well as to reduce the intensity of heat and nonheat treatments. The aims of this study were (i) to compare the extraction of EO from orange peel by different methods (ii) to assess in vitro the antioxidant activity of C. sinensis EO, and (iii) to explore the value-added perspectives of C. sinensis EO for novel applications in egg processing.

2. Material and Methods

The orange peel bark were analyzed by scanning electron microscopy (TOPCON ABT-60. The EOs were analyzed by GC–MS and GC-FID. The antioxidant activity of Eos was determined by DPPH radical-scavenging test. To measure the potential antioxidant capacity of studied EO and to evaluate the extension of the lipid oxidation on the LWE samples, the determination of the amount of the formed 2-thiobarbituric acid-reactive substances (TBARS) was undertaken, according to protocol developed by Djenane et al. [2]. The colour profle was measured using colour space coordinates CIE (L*, a*, and b*) (Mini XE, Portable type, USA) in accordance with the recommendations of the International Commission on Illumination. For sensory analysis a score value higher than 3, denoted that LWE was not acceptable due to undesirable smell of orange: 1=none; 2=slight; 3=small; 4=moderate; and 5=extreme [3].

3. Results

As shown in Table 1, the yields obtained by SFME and HD techniques were identical (0.40%) but the diference is related to the extraction time. Therefore, the SFME is clearly quicker than its conventional counterparts. dditionally, SFME is proposed an "environmentally friendly" extraction method, as it is a very clean method which avoids residue generation (vs. CP) and the use of large quantity of water and voluminous extraction vessels (vs. HD).

Table 1. Chemical composition, extraction time and yield of essential oils obtained by SFME, HD, and CP from Valencia late (Citrus sinensis) peel.

No	Compound	RI[a]	RI[b]	SFME	HD	CP
	Monoterpene hydrocarbons (total)			97.48	98.61	98.32
1	α-Pinene	926	1023	0.43	0.53	0.51
2	Sabinene	961	1121	0.54	0.49	0.54
3	β-Myrcene	988	1165	1.64	1.87	1.82
4	α-Phellandrene	1001	1177	0.15	0.17	0.36
5	Limonene	1030	1206	94.64	95.48	95.06
6	(E)-β-Ocimene	1048	1282	0.02	0.02	0.02
7	γ-Terpinene	1103	1285	0.05	0.03	0.01
8	Terpinolene	1120	1304	0.01	0.02	0.01
	Oxygenated monoterpenes (total)			1.09	0.58	0.55
9	Linalool	1125	1538	0.62	0.30	0.30
10	Citronellal	1167	1478	0.06	0.03	0.04
11	Terpin-4-ol	1191	1590	0.03	0.06	0.02
12	α-Terpineol	1203	1677	0.10	0.06	0.05
13	Nerol	1237	1781	0.10	0.03	0.08
14	Neral	1268	1670	0.05	0.03	0.06
15	Geraniol	1271	1828	0.04	0.01	–
16	Geranial	1284	1714	0.09	0.06	0.11
	Sesquiterpene hydrocarbons (total)			0.06	0.10	0.15
	Oxygenated sesquiterpenes (total)			0.01	–	0.09
	Other oxygenated compounds (total)			0.50	0.27	0.31
	Total oxygenated compounds %			1.60	0.85	0.95
	Extraction time (min)			30	180	60
	Yield %			0.40	0.40	0.16

[a]Kovats retention indices in non-polar HP5MS capillary column
[b]Kovats retention indices in polar Carbowax-PEG capillary column

Table 2. CIE. Lab colour coordinates1 in LWE treated with orange EO

LWE samples	Days of storage	L* (lightness)	a* (redness)	b* (yellow-ness)
Control	0	65.12 ± 1.52^{a}	13.50 ± 2.01^{a}	41.21 ± 0.99^{a}
	3	64.45 ± 0.88^{a}	11.10 ± 1.11^{b}	40.61 ± 1.07^{a}
	5	$60.72 \pm 1.01^{a,b}$	09.20 ± 2.01^{c}	36.58 ± 0.89^{b}
	8	59.52 ± 0.68^{b}	09.04 ± 0.99^{c}	36.02 ± 0.25^{b}
LWE 0.1%	0	65.52 ± 0.54^{a}	13.80 ± 1.01^{a}	41.19 ± 0.99^{a}
	3	64.92 ± 1.88^{a}	13.40 ± 1.31^{a}	40.42 ± 0.49^{a}
	5	63.72 ± 1.31^{a}	11.10 ± 0.05^{b}	37.98 ± 0.69^{b}
	8	63.08 ± 0.81^{a}	10.03 ± 199^{b}	36.10 ± 0.15^{b}
LWE 0.3%	0	65.35 ± 0.32^{a}	13.90 ± 1.01^{a}	41.20 ± 0.21^{a}
	3	65.12 ± 1.08^{a}	13.25 ± 1.41^{a}	40.88 ± 0.32^{a}
	5	65.08 ± 1.25^{a}	13.08 ± 0.55^{a}	40.08 ± 0.60^{a}
	8	63.73 ± 0.51^{a}	$12.23 \pm 0.95^{a,b}$	39.10 ± 0.05^{a}
LWE 0.5%	0	65.12 ± 1.52^{a}	13.50 ± 2.01^{a}	41.28 ± 0.99^{a}
	3	66.22 ± 0.48^{a}	13.62 ± 1.12^{a}	41.42 ± 0.69^{a}
	5	65.09 ± 0.23^{a}	13.59 ± 0.05^{a}	41.55 ± 1.08^{a}
	8	64.69 ± 0.05^{a}	13.50 ± 2.11^{a}	40.89 ± 2.19^{a}

[1]Within each column, means with different superscript uppercase letters (a–c) are different ($p < 0.05$)

After the treatments, red (CIE a*) and yellow (CIE b*) coordinates increased moderately for LWE 0.5% during display if compared with other treatments (Table 2), resulting in more yellowish orange products. Also, the CIE L* value decreased with the exposure time, and results for untreated samples were comparatively darker than the others.

Table 3. Sensory scores (mean±SD) for orange EO odour of LWE during display

Treatments	0-day	3-day	5-day	8-day
Control	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}
LWE 0.1%	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aA}
LWE 0.3%	2.17 ± 0.41^{aB}	2.17 ± 0.41^{aB}	1.17 ± 0.41^{bA}	1.00 ± 0.00^{bA}
LWE 0.5%	3.00 ± 0.00^{aC}	2.83 ± 0.41^{aC}	1.33 ± 0.52^{bAB}	1.17 ± 0.41^{bA}

[1]Within each row, means with different superscript lowercase letters (a–d) are different ($p < 0.05$)

[2]Within each column, means with different superscript uppercase letters (A–C) are different ($p < 0.05$)

It .appeared evident (Table 3) that the intensity of the orange smell decreased with time of display; it was no more perceptible after 5 days ($p<0.05$) and reached acceptable values for all samples. From a practical point of view, the doses used for processing LWE (0.1 to 0.5%) were acceptable for this product.

Table 4. TBA values (mg MDA/kg of LWE) of LWE containing orange EO during storage

Days of storage	Control	LWE 0.1% EO	LWE 0.3% EO	LWE 0.5% EO
0	0.05 ± 0.006^{aW}	0.05 ± 0.006^{aW}	0.05 ± 0.006^{aW}	0.05 ± 0.006^{aW}
3	0.5 ± 0.09^{aX}	0.41 ± 0.06^{abX}	0.45 ± 0.04^{bX}	0.25 ± 0.06^{cX}
5	1.85 ± 0.11^{aY}	1.60 ± 0.08^{abY}	1.09 ± 0.06^{cY}	0.56 ± 0.08^{dXY}
8	2.5 ± 0.07^{aYZ}	2.05 ± 0.13^{abYZ}	1.58 ± 0.09^{bYZ}	1.12 ± 0.12^{cYZ}

[1]Within each row, means with different superscript lowercase letters (a–d) are different ($p < 0.05$)

[2]Within each column, means with different superscript uppercase letters (W–Z) are different ($p < 0.05$)

The initial TBA-RS value of fresh whole eggs was 0.05 mg MDA/kg (Table 4). The activity of the orange EO was found to be dose dependent. The TBA values of control samples increased rapidly throughout the display storage period.

4. Discussion and Conclusions

In this study, EOs from citrus by-products (orange peels) were obtained with a solvent free microwave extraction method and compared to the conventional hydro-distillation and cold pressing methods. The results obtained confrm the efectiveness of this novel process that proved to be a green alternative with the advantages of reducing extraction times, energy and water consumption, solvent use and CO_2 emissions, without detriment to the quality of the EO composition and potential bioactivity. The EO showed a strong antioxidant activity in vitro, which allowed to explore the perspectives for novel applications in egg processing. The efects on sensory (colour and odour) and oxidative stability were studied in liquid whole egg enriched with diferent levels of orange peel EO and stored under simulated commercial conditions. The results obtained indicated that the active compounds of EO preserved the product from undesirable chemical and sensorial changes caused by oxidation. Therefore, EO from orange peel has been characterized as a promising functional food ingredient that could be used in combined processes for food preservation.

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Conflict of Interest:

No confict of interest was reported by the authors.

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Mentha gentilis var. citrata essential oil phytochemical constituents and antiproliferative activity

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Keywords: *Mentha gentilis var. citrata,* essential oil, GC-MS, genotype, antiproliferative activity

Abstract

Mentha gentilis var. citrata (*M. citrata),* also called bergamot, lemon, or orange mint, is classified in the Lamiaceae family, the genus *Mentha*, which includes a diverse group of 31 species and hybrids worldwide. Tea made from the fresh or dried plant leaves has traditionally been used for stomach aches, nausea, parasites, and other digestive disorders, as well as for fevers and headaches. Leaves and flowering plants have analgesic, antiseptic, antispasmodic, carminative, diaphoretic, and vasodilatory properties, as well as insect-repellent properties. This research investigates the chemical composition and antiproliferative activity of *M. citrata* essential oil (EO). The composition analysis of EO (cultivar collected in Isparta province, Turkiye) obtained by hydrodistillation in a Clevenger-type apparatus was carried out by gas chromatography-mass spectrometry (GC-MS). The antiproliferative activity is evaluated by MTT assay against four human cancer cell lines: cervical adenocarcinoma (HeLa), human colon carcinoma (LS174), non-small cell lung carcinoma (A549), and a normal human fetal lung fibroblast cell line (MRC-5). The results were expressed as IC_{50} values (50% cell growth inhibitory concentrations). Based on the chemical composition, linalool (35.40%) is the major component, followed by linalyl acetate (28.60%), 1,8-cineole (6.00%), and geranyl acetate (2.60%). The plant sample is recognized as having a linalool-linalyl acetate chemotype, which is consistent with the previously published studies. Examined EO exerted strong cytotoxicity against cancer cell lines, with IC_{50} values ranging from 3.21±1.92 to 3.86 ± 0.73 μL/mL. The results showed excellent selectivity (SI16) towards the tested normal fibroblast cells (MRC5), which may indicate a good potential for the application of this EO according to its low cytotoxicity on normal human cells.

1. Introduction

The Lamiaceae family is a very important plant family that has been investigated for its medicinal properties. The genus *Mentha*, commonly referred to as mint and classified within the Lamiaceae family, encompasses a diverse group of 31 species and hybrids worldwide [1]. Mint is a very popular herb in many countries and is often consumed in the form of tea, hot or cold beverages, as well as as a spice, as an aroma component, in cosmetics, and in the pharmaceutical industry. *Mentha gentilis var. citrata*, also called bergamot, lemon, or orange mint, traditionally has been used for stomach aches, nausea, parasites, and other digestive disorders, as well as for fevers and headaches. Leaves and flowering plants have analgesic, antiseptic, antispasmodic, carminative, diaphoretic, and vasodilatory properties, as well as insect-repellent properties. The Mentha essential oils are known to contain numerous monoterpenoids, with piperitone oxide, piperitone, piperitenone, pulegone, d-limonene, carvone, menthone, β-caryophyllene, 1,8-cineole, and menthol as dominating compounds. However, there have been some variations in the constituents of this oil from different countries, and a chemogeographical variation has been observed in the essential oil composition of *Mentha* species [2, 3].

The aim of the presented work was to determine chemical composition and to investigate the EO antiproliferative activity of *M. citrata* cultivated in Isparta area, Republic of Türkiye.

2. Material and Methods

Plant material: *M. citrata* plant material was cultivated during the 2021 vegetation period. Agricultural procedures (irrigation, fertilization, weed control, etc.) were carried out pre-harvest. The plant was harvested

during the flowering stage, and subsequently, the plant was subjected to a drying process under room conditions until a constant weight was achieved.

Isolation of essential oil (EO): The extraction of essential oil from the dried plant sample was performed using the Clevenger apparatus. Specifically, 50 g of plant material was utilized with 1000 mL of water in a 1:20 ratio for water distillation, a process that spanned a duration of two hours. The obtained distilled essential oil was dried over anhydrous sodium sulfate and preserved at +4 °C until subjected to Gas Chromatography (GC) analysis.

Gas chromatography and mass spectrometry analysis of volatiles: GC/MS analysis was performed on a gas chromatograph (GC) Agilent 7820A model (Palo Alto, CA, USA) using an HP-Innowax FSC column (60 m x 0.25 mm Ø, film thickness 0.25 μm) and a mass spectrometer (MS) Agilent 5977B. The GC conditions were set up 2 min isothermally at 70 °C, then raised up to 220 °C at a rate of 4 °C/min; injector temperature was 240 °C; split ratio was 1:50; gas carrier was He (1 mL/min). The MS conditions were: EI mode at 70 °C; ion source temperature was 250 °C; the mass range was 30-350 amu. The identification of compounds was performed using GC peaks by comparing their retention indices (RI) relative to C9-C25 n-alkanes and by comparing their mass spectra with those from the Wiley 275 (Wiley, NY, USA) and NIST02 (Gaithersburg, MD, SAD) libraries. The quantification (%) of EO components was calculated from the GC peak areas (average of duplicate analyses) applying the normalization method with no correction factors.

Antiproliferative activity

Cell culture: The cell lines used in this study were: cervix adenocarcinoma (HeLa), human colon carcinoma (LS174), non-small cell lung carcinoma (A549), and a normal cell line, the human fetal lung fibroblast cell line (MRC-5).**Treatment of cells:** Target cells HeLa (2000 cells per well), LS174 (7000 cells per well), A549 (5000 cells per well), and MRC-5 (5000 cells per well) were seeded into wells of a 96-well flat-bottomed microtiter plate. Twenty-four hours later, after the cell adherence, different concentrations of investigated EO were added to the wells, except for the control cells, to which only nutrient medium was added. Final concentrations reached in treated wells were in the range of 1 μL/mL to 50 μL/mL (1.00; 6.25; 12.50; 25.00; 50.00 μL/mL). All investigated concentrations were set up in triplicate. A nutrient medium with corresponding concentrations of investigated compounds but without cells was used as a blank, also in triplicate. The cultures were incubated for 72 hours.

Determination of IC_{50} values: The effect of the investigated extracts on the viability of the specified cell lines was determined by the microculture tetrazolium test (MTT) according to Mosmann (1983) [4] with modification by Ohno and Abe (1991) [5] 72 h after addition of the EO as described earlier. Briefly, 20 mL of MTT solution (5 mg/mL phosphate-buffered saline) was added to each well. Samples were incubated for a further 4 hours at 37 °C in a humidified atmosphere of 95% air/5% CO_2 (v/v). Then 100 μL of 100 g/L sodium dodecyl sulfate was added to dissolve the insoluble product formazan resulting from the conversion of the MTT dye by viable cells.The absorbance (A) at 570 nm was measured 24 hours later. The number of viable cells in each well was proportional to the intensity of the light's absorbance, which was read on an enzyme-linked immunosorbent assay (ELISA) plate reader to determine cell survival (%). The A of a sample with cells grown in the presence of various concentrations of the investigated extracts was divided by the control optical density (the A of control cells grown only in nutrient medium) and multiplied by 100. In each experiment, the A of the blank was always subtracted from the A of the corresponding sample with target cells.

IC_{50} is defined as the concentration of an agent inhibiting cell survival by 50% compared with a vehicle-treated control. All experiments were done in triplicate.

3. Results

Results of chemical composition determination of *Mentha gentilis var. citrata* essential oil by GC/MS analysis were showed in Table 1. Results of antiproliferative activity of EO are presented in Table 2.

Table 1 Chemical composition of *Mentha gentilis var. citrata* essential oil

Number of components	RI	Components	*M. citrata* Citra-1
1	1203	Limonene	0.50
2	1213	1.8-Cineole	6.00
3	**1553**	**Linalool**	**35.40**
4	**1565**	**Linalyl acetate**	**28.60**
5	1612	β-Caryophyllene	1.70
6	1638	Menthol	0.70
7	1765	Geranyl acetate	2.60
8	1857	Geraneol	2.60
Total identfied (%)			78.10

Table 2 Antiproliferative activity of *Mentha gentilis var. citrata* essential oil

Clone-Cultivar/*Specie*	Chemotype	$IC_{50} \pm SD$ (µL/mL)				
		HeLa	LS174	A549	MRC5	SI
Mentha gentilis-citrata / Lemon mint	Linalool/ Linalyl acetate	**3.86 ± 0.73**	**3.41±1.32**	**3.21±1.92**	>50	16

The EOs samples were incubated with cells for 72 h.
HeLa- cervix adenocarcinoma cell line; (HeLa), LS174- human colon carcinoma;
A549- non-small cell lung carcinoma; MRC-5- normal cell line (human fetal lung fibroblast cell line); SI- Selectivity Index

4. Discussion and Conclusions

Based on the chemical composition, linalool (35.40%) is the major component, followed by linalyl acetate (28.60%), 1,8-cineole (6.00%), and geranyl acetate (2.60%). The plant sample is recognized as a linalool-linalyl acetate chemotype, which is consistent with the previously published studies [6].

Examined EO exerted strong cytotoxicity against malignant cell lines, with IC_{50} values ranging from 3.21±1.92 to 3.86 ± 0.73 µL/mL. The results showed that the tested EO sample exhibited strong and similar cytotoxic effect against all tested cell lines (HeLa, LS174 and A549). The results showed excellent selectivity with selectivity index (SI) of 16, towards the tested normal fibroblast cells (MRC5). Presented data may indicate a good potential for the application of this EO according to its low cytotoxicity on normal human cells.

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Conflict of Interest

The authors declare no conflict of interest.

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Essential oils of garden-growing lavender species: *in vitro* antimicrobial activity

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Keywords: Lavandula angustifolia, Lavandula latifolia, essential oil, antimicrobial activity, Micrococcus luteu**s,** Candida albicans.

Abstract

Lavender is a widespread aromatic and medicinal plant whose flower and essential oil are primarily used in the fragrance and toiletry industries, aromatherapy, and traditional medicine to treat a variety of gastrointestinal and rheumatic disorders as well as headaches, depression, and anxiety. Samples of *Lavandula angustifolia* Mill. (*Lavandula officinalis,* or true lavender) and *Lavandula latifolia* Vill. (Spike lavender) were harvested from Sarajevo gardens, where they serve as decorative fragrant plants. The research was conducted to investigate the *in vitro* antimicrobial activity of EOs on the Gram-(+) bacterial strain *Micrococcus luteus*, ATCC 10240, which is reported to possibly cause infections in immunosuppressive patients. The effect of both EO samples on the fungus *Candida albicans,* ATCC 10231, was also investigated. Diameters of inhibition zones (DIZ/mm±STDEV) were determined by the agar disk diffusion assay. Minimum inhibitory (MIC) and bactericidal concentrations (MBC) were determined by the microdilution method. The findings demonstrated the tested EOs' potent antibacterial activity against *M. luteus* strains, with DIZ values of 27.96 ± 2.2 for *L. angustifolia* and 21.73 ± 1.27 for *L. latifolia*; however, the low antifungal activity against *C. albicans* was indicated by DIZ values of 6.66 ± 0.15 for *L. angustifolia* and 6.7 ± 0.2 for *L. latifolia*. Determined MIC values (mL/mL) were 20.00 for *L. angustifolia* and 2.08 for *L. latifolia* on *M. luteus*, and for *C. albicans*, 20.00 for both tested EOs. The determined MBC values (mL/mL) were in the range of 33.3 to 80.00. Tested EOs obtained from decorative lavender from Sarajevo gardens are in the same rank in terms of demonstrated intensity of antimicrobial activity compared with EOs obtained from cultivated, purpose-grown lavender presented in the literature.

1. Introduction

Since ancient times, spices and scented herbs have been used as food flavors, and their effects on human health are still being investigated to understand the roles of their chemical components. Lavender essential oil is popular as a complementary medicine and as an additive to many cosmetic products [1–3]. Nowadays, the use of essential oils as alternative therapies has gained worldwide concern due to various biological activities. Considerable attention has been devoted to lavender oil, which is widely used for its medicinal actions, including antispastic, anti-inflammatory, sedative, antimicrobial, and general tonic actions. Traditionally, it is believed to be antiseptic, antibacterial, antifungal, anti-inflammatory, carminative, sedative, anti-depressive, and effective for burns and insect bites. As major compounds, it contains linalool (20–45%), linalyl acetate (25–46%), monoterpenes, alcohols, and esters [2–4]. EO chemical composition is highly complex, and it can vary considerably depending on several factors, such as the cultivation area, environmental conditions, morphological characteristics, and processing techniques of the plant [5]. Moreover, the chemical composition influences the way in which EOs exert their biological activity [6].

In this context, the aim of this work was to evaluate the antimirobial activity of *Lavandula angustifolia* Mill. (*Lavandula officinalis* or true lavender) and *Lavandula latifolia* Vill. EOs on the Gram-(+) bacterial strain *Micrococcus luteus*, ATCC 10240, and on the fungus *Candida albicans,* ATCC 10231.

2. Material and Methods

Plant material and essential oil hydrodistillation: Flowers of *L. angustifolia* and *L. latifolia* were collected in late July in Sarajevo flower gardens. Voucher specimens were deposited in the herbarium of the Faculty of

Pharmacy- University of Sarajevo (Bosnia and Herzegovina). The essential oil was prepared by hydrodistillation for 2 hours using a Clevenger-type apparatus, according to the method recommended in the European Pharmacopoeia (1997) [7].

Testing of antimicrobial activity: The antimicrobial activity of the essential oil was tested using the agar diffusion method. In this work, Tryptic Soy Agar (TSA, Manufacturer Liofilchem S.r.l., Italy) was used for testing activity against bacteria, and Sabouraud Dextrose Agar (SDA, Manufacturer Liofilchem S.r.l., Italy) was used for testing fungi. The microorganisms used in the work were *Micrococcus luteus,* ATCC 10240, and *Candida albicans*, ATCC 10231, prepared from basic suspensions (fourth to fifth passage). In accordance with antimicrobial susceptibility testing using the EUCAST disc diffusion method (Version 11.0, January 2023) [8], from a 24-hour culture, an inoculum corresponding to a 0.5 McFarland suspension was prepared in physiological solution (by comparison with the standard photometrically at 625 nm, 1 cm, absorbance 0.08–0.13). In this way, an inoculum with approximately 1.5 x 10^8 cfu/mL is obtained for bacteria, or 5 x 10^6 cfu/mL for fungi [9].

In aseptic conditions, the previously dried agar surface (TSA for bacteria, SDA for fungi) is inoculated by evenly applying the prepared microorganism suspension, using a sterile swab that has previously been deeply immersed in the prepared microorganism suspension, and gently drained (by pressing the soaked swab against the walls of the test tube). In aseptic conditions (laminar chamber), wells are drilled in the inoculated agar with a sterile drill (Φ -6 mm). 50 µl of essential oil was placed in the wells, and for each sample and each microorganism, the test was performed in triplicate. After applying the samples, the plates were closed and left at room temperature for the oil to diffuse into the substrate for 1 hour, after which they were incubated at 35 ºC for 24 h for bacteria and at 30 ºC for fungi. After the prescribed incubation time, the inhibition zones were read (automatic inhibition zone reader, Scan® Interscience 4000). Diameters of inhibition zones (DIZ/mm±STDEV) were determined by the agar disk diffusion assay. Minimum inhibitory (MIC) and bactericidal concentrations (MBC) were determined by the microdilution method.

3. Results

In Table 1, the results of the antimicrobial activity of *Lavandula angustifolia* Mill. and *Lavandula latifolia* Vill. essential oils against the bacterial strain *Micrococcus luteus* and the fungus *Candida albicans* were presented.

Table 1. Antimicrobial activity of *Lavandula angustifolia* Mill. and *Lavandula latifolia* Vill. essential oils against *Micrococcus luteus* and *Candida albicans*

Essential oil	*Lavandula angustifolia* Mill.			*Lavandula latifolia* Vill.		
	IZ /mm ±STDEV	MIC /mL/mL ±STDEV	MBC /mL/mL ±STDEV	IZ/mm ±STDEV	MIC /mL/mL ±STDEV	MBC /mL/mL ±STDEV
***Micrococcus luteus*, ATCC 10240**	27.97±2.20	20.00±0.00	80.00±0.00	21.73±1.27	2.08±0.72	33.33±11.57
***Candida albicans*, ATCC 10231**	6.66±0.15	20.00±0.00	40.00±0.00	6.70±0.20	20.00±0.00	40.00±0.00

4. Discussion and Conclusions

Despite having a smaller zone of inhibition against the bacterial strain *Micrococcus luteus*, EO of *L latifolia* exhibits stronger antibacterial efficacy than *L. angustifolia* EO due to its lower MIC (2.08±0.72) and MBC (33.33±11.57) values. Both EOs showed similar antifungal activity against the fungus *Candida albicans*, with the same values of MIC 20.00±0.00 mL/mL and MBC 40.00±0.00, and almost the same inhibition zone diameter of 6.7 mm. Essential oils have a wide variety of bioactivities and play an important role as ideal natural sources of antimicrobial, antioxidant, and chemopreventive agents. In particular, thanks to their interesting physicochemical characteristics, Lamiaceae EOs were utilized in the industrial and medical research sectors as natural products. Most studies have focused on the two main constituents of most lavender essential oils (linalool and linalyl acetate), although other less abundant essential oil constituents (e.g., camphor, 1,8-cineole, carvacrol, etc.) have also been evaluated and showed synergistic effects along with the main components [10, 11, 12]. This research is aimed at determining the optimal concentrations of essential oils in preparations for combating bacterial infections, which can be used on daily basis. Given that the chemical profile of both examined lavender EOs does not differ significantly from the EOs of cultivated, purpose-grown lavender, the results of this research would easily find their application considering the ubiquity of lavender as an available ornamental plant.

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Conflict of Interest

The authors declare no conflict of interest.

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P-29

New advances from the Corsican Liverwort *Porella arboris-vitae*

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Keywords: *Porella arboris-vitae;* essential oils; allelopathy.

Bryophytes are the second largest group of extant land plants [1], distributed all over the world comprising approximately about 25000 species, they estimated at 1800 in Europe and 1400-1300 species in France. Based on morphological and molecular traits, bryophytes are classified into three coordinate phyla: Marchantiophyta (liverworts), Bryophyta (mosses), and Anthocerophyta (horworts) [2].

However, because of their small size and low biomass production, they are often neglected. Until now, less than 10% of bryophyte chemistry has been studied, and various terpenoids, bibenzyls, flavonoids, alkaloids, and other novel compounds have been isolated. To our knowledge, the bryophytes of Corsica have been the subject of only few phytochemical studies carried out earlier by our group [3,4,5].

The family Porellacea includes about 60 species of liverworts with a worldwide distribution. The large genus is represented in Corsica by four species: *P. arboris vitae* (=*P. laevigata*), *P. platyphylla*, *P. obtusata*, and *P. cordaeana* [6]. Our study focused on *Porella arboris-vitae*, a liverwort, due to its availability and accessibility.

To the best of our knowledge, the essential oil and hydrosol of *P. arboris-vitae* have not been previously studied. As part of our chemical investigation of Corsican bryoflora, this study focuses on the volatile components of *P. arboris-vitae* and examines its allelopathic potential.

Three sample preparation techniques, including hydrodistillation, hydrosol extraction, and cold maceration in diethyl ether, were used to produce exhaustive volatile extracts.

The identification of components began with a methodology that compared retention indices, on polar and apolar columns, and those contained in the in-house library or commercial libraries. Following this preliminary analysis, components matched by standards from the in-house library were considered as definitely identified while components matched only by commercial library database needed identification-confirmation. So, column chromatography and additional NMR experiments were carried out to achieve an unambiguous compound identification, as well as the complete NMR assignment.

*P. arboris-vita*e chemical composition analysis identified 59 metabolites, mainly oxygenated terpenes, particularly drimanes and pinguisanes compounds. Among its metabolites, we described a new alcohol as a new natural compound for the first time.

A study of the allelopathic activity of the extracts revealed the presence of phytotoxins in this plant. Sesquiterpenes lactones present in the essential oil, hydrosols and ether extracts seem to play a predominant role in its activity, though other terpenes and molecules are also involved.

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P-30

Comprehensive assessment of hydrolates produced from different medicinal plants

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Keywords: GC-HRMS, hydrolates, SPME, volatiles

Abstract

Hydrolates, also known as hydrosols or floral waters, are by-products of the production of essential oils from aromatic plants through steam distillation or hydrodistillation. This study focused on the comprehensive assessment of hydrolates produced from six medicinal plant represented by peppermint (*Mentha piperita*), feverfew (*Tanacetum parthenium*), lavender (*Lavandula angustifolia*), savin juniper (*Juniperus sabina*), common agrimony (*Agrimonia eupatoria*), and common wormwood (*Artemisia absinthium)*. To identify potential hydrolates uses, their composition and biological activities were tested. The experiments involved developing and optimizing the analytical process, testing various extraction techniques followed by gas chromatography coupled to high-resolution mass spectrometry (GC–HRMS), assessing the biological activity of hydrolates, and monitoring the stability of these products during storage.

1. Introduction

Hydrolates (also hydrosols or flower waters) are interesting secondary products of the distillation and/or hydrodistillation processes used for the isolation of essential oils from aromatic plants. [1, 2]. Characterized by unique organoleptic properties, some of them can show biological activity as well. With regards to these facts, several application such as in food industry for removing biofilms (hydrolates from bitter orange (*Citrus aurantium*) and/or thyme (*Thymbra capitata*) [3, 4] or in cosmetics hydrolates from lavender (*Lavandula angustifolia*) and/or peppermint (*Mentha piperita*) [5].

While essential oils contain mainly non-polar and some medium polar compounds, in hydrolates monoterpene oxygen compounds (terpene alcohols, aldehydes and ketones) are typically present, often together with some sesquiterpene alcohols. In any case, to understand hydrolates organoleptic properties as well as their bioactivities, deep understanding of their chemical composition is needed. The most widely used approach for isolation of interesting compounds occurring in hydrolates is liquid-liquid extraction (LLE) by organic solvents of a different polarity (e.g. n-hexane, petroleum ether, ethyl acetate)[1, 6-8]. Alternatively, solvent-free techniques such as "head space" (HS) [6, 9], solid phase microextraction (SPME) [4, 10] and "purge and trap" automatic thermal desorption (P&T ATD) [7] are applicable for this purpose. For separation of complex volatile mixtures occurring in essential oils /hydrolates, gas chromatography (GC) technique either with flame ionization detector (FID) [11, 12] or a mass spectrometric detector (MS) is used [1, 8, 13, 14].

While a countless number of publications on the composition of essential oils and their uses has been published [15], significantly less attention has been paid in this respect to hydrolates, moreover, studies concerned with correlation between these two attributes are rare [1, 16].

In this study, we aimed to investigate in depth the volatile profiles of hydrolates obtained from medicinal plants representing various families/genera. For this purpose, SPME-GC-HRMS technique was applied. Attention was also paid in the dynamics on volatiles transfer into hydrolates during hydrodistillation. To get a deeper insight into the quality and potential uses of hydrolates, their biological activity of hydrolates was tested, too.

2. Material and Methods

Hydrolates used for investigation were produced by industrial partner from six medicinal plant represented by peppermint (*Mentha piperita*), feverfew (*Tanacetum parthenium*), lavender (*Lavandula angustifolia*), savin juniper (*Juniperus sabina*), common agrimony (*Agrimonia eupatoria*), and common wormwood (*Artemisia absinthium)*. All samples were represented by fractions taken at regular time intervals for the purpose of monitoring the composition of volatile substances during the production process.

In this study two extraction techniques were applied: head-space solid-phase microextraction (HS-SPME) and

solid phased extraction (SPE) to methanol using HLB sorbent.For the HS–SPME extraction, SPME fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm, 1 cm) from Supelco (Bellefonte, USA) was used. For the solid phase extraction (SPE) Oasis HLB 6cc (500 mg) LP Extraction Cartridge (Waters, USA) were employed. The Agilent 7200B system consists of an Agilent 7890B gas chromatograph equipped with a multimode inlet, PAL RSI 85 for automated headspace–solid phase microextraction (HS–SPME) and direct injection, and quadrupole – time of flight mass spectrometer (Q-TOF) (Agilent Technologies, Palo Alto, California, USA) was used. Chromatographic separation was performed at the HP–5MS (30 m x 250 μm x 0.25 mm) capillary column.

The antimicrobial activity of hydrolates was assessed using the microdilution method, which allowed for determining the minimum inhibitory concentration (MIC) and the concentration that inhibits activity by half (IC50). A collection of microorganisms, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Propionibacterium acnes*, was employed to evaluate antimicrobial activity. Consequently, the next phase of the experiment involved testing the hydrolate samples for their ability to inhibit the germination of spores of micromycete fungi, specifically *Aspergillus niger*.

3. Results

In the first part of the experiments, an analytical method was developed and optimized for characterization of bioactive compounds occurring in hydrolates produced from selected medicinal plants. During the optimization of procedure of volatiles isolation / pre-concentration, two approaches were compared: (i) solid-phase microextraction (SPME) and (ii) solid-phase extraction (SPE) using HLB sorbent. GC-HRMS analysis was subsequently performed for both extraction methods.

To learn more about the volatiles transfer from raw material into hydrolate and to find out optimal conditions, all fractions collected during hydrodistillation process were analysed using the developed SPME-GC-HRMS method. The number of volatile compounds was detected in the fractions of hydrolates from wormwood (n=855) and lavender (n=450). When comparing volatile profiles of the individual fractions, then the optimal distillation times (based on the amount of volatiles as criterion), fairly differed depending on processed plant material. For example, for wormwood hydrolate, the highest number of volatiles was detected in the fraction taken after 120 minutes of the production process (n=855), while for peppermint, the highest amount of volatiles (n=310) was detected in the fraction taken after 20 minutes of the distillation.

As mentioned in the Introduction, hydrolates are believed to possess a number of beneficial bioactivities, antimicrobial being was one of them involved in our testing. Selected microorganisms, *Staphylococcus aureus, Pseudomonas aeruginosa*, *Candida albicans and Propionibacterium* representing various genera were used, however, no distinct inhibition activity was observed, probably due to a low concentration of bioactive compounds. For this reason, their pre-concentration using SPE method was performed. Under such conditions, weak antimicrobial activity against *Propionibacterium acnes* was detected in the case of lavender extract. In addition, inhibition of spores' germination was observed, the strongest effect was also found for lavender.

4. Discussion and Conclusions

The combination of a knowledge about the chemical composition with results of bioactivities testing is essential for a complex assessment of the hydrolate quality and understanding its potential beneficial effects.

In the follow-up experiments, not only extended antimicrobial activities of various hydrolates will be tested, but also a range of other bioassays will be applied to search for correlation with the bioactive compounds occurring in respective hydrolate.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-31

Effects of different preservation methods on the active substances and colour properties of Lemonbalm (*Melissa officinalis* L.) leaves

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Keywords: drying, lyophilization, freezing, microwave, essential oil

Abstract

The aim of this study was to determine the effect of different preservation methods (shade drying, oven drying at 40 and 60ºC, lyophilization, microwave drying at 250 and 700 W, freezing) on the active substances (EO content and composition, TPC, TAC) and colour characteristics of *Melissa officinalis* leaves. Variety 'Lemona' was used for the experiment. According to the results, it was established that the gentle convective drying methods (oven drying at 40°C and shade drying) preserved the EO content very well, the TPC and TAC relatively well, but caused some degradation in colour. Freezing also retained the volatiles and colour of leaves perfectly, but reduced the TPC and TAC to a considerable extent. Microwave drying methods resulted in an almost complete loss in volatile content and significant changes in the composition, but were able to conserve the phenolic compounds, especially at higher power (700 W). Drying at 60°C significantly reduced both the EO content and the amount of antioxidant phenolic compounds, furthermore leaves almost completely lost their green colour, so this method was definitely not recommended for preservation. In contrast, lyophilization proved to be a very successful technique in all aspects, being able to keep not only the active ingredients of lemonbalm leaves, but also its original green colour.

1. Introduction

Melissa officinalis L. is one of our oldest medicinal plants belonging to the *Lamiaceae* plant family. Primarily its pleasant, lemon-flavoured leaves (*Melissae folium*) are used in the medicine and gastronomy. The leaves contain 0.1-0.5% essential oil (EO) in external glandular hairs, but also contain flavonoids, hydroxycinnamic acid derivatives and triterpenes. The aromatic leaves are generally used in dried form, but the method of preservation can significantly affect their nutritional and organoleptic properties. The aim of this study was to find the best methods to conserve the colour characteristics and the most important active substances present in lemonbalm leaves.

2. Material and Methods

Plant material

Melissa officinalis variety 'Lemona' was used for the research. The 2-year-old plant stand established in the Experimental Field of the Department in Soroksár, Hungary was examined in 2021. Approximately 5 kg of fresh leaves were harvested at the end of flowering (in August). The homogeneous plant material was divided into eight parts for the different treatments.

Applied preservation methods

In the experiment, seven preservation methods (drying in shade, oven drying at 40 and 60ºC, lyophilization, microwave drying at 250 and 700 W, freezing) were investigated in comparison to the freshly harvested plant material (control). For shade drying, the air temperature was 24-30°C during the day and 17-22°C at night.

Chemical analyses

In case of EO content determination, 50 g fresh and frozen leaves, furthermore 20 g of each dried samples were hydro distilled for 2 hr in a Clevenger-type apparatus in 3 replications.

The EO composition was carried out in three replications per treatment using an Agilent Technologies 6890N instrument equipped with an HP-5MS capillary column and an Agilent Technologies MS 5975 inert mass selective detector. Te appiled temperature program was: initial temperature 60°C, heating at a rate of 3°C/min up to 240°C, the final temperature was kept for 5 min. Carrier gas was helium (constant flow rate: 1 $mLmin^{-1}$).

The total phenolic content (TPC) was determined from the aqueous extracts prepared from lemonbalm samples by using the modified method of Singleton and Rossi (1965). The total antioxidant capacity (TAC) of the same extracts was measured using FRAP method according to the modified method of Benzie and Strain (1996). Three

parallel measurements were performed from the three biological replications for each treatments. Values obtained in each chemical analyses were referenced to the dry matter content of the samples.

Colour measurement

Colour of leaves was measured in six replications using a Konica Minolta CR-410 tristimulus colorimeter. L* (lightness), a* (± red/ green) and b* (± yellow/ blue) values were recorded and a*/b* data was calculated.

3. Results

In fresh lemonbalm leaves 0.50 ml/100 g EO content was found (Figure 1). Lyophilization, the gentle drying methods using low temperatures (drying in shade and at 40°C), furthermore freezing could preserve the original EO content very well. However, drying at 60°C and by microwaves at 250 and 700 W reduced the amount of volatiles by 72-95%.

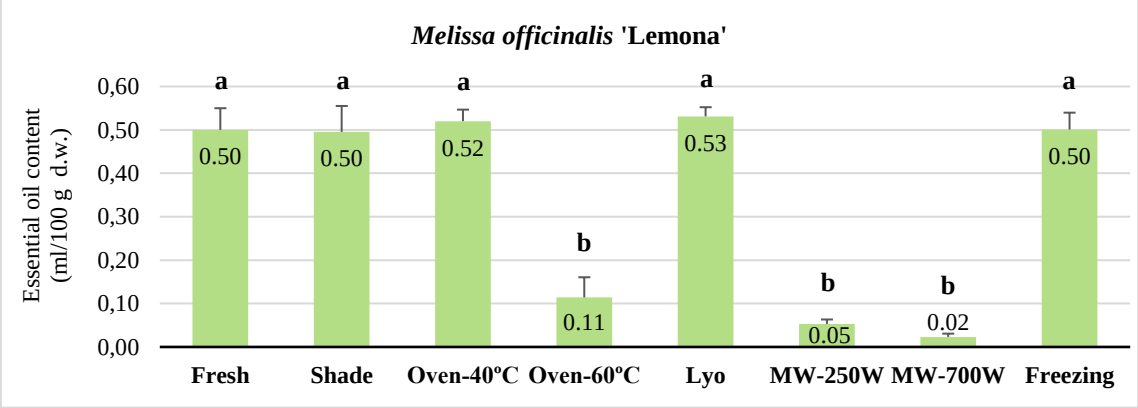

Figure 1 Essential oil content of fresh and preserved leaves of *Melissa officinalis* L. 'Lemona'

†Different letters indicate significant differences between means at $p < 0.05$. †Fresh = Fresh leaves; Shade = Shade drying; Oven-40°C = Oven drying at 40°C; Oven-60°C = Oven drying at 60°C; Lyo = Lyophilization; MW-250W = Microwave drying at 250 W; MW-700W = Microwave drying at 700 W.

In fresh lemonbalm leaves, the major volatile compounds identified were geranial (40.66%), neral (29.60%), citronellal (6.31%), geraniol (5.59%) and citronellol (4.60%) (Table 1). Examining the effect of preservation methods, every applied treatments significantly reduced the ratio of citronellol and geraniol (by 77-95% and 88-99%, respectively), and most of the treatments increased the proportion of citronellal (by 33-62%) too.

According to data, microwave drying methods caused the most spectacular changes in the volatile composition. Both the applied wattages (250 and 700 W) significantly reduced the ratio of citronellol, citronellal and geraniol, but notably increased the proportion of sesquiterpenes (e-caryophyllene, caryophyllene oxide) (Table 1).

Table 1 Ratio of the main EO compounds of fresh and preserved leaves of *Melissa officinalis* L. 'Lemona'

Compounds	LRI	Total GC area percentages (%)							
		Fresh sample	Shade drying	Oven drying at 40°C	Oven drying at 60°C	Lyo-phili-zation	Micro-wave at 250W	Micro-wave at 700W	Free-zing
Citronellal	1155	**6.31**	**10.12**	**10.91**	**5.53**	**8.38**	**3.18**	**2.07**	**10.21**
E-Isocitral	1178	1.21	1.10	1.56	1.02	1.50	1.05	0.68	1.26
Citronellol	1223	**4.60**	**0.24**	**0.80**	**1.05**	**0.49**	**0.39**	**0.35**	**0.44**
Neral	1238	**29.60**	**33.26**	**33.11**	**32.37**	**34.41**	**28.18**	**26.60**	**32.07**
Geraniol	1252	**5.59**	**0.05**	**0.60**	**0.66**	**0.38**	**0.40**	**0.32**	**0.19**
Geranial	1268	**40.66**	**46.11**	**43.33**	**46.73**	**43.75**	**40.20**	**40.44**	**45.24**
8-Hydroxyneomenthol	1324	2.08	1.25	1.51	1.19	1.13	1.34	0.74	1.66
Citronellyl acetate	1354	1.29	0.77	0.80	0.59	0.61	0.89	0.11	0.94
Geranyl acetate	1388	1.01	0.65	0.79	1.43	0.79	1.48	1.33	0.88
e-Caryophyllene	1420	1.64	1.30	1.02	1.32	1.40	**12.98**	**15.72**	1.20
Caryophyllene oxide	1590	1.09	0.81	0.57	2.28	0.44	**3.82**	**5.42**	0.76

Note: Major compounds are shown in bold.

The TPC in fresh lemonbalm leaves of variety 'Lemona' was measured to be 280.1 mg GAE/g d.w. (Figure 2). Microwave drying at 700 W, lyophilization and drying in shade proved to be the best methods in preserving the

phenolic compounds (160.7-205.2 mg GAE/g d.w.). The worst techniques was found to be microwave drying at 250 W, oven drying at 60°C and freezing, which caused 67-74% loss.

The TAC of aqueous extract made from fresh leaves was 286.0 mg AAE/g d.w. (Figure 2). In this case shade drying, microwave dryings (700 W, 250 W) and lyophilization resulted in the highest values (200.8-282.2 mg AAE/g d.w.), whereas oven drying at 60°C, furthermore freezing reduced the samples' TAC the most (by 64-89%). A strong positive, linear relationship (r = 0.80) was observed between TPC and TAC.

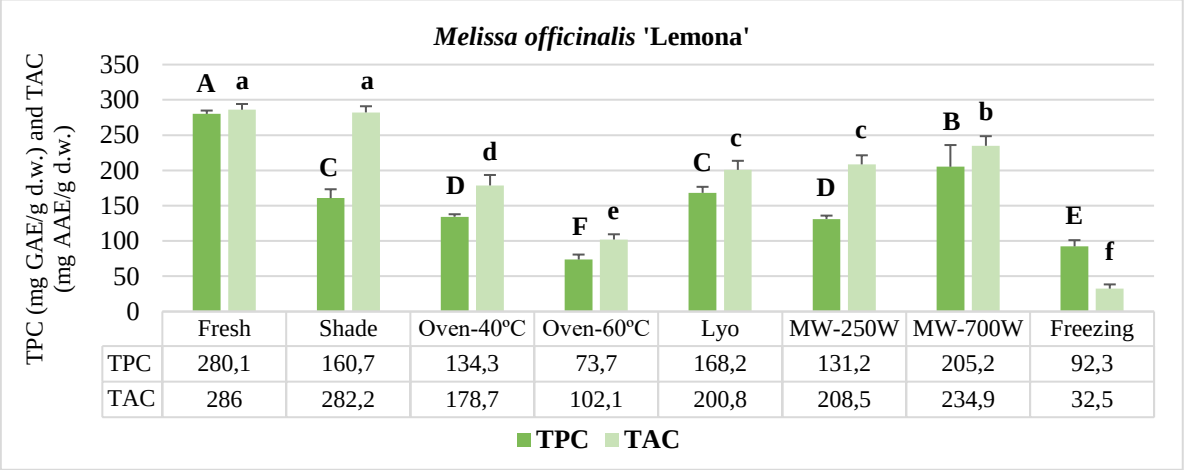

Figure 2 Total phenolic content (TPC) and total antioxidant capacity (TAC) of fresh and preserved leaves of *Melissa officinalis* L. 'Lemona' †Different letters indicate significant differences between means at $p < 0.05$. †Fresh = Fresh leaves; Shade = Shade drying; Oven-40°C = Oven drying at 40°C; Oven-60°C = Oven drying at 60°C; Lyo = Lyophilization; MW-250W = Microwave drying at 250 W; MW-700W = Microwave drying at 700 W.

Examining the colour changing, lyophilization resulted in the highest L* value (37.5) for lemonbalm variety 'Lemona', indicating that lyophilized leaves became a little lighter in appearance than in fresh state (L*=33.4). Oven drying at 60°C and freezing had the lowest L* values, suggesting darker hues (Table 2).

The calculated a*/b* ratio shows the proportion of the two coordinates. The larger and more negative the ratio, the more intense is the green colour of the sample. In the experiment, lyophilization (-0.4) and freezing (-0.4) maintained the original hue (-0.5) the most. Oven drying at 40°C, furthermore microwave drying at 700 W also proved to be good at preserving the natural colour. In contrast, drying at 60°C resulted in the highest a*/b* value, causing the biggest colour degradation.

Table 2 Colour characteristics of fresh and preserved leaves of *Melissa officinalis* 'Lemona'

Colour characteristics	Fresh sample	Shade drying	Oven drying at 40°C	Oven drying at 60°C	Lyophi-lization	Micro-wave at 250 W	Micro-wave at 700 W	Free-zing
L* (lightness)	33.4^{b}	28.5^{cd}	29.6^{cd}	27.6^{d}	37.5^{a}	28.7^{cd}	31.9^{bc}	26.6^{d}
a* (red/green)	-7.8^{a}	0.3^{e}	-0.1^{e}	2.0^{f}	-4.5^{bc}	0.3^{e}	-1.5^{d}	-3.6^{c}
b* (yellow/blue)	15.0^{a}	8.0^{d}	7.7^{d}	7.6^{d}	11.6^{b}	9.5^{bcd}	11.4^{b}	9.2^{cd}
a*/b*	-0.5^{a}	0.1^{d}	-0.1^{cd}	0.3^{e}	-0.4^{b}	0.1^{d}	-0.1^{c}	-0.4^{b}

†Different letters indicate significant differences between means at $p<0.05$ within characteristics (within rows).

4. Conclusions

Based on our results, lyophilization was found to be the best preservation method for lemonbalm leaves, as it retained both the volatile components and phenolic compounds very well, and even kept the original green colour of leaves in high level. However, drying at 60°C cannot be recommended in any way, as it had a negative effect on all the characteristics studied. The other preservation methods had advantages and also disadvantages, so their applicability depends on the quality of the desired final product.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-32

In vitro antimicrobial evaluation of *Salvia sclarea* and *Origanum vulgare* essential oils in combination against sinusitis pathogens

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Keywords: Anti-sinusitis, essential oil combination, synergy

1. Introduction

Sinusitis is a common pathology that arises from a variety of causes including microbial infections within the upper respiratory tract. In this present study, commercial *Salvia sclarea* L. and *Origanum vulgare* L. essential oils, which are documented to have ethnobotanical were used. Various systematic essential oil combinations were prepared for targetted antimicrobial evaluation.

2. Material and Methods

The commercially available essential oils were analysed by GC-MS and GC-FID, for their quality. *In vitro* antimicrobial evaluation of the oils against methicillin-resistant *Staphylococcus aureus, Staphylococcus aureus* ATCC 23235, *Moraxella catarrhalis* ATCC 43617 and *Streptococcus pneumoniae* ATCC 6313, were performed using a microdilution assay. Combinations of the essential oils were evaluated using the checkerboard method. Active samples were then prepared for nasal spray formulations and the effects were challenged and compared also using the agar-well diffusion method. The in vitro safety of the essential oil nasal formulation was evaluated using the MTT method against L929 cell lines.

3. Results and Discussion

Analyses of *O. vulgare* essential oil resulted in 73% carvacrol as the major component, whereas the main component of *S. sclarea* oil was 67% linalyl acetate among other components determined, respectively. The calculated fractional inhibition concentration index (FICI) of combinations against *M. catarrhalis* resulted as synergistic (FICI value: 0.259), while the FICI value of the combinations against methicillin-resistant *S. aureus, S. aureus* ATCC 23235, and *S. pneumoniae* ATCC 6313, were found additive with 0.51, 0.509 and 0.508 FICI values, respectively. The tested two essential oils were relatively more effective in combination against all tested pathogens. No in vitro toxic effects of the formulation up to a concentration of 2 mg/mL was observed on healthy cell line evaluation. To the best of our knowledge, this is the first oregano-clary sage combination with the potential as nasal anti-sinusitis formulation.

4. Conclusions

The initial promising in vitro results of the sinusitis preparation needs to be further optimized and developed, confirmed by *in vivo* and clinical studies as well.

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P-33

Terpenoids diversity of Cannabis essential oil

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Keywords: GC/MS, Cannabis, essential oil, terpenes

Introduction

The GC/MS analysis of Cannabis essential oil has provided valuable insights into its chemical composition, highlighting the abundance and diversity of terpenoids. These compounds which exhibit a broad spectrum of biological and pharmacological activities such as antifungal, antiviral, anticancer, anti-inflammatory, antihyperglycemic, antiparasitic, antioxidant, and antimicrobial properties, play a significant role in the oil's therapeutic potential [1].

Material and Methods

Hydrodistilation of Cannabis essential oil isolated from dried flowers was performed in Clevenger apparatus according to the method 2.2.13 given in the European Pharmacopeia 11.0. An optimized GC/MS method was used to determine the chemical composition of the essential oil.

Results

GC/MS analyses resulted in identification of 35 components representing 99.02% of the total essential oil amount. The chemical composition was primarily categorized into three fractions: monoterpenes (up to 79.40%), sesquiterpenes (18.17%), and diterpenes (1.45%). Each fraction consists of various compounds with distinct chemical structures and properties. Among the monoterpenoids, β-myrcene emerged as the most abundant compound, constituting 42.69% of the monoterpenoid fraction and standing out as the most prevalent terpenoid overall. α-Pinene (29.58%), limonene (3.60%) and β-pinene (1.24%) while present in lower proportions compared to β-myrcene, still contributed significantly to the total composition of the monoterpenoid fraction. The most abundant sesquiterpene was germacrene D (12.42%), followed by α-humulene (1.10%) as a second predominant compound, all other were below 1%. Identified components within the diterpenoids fraction (all bellow 1%) were: pimaradiene, manool oxide, abietatriene, sandaracopimarinal and sandaracopimarinol, with percentage ranging from 0.07% to 0.73% for abietatriene and sandaracopimarinal, respectively.

Conclusions

Detecting specific terpenoids as well as determining their percentage representation in Cannabis essential oil provides researchers crucial knowledge for chemical isolation of predominant compounds. Furthermore, this leads not only to the clarification of the biological activity and pharmacological effects of Cannabis essential oil, but also to optimization of its applications in pharmaceuticals, cosmetics, and aromatherapy.

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P-34

Antibacterial and antibiofilm effect of fennel essential oil, fennel honey and their combination

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Keywords: fennel, antibiotic resistance, essential oil, honey, antibiofilm effect

Abstract

The irresponsible overuse of antibiotics has increased the occurrence of resistant bacterial strains, which represents one of the biggest patient safety risks today. Due to antibiotic resistance and biofilm formation of bacteria, it is becoming increasingly difficult to suppress the bacterial strains responsible for various chronic infections. *P. aeruginosa* is an opportunistic, nosocomial, biofilm-forming pathogen. It does not attack the body of a healthy person, or very rarely, but poses a threat to a weakened or immune-deficient body or a body suffering from a chronic disease, so it can cause an extremely large problem in health institutions. It is important to find an alternative, complementary therapy that can degrade biofilms and reduce the spread of resistance.

Many essential oils and honeys have antibacterial and antibiofilm properties, so we aimed to explore the antibacterial and antibiofilm effects of fennel essential oil (FEO), fennel honey (FH) and their combination.

We first determined the minimum inhibitory concentrations (MIC) for FH (142.86 mg/mL) and FEO (2.5 mg/mL). Gentamicin was used as a positive control, with a MIC of 2µg/mL. FH displayed a biofilm inhibition rate of 75.3%, FEO resulted 79.4% inhibition, and the combination of FH and FEO 83.9% against bacterial biofilms formed by *P. aeruginosa*.

In summary, the antibacterial and biofilm-inhibiting effects of FEO and FH were supported by *in vitro* microbiological methods. The combined use of fennel essential oil and honey may be suitable for the prevention of nosocomial diseases and the control of the spread of *P. aeruginosa*.

1. Introduction

Today antibiotic resistance, which is related, among other things, to the biofilm-forming property of bacteria, causes a lot of problems in healthcare. When bacteria form a biofilm, they are better protected against external influences, and are much more resistant to different factors, e.g. disinfectants and antibiotics [1]. *Pseudomonas aeruginosa* is an opportunistic, biofilm-forming pathogen known to cause a variety of infections, particularly in immunocompromised individuals. Being a Gram-negative bacterium, there is a lipopolysaccharide (LPS) layer in its cell wall, which increases the negative charge of *P. aeruginosa* cells and gives them structural integrity, making them more resistant to various chemicals [2]. It does not, or very rarely, attacks the body of a healthy person, but it poses a threat to a weakened or immunodeficient body or a body suffering from a chronic disease, so it can cause an extremely large problem in healthcare institutions. *P. aeruginosa* can cause inflammation in almost all tissue types. The pathogen typically enters the body by means of carrier objects, contaminated drinking water or food. In most cases, it causes inflammation of the ear canal and of hair follicles, but infection can also occur when using contaminated contact lenses. During hospital treatments and interventions, the patients most at risk are those on a ventilator or having a catheter, a surgical or burn wound. During infection, meningitis and blood poisoning can occur. Bacteria introduced by catheters can cause urinary tract infections, while infections acquired during intubation and in intensive care units can cause severe pneumonia [3]. Eradication of the bacterium is possible with antibiotic treatment, but due to its resistance and biofilm-forming ability, the infection can cause serious therapeutic problems [4]. During our research, we aimed to study the antibacterial and antibiofilm effects of fennel essential oil (FEO), fennel honey (FH) and their combination against *P. aeruginosa* ATCC 27853.

2. Material and Methods

For the analysis of the chemical constituents of essential oils, the European Pharmacopoeia VIII prescribes gas chromatography (Ph. Eur. 10.0). In order to determine the composition of FEO, GC-MS (gas chromatography

coupled to a mass spectrometer) was performed. To determine the composition, retention times and mass spectra were used for each component, which were compared with standard values and data from the NIST 2.0 library. Area normalization was used to determine percentages [5]. The parameters of the GC-MS are summarized in Table 1.

Table 1. Parameters for GC-MS analysis

	GC-MS
Appliance	Agilent 6890N
Column	30x0.25 mm i.d, Agilent SLB-5MS (film thickness 0.25 μm)
Program	60°C 3 min, 8°C/min 60-250°C, 250°C 1 min
Carrier gas	high purity helium 6.0, 1.0 mL/min, (37cm/s), constant flow
Injector	250°C
Injection	Split ratio 1:50
Detector	5973N (MS)

The botanical origin of fennel honey was confirmed by melissopalynological analysis, performed in the same manner as described in Balázs et al. [6]. The pollen preparations were examined with a Nikon Eclipse E200 microscope (Auro-Science Consulting Kft., Budapest, Hungary) at 400× magnification. The quantitative assessment was carried out by counting at least 500 pollen grains, identifying the source plant at species, genus or at least family level. The reference tool used was the Bee Pollen Atlas 1.0, which was developed specifically for Hungarian honey samples. The percentage of pollen types was calculated.

During the microbiological tests, *P. aeruginosa* was incubated in a shaker incubator at 37°C and at a speed of 60 rpm for 12 h in Brain Heart Infusion (BHI) [7]. The minimum inhibitory concentrations (MIC) were determined using the microdilution method on 96-cell microtiter plates (10^5 CFU/mL) [8]. After the incubation period, absorbance values were measured at 600 nm using a microplate reader (BMG Labtech). Antibiotic (gentamicin) was used as positive control. Bacterial biofilms were formed on 96-cell polystyrene microtiter plates. 200 μL of a cell suspension with a cell count of 10^8 CFU/mL was measured into each cell. After incubation (4 h, 37°C), planktonic cells were washed with physiological saline and the sample to be tested (FEO, FH, combination of FEO and FH) was added to the biofilms (MIC/4). In order to solve the FEO in BHI, Tween40 emulsifier was used (1%). After the treatment (24 h, 37°C), 0.1% crystal violet (CV) solution was measured into the cells [9]. CV binds to negatively charged surface molecules and polysaccharides within the extracellular matrix of biofilms, thus allowing measurement of the total biomass of the biofilm in the cell of the microtiter plate. After incubation time of 20 min, absorbance was measured at 595 nm using a plate reader.

3. Results

The GC-MS results showed that the main component of FEO was anethole (73.1%), and a large amount of fenchone (11.5%) was detected. 14 components were detected in FEO, the components above 1% are summarized in Table 2.

Table 2. The results of GC-MS in case of FEO

Compounds	**KI**	**tR (min)**	**Ratio of the compounds**
α-Pinene	923	5.1	3.9%
Limonene	1020	7.2	3.0%
Fenchone	**1083**	**8.6**	**11.5%**
α-Terpineol	1191	10.9	4.5%
Anethole	**1292**	**12.9**	**73.1%**

KI: Kovats-index, tR: retention time

From the pollen spectrum of FH, it can be seen that fennel (*Foeniculum vulgare*) pollen was present as the dominant pollen, while linden (*Tilia* sp.), sunflower (*Helianthus annuus*) and rape (*Brassica napus*) pollen were also observed in the sample (Table 3).

Table 3. Pollen spectrum of fennel honey

F. vulgare	*Tilia* sp.	*H. annuus*	*B. napus*	Other
42.5	27.4	12.9	8.1	9.1

The minimum inhibitory concentration of the essential oil was 2.5 mg/mL, the MIC value of honey was 142.86 mg/mL against *P. aeruginosa*. The MIC value of the antibiotic gentamicin, which served as a positive control, was 2 μg/mL. The antibiofilm activity of FEO was more pronounced (79.4%) compared to FH (75.3%). The highest inhibition rate was achieved with the combination of FH and FEO (83.9%). The inhibition rate values are illustrated in Figure 1.

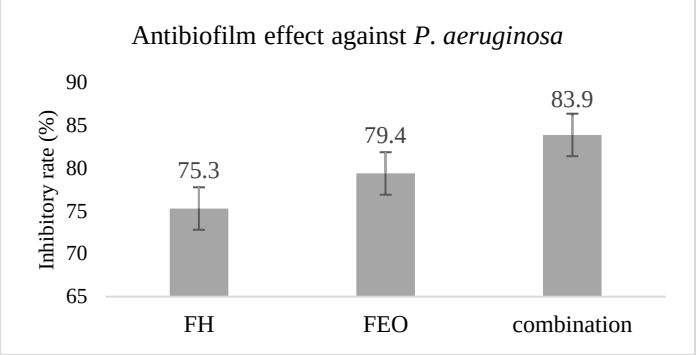

Figure 1. Antibiofilm effect of fennel honey (FH), fennel essential oil (FEO) and their combination against *P. aeruginosa*. Inhibitory rate = (1 - S/C) ×100% (C and S were defined as the average absorbance of control and sample groups, respectively) [9].

4. Discussion and Conclusions

Fennel essential oil and fennel honey have proven antibacterial properties [10,11]. Most of the research has focused on essential oils, with little evidence so far available for honey. To date, no literature has been available on the anti-*Pseudomonas* effect of FEO and FH. There are currently no data on the biofilm degrading effect of honey and the antibacterial and biofilm inhibitory effect of essential oil-honey combinations. Our research team is the first to investigate the biofilm inhibitory effect of FEO, FH and their combination. Our results show that they have an inhibition rate individually, but the highest antibiofilm effect was achieved when they were combined. Nowadays, when antibiotic-resistance is a worldwide problem, it is extremely important to study natural substances and their mode and points of action in/on bacterial cells.

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Conflict of Interest

The authors declare no conflicts of interest.

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P-35

Diversity of aroma characteristics of *Alpinia zerumbet* and differences from its closely related species

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Keywords: Alpinia zerumbet, Alpinia zerumbet var. excelsa, Alpinia formosana, chemotype

Abstract

Alpinia zerumbet (AZ) is a perennial herb of the *Zingiberaceae* family, widely distributed in tropical and subtropical regions worldwide. The essential oil of *Alpinia spp*., produced in Okinawa, includes AZ and its variants: *A. zerumbet* var. *excelsa* (AZe) and *A. formosana* (AF). Recent reports indicate natural hybridization within this genus in Taiwan. The study revealed significant variation in aroma characteristics among individual plants, with numerous chemotypes identified within each species. This study aimed to determine the chemical characteristics of these species based on the aromatic properties of AZ and its related species, derived from a wide range of intrinsic aromatic properties. *Alpinia spp*. were collected from 339 individual plants across 21 Ryukyu Islands, and their aroma characteristics were analyzed using DHS-TD-GC/MS, principal component analysis (PCA), and hierarchical cluster analysis (HCA). The aroma profiles of AZ, AZe, and AF varied significantly among individuals and were largely independent of regional and weather conditions. Compounds such as α-thujene, sabinene, α-terpinene, γ-terpinene, and terpinen-4-ol were particularly abundant in AZe, distinguishing it from AZ and AF. Detailed analysis of individual aroma characteristics allowed for the identification of species and the detection of contamination through the presence of specific essential oil components.

1. Introduction

Alpinia zerumbet (Pers.) Burtt and Smith, perennial herbs of the *Zingiberaceae* family, are widely distributed in tropical and subtropical regions, including India, Malaysia, Taiwan, Brazil, and Japan. In Japan, the species is prevalent from southern Kyushu to the Ryukyu Islands. Characterized by their fragrant flowers, leaves, and rhizomes, the essential oils extracted from them are used as fragrance and cosmetic ingredients due to their pleasant aroma and strong antioxidant effects. The essential oils of Alpinia spp., produced in Okinawa, are derived from *A. zerumbet* (AZ) and its variants, *Alpinia zerumbet* var. *excelsa* (AZe)—noted for its slightly larger flowers and longer stems [1] than AZ [1], and *Alpinia formosana* (AF). These oils find extensive use in the food and cosmetic industries, as well as in folk medicine, for their aromatic and antioxidant properties. Recent studies have reported that five species of *Alpinia spp*. naturally hybridize within the genus in Taiwan [2]. The results demonstrate considerable variability in aroma characteristics among individual plants, with multiple chemotypes present within each species [3]. Due to this variability and the diverse morphologies of flowers, leaves, and other traits, identifying species based solely on aromatic properties is particularly challenging. Thus, this study aims to elucidate the chemical characteristics of these species by examining the extensive range of individual-specific aroma characteristics.

2. Material and Methods

AZ was collected from 225 individual plants across 21 Ryukyu Islands between May 2017 and March 2023. For comparison, data on the aromatic characteristics of AZ cultivated in Tokushima Prefecture in June 2022 were also used. AZe was collected from 52 individual plants across five Ryukyu Islands between February 2018 and March 2024. AF was collected from 52 individual plants across four Ryukyu Islands between July 2017 and June 2019. After collection, the leaves and stems were separated, cut, and dried at 45 °C until the moisture content reached 10% or less. Then, 0.2 g of each leaf and stem sample was placed in a 27 mL vial and heated at 60 °C for 10 min. Air was then forced through activated carbon at 100 mL/min for 10 min to capture the aroma in TENAX TA-filled glass tubes. The dynamic headspace method and thermal desorption-gas chromatography–mass spectrometry (DHS-TD-GC/MS; GCMS- QP2010Plus, TD-20, column: DB-WAX 60 m × 0.32 mm i.d., 0.5 μm) were employed to analyze the collected aromatic components. The identified aromatic components were subjected to principal component analysis (PCA) and hierarchical cluster analysis (HCA) using SIMCA (ver. 13.01; UMETRICS) to determine the chemical composition of the fragrances of the *Alpinia spp*. Volatile components were identified by comparing their retention indices and mass fragmentation patterns with MS libraries (NIST05 and FFNSC Library

ver. 1.2; Shimadzu, Kyoto, Japan). Linear retention indices were determined for all constituents using a homologous series of *n*-alkanes (C8–C24) injected under the same chromatographic conditions as the samples.

3. Results and discussion

Previous studies have demonstrated that the aroma characteristics of AZ, AZe, and AF differ among individuals and are almost independent of regional and weather conditions. AZ, AZe, and AF were classified into 19, 5, and 9 groups, respectively, based on PCA and HCA of aroma characteristics for individuals collected from 21 islands in the central and southern Ryukyu Islands. Figure 1 shows representative chromatographs of AZ, AZe, and AF. The number and concentration of components in AZ, AZe, and AF varied widely among individuals, as did flower morphology. Of the 112 components found in more than 0.1% of AZ samples, 105 were not present in the other plants. Conversely, 76 components in AZe and 106 in AF were commonly found in AZ. The 37 components absent in AZe were also missing in some AZ individuals, making it impossible to identify species or varieties based solely on the presence or absence of specific components. Thus, from the analysis of 329 individuals, the minimum, maximum, and mean values of each component were determined for each species, and components with clear differences between the maximum and minimum values were extracted (Table 1).

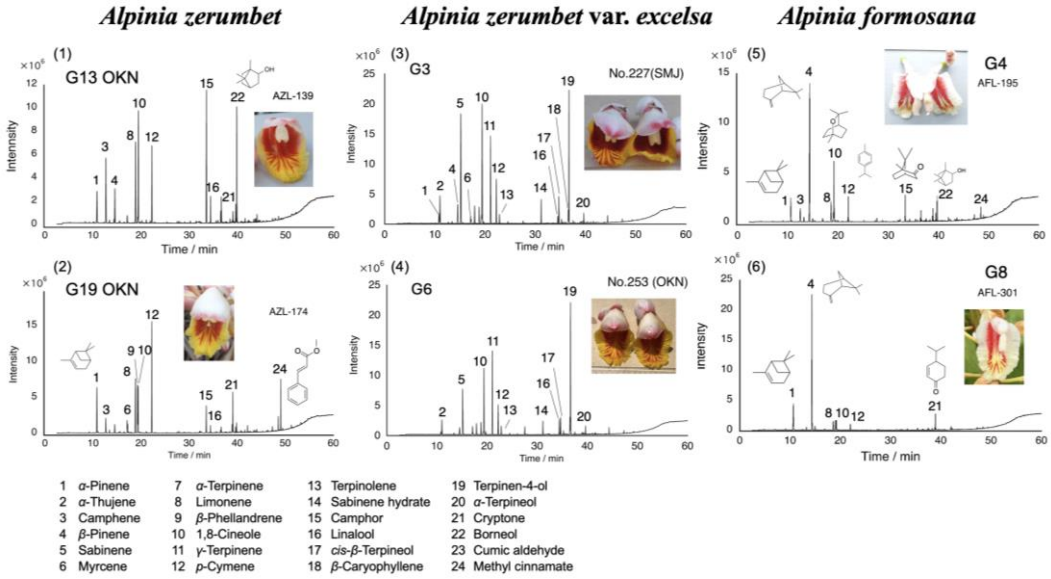

Figure 1 Chromatogram of (1), (2); *A. zerumbet*, (3), (4); *A. zerumbet* var. *excelsa*, (5), (6); *A. formosana*.

α-Thujene, sabinene, α-terpinene, γ-terpinene, and terpinen-4-ol were particularly abundant in AZe, suggesting they can distinguish AZe from AZ and AF based on their content. γ-Terpinene was also notably prevalent in AZe, indicating its potential use in distinguishing the essential oils of AZe from other species. The mean β-pinene content in AZ is 2.20%, whereas in AF it is 35.05%; AZe contains a maximum of only 2.92%, suggesting that AF can be easily distinguished from other species. Species discrimination is particularly straightforward for AF, as its floral morphology clearly differs from that of the other two species. While 4 out of 225 AZ individuals had β-pinene concentrations above 10%, and 4 out of 52 AF individuals had concentrations below 10%, it is necessary to mitigate contamination among individuals to stabilize the essential oil composition.

Table 1. Volatile compounds characterizing Alpinia spp**..**

RI†	Compound	*Alpinia zerumbet*			*Alpinia zerumbet* var. *excelsa*			*Alpinia formosana*		
		Relative Conc. (%)								
		Min.	Max.	Ave.	Min.	Max.	Ave.	Min.	Max.	Ave.
1029	α-Thujene	0.00	0.54	0.23	**2.44**	**7.91**	**3.42**	0.00	0.32	0.16
1113	β-Pinene*	0.43	**12.11***	2.20	0.20	2.92	1.29	**2.35****	**73.51**	**35.05**
1126	Sabinene	0.00	1.10	0.06	**4.13**	**27.49**	**12.04**	0.00	3.98	1.65
1186	α-Terpinene	0.00	0.27	0.03	**0.96**	**5.30**	**2.63**	0.00	0.24	0.05
1253	γ-Terpinene	0.00	0.88	0.17	**5.17**	**17.54**	**13.24**	0.00	0.42	0.16
1620	Terpinen-4-ol	0.00	3.55	1.47	**4.19**	**26.97**	**16.13**	0.00	2.48	0.89

†; RI: Retention index relative to *n*-alkanes on the DB-WAX column.

*AZ; 4 of 225 indivisual plants with *β*-Pinene concentration greater than 10%.

**AF; 4 out of 52 indivisual plants with *β*-Pinene concentrations below 10%.

4. Conclusions

Since 2015, a chemotaxonomic study has been conducted on the scent characteristics between individuals of *A. zerumbet* in the Ryukyu Islands. An examination of the leaf scent of *Alpinia spp*. has revealed significant differences in aroma characteristics between chemotypes and distinct sensory differences. These findings corroborate the occurrence of reticulate natural hybridization as described by Liu et al. in Okinawa. A detailed analysis of the aroma characteristics of individuals has enabled the determination of species identity and the presence of contamination by focusing on several essential oil components. Despite the challenges in stabilizing the chemical composition of the essential oils of *Alpinia spp.* due to their low content and diversity, understanding these diverse fragrance properties and selectively harvesting individuals with pleasant fragrances can lead to the production of essential oils of stable quality. This strategy will not only stabilize quality but also facilitate branding and the creation of more functional essential oils that capitalize on regional and fragrance characteristics.

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We thank the many individuals for their cooperation. Specifically, we are grateful to Dr. Kenta Watanabe for his advice on the identification and ecology of *A. zerumbet*, Ms. Shina Nakata for her assistance in extracting the essential oil of *A. zerumbet*, and Mr. Hironobu Kawamitsu for his cooperation in sampling from Iriomote Island.

Conflict of Interest

The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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P-37

Biological activities, GC/MS analysis and Computational approaches of rhizome *Homalomena aromatica* (Spreng.) Schott essential oil - an indigenous plant to Northeast India

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Keywords: *Homalomena aromatica*, Bioactivity, Molecular Docking, Target Prediction

Abstract

Introduction: *Homalomena aromatica* (Spreng.) Schott is a valuable medicinal aromatic plant, having wide range of uses in ethnobotany, pharmacology, perfumery and flavor industry [1]. Objective: This study aimed to evaluate the composition of essential oil, investigate biological activities and perform in-silico analysis.Methods: Dry rhizome of *H. aromatica* was taken and essential oil was extracted. Followed by GC/FID and GC/MS profiling was performed. Followed by biological activities was performed. To validate the findings, *in-silico* methods such as the PASS tool, target prediction and molecular docking were employed. Results: The *H. aromatica* rhizome essential oil (HAEO) yield was 1.09±0.641% (v/w). Through GC/FID and GC/MS 12 compounds were identified and major compound was linalool (76.29%). Anti-oxidant activity result showed IC50 value for DPPH (50.12µL/mL), ABTS (33.05µL/mL) and metal chelating (35.23µL/mL). Anti-inflammatory activity for protease inhibitory (19.59µL/mL) and albumin denaturation assay (32.16µL/mL), α-amylase inhibitory activity showed (29.84µL/mL), Tyrosinase inhibitory activity 73.62µL/mL, Acetylcholinesterase inhibitory (AChEase) activity 38.13µL/mL. Antimicrobial activity showed MIC values of 10±0.47µg/mL, and 45±0.47µg/mL against Staphylococcus aureus and Candida albicans. Genotoxicity analysis showed HAEO has moderate toxic effect. *In-silico* results identified six potential targets via swisstarget prediction: (ACHE, CYP51, PPAR, COX-2, TYRP1, and PRDX5). Molecular docking studies showed a better docking score for Linalool -7.4 kcal/mol, Spathulenol -8.5 kcal/mol, Aromadendrene oxide-(2) -8.3 kcal/mol. Conclusions: For the first time, this study reports on anti-diabetic, anti-tyrosinase, acetylcholinesterase inhibitory activities, and genotoxicity of HAEO. The *in-silico* investigation aligns with these biological activities, evaluating the binding affinities, interactions between the compounds and their respective targets through molecular docking studies.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-38

Essential oil from basil genotypes: fertilizers, seasons and growing conditions

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Keywords: *Ocimum basilicum* L., fertilization, seasonality, greenhouse

Abstract

The objective was to evaluate the essential oil basil (*Ocimum basilicum* L.), cultivars "Sweet Dani" and "Cinnamon", simultaneously in the open field and greenhouse, in autumn-winter and spring-summer of the Uberlândia-MG, Brazil, with fertilization with mineral, organic and organomineral. The experimental design used was DBC, in factorial (2 x 4), with two genotypes and four types of fertilizer including the control, with three replications. Two harvests were carried out, in July (autumn-winter) and March (spring-summer). In spring-summer cultivation, "Cinnamon", grown in a greenhouse and organic fertilization, presents an oil yield 95% (1.72 g plant-1) higher than Organomineral (0.88 g plant-1) and 258% higher than Control (0.48 g plant -1). In greenhouse cultivation and organic fertilization, "Cinnamon" presented an oil yield 192% higher than "Sweet Dani". The main compounds found in the "Sweet Dani" were geranial and neral and for Cinnamon were linalool and (E)-methyl cinnamate. In autumn-winter plants grown in the field and fertilized with organomineral showed an oil yield 112% higher than in greenhouse. In field cultivation, organomineral fertilization resulted in an oil yield 33% higher than organic fertilization and 53% higher than the absence of fertilization. For oil yield there was no difference between the varieties within each cultivation system. The "Sweet Dani" variety revealed 60% higher oil yield in field cultivation. The main compounds found in the "Sweet Dani" variety both in the field and in the greenhouse and among the types of fertilizer were neral, geranial and α-trans-bergamotene. For "Cinnamon" were (E)-methyl cinnamate, α-muurolol and linalool.

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Conflict of Interest

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P-39

Characterization of yield, chemical profile and antioxidant capacity of *Salvia rosmarinus* Spenn. essential oil

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Keywords: rosemary, ORAC, steam distillation, chemical composition, pilot plant

Abstract

This study evaluated the yield, chemical composition, and antioxidant capacity of rosemary essential oil from wild shrubs in central Spain. Rosemary twigs underwent steam distillation at a pilot scale for 4.5 hours, yielding three essential oil fraction samples. Yield was measured as the weight of essential oil per dried plant material (% w./d.w.), and composition was analysed using GC (FID)-MS. Antioxidant activity was assessed by the ORAC assay and expressed as IC_{50} (µg/mL). The results showed that the first distillation period produced the highest essential oil yield (0.29% w./d.w.), with subsequent fractions yielding less. The first fraction was rich in α-pinene, camphene, β-pinene, and 1,8-cineole, while the final fraction had higher amounts of camphor, bornyl acetate, and trans-β-caryophyllene. Antioxidant capacity was highest in the last fraction, with IC50 value173.49 ± 23.27 µg/mL.

Introduction

Rosemary, a member of the Lamiaceae family, predominantly originates from the western Mediterranean region, where three main species are found. Traditionally valued for its fragrance within the cosmetic industry and as a food flavouring, rosemary has gradually gained recognition across the food and pharmaceutical sectors due to its antioxidant and antibacterial attributes [1].

Objective

This study aimed to enhance understanding of essential oil production at a pilot scale. It focused on evaluating yield, chemical composition, and antioxidant capacity of rosemary essential oil fractions derived from foliage biomass of wild shrubs in forestry enhancement operations.

Materials and Methods

Fresh twigs of *Salvia rosmarinus* Spenn. (Laminaceae), manually collected in central Spain, underwent steam distillation in a 1.8 m^3 stainless steel still using steam produced in an electric boiler (38.3 kg/h, 50 kPa). One distillation test was carried out with 412 kg of 20 mm milled material for 4.5 h with constant temperature of 98 ˚C inside the still. Three essential oil samples obtained at different moments of distillation were separated. The essential oil yield was calculated as a percentage, by measuring the weight of essential oil extracted per weight of dried plant material (% w./d.w.) and their composition (% relative area) was analysed and identified with GC (FID)-MS as described by Mediavilla et al. [2]. The antioxidant activity was determined by the ORAC assay, based on the methodology of Ou et al. [3] and a slightly modified procedure from Rondevaldová et al. [4]. The results were obtained as an average value of three repetitions and expressed as IC_{50} (µg/mL).

Results

As can be seen in the Table 1 below, the first distillation period yielded the most of essential oil, while the second and third periods had minimal yields. Each fraction had a different composition, with the first fraction having higher percentages of α-pinene, camphene, β-pinene, and 1,8-cineole, and the last fraction reporting higher proportion of camphor, bornyl acetate, and *trans*- β-caryophyllene. These compositional differences also impacted the antioxidant capacity; the first fraction exhibited the lowest capacity and the last fraction the highest antioxidant ability.

Table 2. Results of yield, chemical composition and antioxidant capacity of studied rosemary's *fractions*

	1st fraction	2nd fraction	3rd fraction
Yield (% w./d.w.)	0.29	0.04	0.01
Chemical composition (> 5%)			
α-Pinene	17.52	3.47	2.19
Camphene	9.04	1.91	1.02
β-Pinene	5.06	1.29	0.62
1,8-Cineole	28.95	21.17	5.74
Camphor	13.2	37.48	41.2
Bornyl acetate	0.78	2.62	5.02
trans-β-Caryophyllene	1.22	4.44	8.92
Antioxidant capacity IC_{50} (µg/mL)	>256.00	209.78 ± 12.02	173.49 ± 23.27

Discussion and Conclusions

α-Pinene, 1,8-cineole and camphor were the main essential oil constituents, which corresponds to rosemary of Spanish origin [5]. As reported by others [6], more volatile compounds were found in first fraction and the less volatile components remained in the last fraction. The strongest antioxidant capacity of the last fraction could be attributed to higher amount of camphor [7] and lower amounts of α-pinene [8].

The yield, chemical composition and antioxidant capacity of rosemary essential oil obtained via steam distillation at pilot scale varied among the studied fractions. These findings highlight the importance of selecting the appropriate distillation period to obtain rosemary essential oil with the desired characteristics for specific applications.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-40

Vapour phase anticandidal activity from *Pimenta pseudocaryophyllus* (Gomes) Landrum essential oil

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Keywords: *Pimenta pseudocaryophyllus*, anticandidal activity, vapour phase, fumigant, environmental disinfectant

Abstract

Pimenta pseudocaryophyllus (Gomes) Landrum is a native species that is used for culinary and medicinal purposes whose essential oils have already demonstrated antimicrobial activity. The essential oil (EO) obtained via hydrodistillation yielded 2.7%. The major components of the EO were eugenol (31.5%), isoeugenol (14.8%), and methyl-eugenol (12.2%). In this study, the antifungal activity of *P. pseudocaryophyllus* oil was evaluated against *Candida albicans* (ATCC 10231), a prominent fungus responsible for fungal sepsis in hospitals. The minimum inhibitory concentration (MIC) of the EO against *C. albicans* was 43.2 µg/mL in the liquid phase and 106.5 µg/L in the vapor phase. These results highlight the promising antifungal activity of the oil, particularly as an environmental disinfectant in hospitals. Given the escalating issue of fungal sepsis in intensive care units, the vapor phase's efficacy is particularly noteworthy. Furthermore, *P. pseudocaryophyllus* EO holds potential for use by the pharmaceutical industry in developing new drugs for candidiasis treatment."

1. Introduction

Community-based infections have a strong impact on the most fragile populations, such as children, elderly, and immunosuppressed people. Although conventional products such as bleach are widely used, the search for other less hazardous and environmentally friendly disinfectants grows. Essential oils have a recognized range of antimicrobial activities against bacteria, Gram-negative and Gram-positive, and pathogenic fungi. These oils have already demonstrated *in vitro* activity in both liquid and vapour phases. Lately, essential oil vapours gained some interest because they can be an interesting alternative treatment of hospital environment due to their ability in preventing biofilm formation [1].

Pimenta pseudocaryophyllus (Gomes) Landrum is a tree, belonging to the Myrtaceae family, that produces essential oils that provide a tea much appreciated by local communities which is used as a diuretic and against colds, flu, and fatigue. Its leaves are also used for the preparation of inhalations, alcoholic solution for massage. Earlier studies indicated the presence of different chemotypes that may be genetically determined, and its essential oils have already demonstrated antimicrobial activity [2]. Thus, the objective of this study was to evaluate the antifungal activity of *P. pseudocaryophyllus* essential oil in the liquid and vapour phases using the yeast *Candida albicans* as test microorganism.

2. Material and Methods

Plant material: The leaves of *Pimenta pseudocaryophyllus* were collected at the State Park Ilha do Cardoso 25º03'05" - 24º18'18"S e 47º53'48" - 48º03'05"W, Cananéia - SP. The vouchers (MORENO 34) were identified by Dr. Inês Cordeiro and deposited at the Herbarium of the Instituto de Botânica - São Paulo.

Oil Isolation: The oils were obtained from the dried leaves (70-150 g) by hydro-distillation for 4 h using a Clevenger-type apparatus [2].

Chemical analysis: The oil was analysed by GC and GC/MS. GC analysis was performed in a chromatograph (Varian CP-3380) using the method previously described by Lima *et al.* [2].

Antimicrobial Assay: The antimicrobial activity was evaluated against *Candida albicans* (ATCC 10231) using the microdilution method after reading the absorbance at λ=630 nm, after 24 h of incubation [3]. The vapour phase activity was assessed by the inverted plate method (Fig 1.) [3]. Formaldehyde was used as a reference of an active substance in the vapour phase.

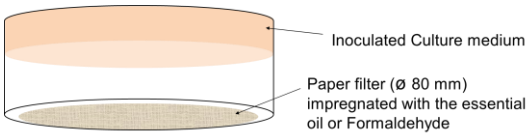

Figure 1. Scheme for testing the Antimicrobial Activity in the Vapour Phase using the inverted plate method.

3. Results

The average essential oil yield was 2.7 % (w/w), presenting as major components eugenol (31.5%), isoeugenol (14.8%), and methyl-eugenol (12.2%).

The minimum inhibitory concentrations (MIC) for the vapour phases from *P. pseudocaryophyllus* EO and formaldehyde were calculated by the ratio between the amount of oil or formaldehyde applied and the expansion volume of the vapours inside the plate (0.054L). The antifungal activity for the oil is presented in Table 1.

Table 1 Anfungal activity of the essential oil from the leaves of *P. pseudocaryophyllus* in liquid and vapour phases against *Candida albicans* (ATCC 10231)

Phase	MIC (mg/L)	Formaldehyde (MIC mg/L)
Liquid	42.3	2.56
Vapour	0.11	0.82

4. Discussion and Conclusions

P. pseudocaryophyllus is the only species native to Brazil within this genus. The chemotype specimen, collected on Cardoso Island, was found to be rich in eugenol derivatives. Eugenol has been recognized as an antifungal agent against *C. albicans* [4]. The MIC values obtained against *C. albicans* in both phases indicate promising antifungal activity for the oil [2], particularly as an environmental disinfectant in hospitals. A potential explanation for the heightened activity observed in the vapour phase could be that the oil is solvated in the liquid phase. However, in the vapour phase, it is free from the solvent envelope. This freedom allows it to bind more readily to the membrane of microorganisms, due to its lipophilic characteristics. Oil vapours have the advantage of being sanitizers that do not require direct application on surfaces, making them suitable for use as room disinfectants and air decontaminants, even in inhabited areas, due to their lower toxicity compared to formaldehyde. *P. pseudocaryophyllus* EO can also be used to produce ointments for treating candidiasis, primarily the vulvovaginal form.

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Conflict of Interest

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Investigation of the anti-biofilm and anti-QS effect of selected essential oils tested on nosocomial bacteria

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Keywords: nosocomial infection, antibiotic resistance, essential oils, anti-biofilm effect, MTT assay

Abstract

In parallel with the spread of antibiotic resistance, it is vital to look for new antibacterial compounds, even those of plant origin, in order to be able to use them for therapeutic purposes. The over-prescribing and over-use of antibiotics has significantly increased the number of resistant strains. This is particularly problematic in hospitals where nosocomial infections develop. We aimed to explore the antibacterial and anti-biofilm effects of peppermint, cinnamon bark, clove and thyme essential oils (EOs) and their main components. We experienced significant anti-biofilm effect of cinnamon bark (59.2-72.6% inhibitory rates) and thyme (72.9-81.5% inhibitory rates) EOs against *E. coli*, MRSA, *S. pneumoniae* and *P. aeruginosa*. Moreover, all of the EOs and their main components (menthol, cinnamic aldehyde, thymol, and eugenol) showed anti-quorum sensing activity at 10 mg/mL. The effect of the EOs of peppermint and clove cannot be neglected, as they also inhibited the growth of the above-mentioned bacteria and their biofilm formation in *in vitro* assays. In conclusion, both cinnamon bark and thyme EO may be suitable for the prevention of nosocomial diseases and control the spread of nosocomial bacteria.

1. Introduction

Today antibiotic resistance, which is related, among other things, to the biofilm-forming property of bacteria, causes a lot of problems in healthcare. In order to reduce it, it is necessary to research new, effective materials e.g. essential oils (EOs) with antimicrobial activity [1]. When bacteria form a biofilm, they are much more resistant to different factors, e.g. disinfectants and antibiotics. In biofilms, bacterial cells are better protected against unfavorable changes in pH and extreme fluctuations in temperature [2]. During our research, we aimed to study the antibacterial, the anti-biofilm and anti-quorum sensing (anti-QS) effects of peppermint, cinnamon bark, thyme, clove EOs and their main components (menthol, cinnamaldehyde, thymol and eugenol) against some relevant nosocomial bacteria. These strains were the follows: *Pseudomonas aeruginosa* ATCC 27853, methicillin-resistant *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Streptococcus pneumoniae* DSM 20566.

2. Material and Methods

In order to determine the composition of EOs, GC-MS was performed. The n-alkane homologue series (C7-C30; Supelco 49451-U) was used, samples were measured in 1:1000 dilution. The device was an Agilent 7890B gas chromatograph connected to an Agilent 7010B triple quadrupole mass spectrometer, wich was used in MS1 scan and EI (electron ionisation) mode.

During the microbiological tests, each bacterium was incubated in a shaker incubator at 37°C and at a speed of 60 rpm for 12 h [3]. The minimum inhibitory concentrations (MIC) were determined using the microdilution method on 96-cell microtiter plates [4]. In order to describe the mechanism of action of the EOs, anti-biofilm with MTT assay and anti-QS assays were carried out. Bacterial biofilms were formed on 96-cell polystyrene microtiter plates. 200 µL of a cell suspension with a cell count of 10^8 CFU/mL was measured into each cell (4 h, 37°C). After the treatment (24 h, 37°C) 0.1% crystal violet (CV) solution was measured into the cells. CV binds to negatively charged surface molecules and polysaccharides within the extracellular matrix of biofilms, thus allowing measurement of the total biomass of the biofilm in the cell of the microtiter plate. In order to determine the amount of viable bacterial cells in the biofilm, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye was used. Mitochondrial dehydrogenases reduce yellow MTT to blue formazan crystals. After incubation time absorbance was measured at 595 nm using a plate reader. Anticiotics (gentamicin and imipenem) were used as positive controls. In order to detect the anti-QS effect, a disc diffusion method was applied. The inhibition of the

QS mechanism can be clearly illustrated with pigment-producing bacteria, so we performed our tests with the model bacterium *Chromobacterium violaceum* SZMC 6269. The test was performed with both concentrated and diluted samples (2.5, 10 mg/mL).

3. Results

The GC-MS results showed that the main component of peppermint oil was menthol with 50.4%, and with a large amount of menthone (19.8%). The main component of cinnamon bark EO was *trans*-cinnamic aldehyde, which was present in 64.7%. The clove EO consisted of only seven components, it contained eugenol (78.8%) as main component, and β-caryophyllene, which was detected in 13.5%. Thyme EO was the most complex sample, as we detected more than twenty components. Its main components were thymol (39.8%) and *p*-cymene (19.2%).

The MIC values of EO samples are summarized in Table 1. Thyme proved to be the most effective EO. The most sensitive pathogen was *S. pneumoniae*.

Table 1. MIC values of the tested EOs and antibiotics (mg/mL for EOs; μg/mL for antibiotic)

Samples	Bacterial strains			
	1	2	3	4
cinnamon bark	0.12	0.15	0.78	0.06
clove	0.25	0.20	1.02	0.25
peppermint	0.65	1.75	1.96	0.35
thyme	0.31	0.20	0.46	0.12
antibiotic	2.0	12.5	2.0	0.4

1: *E. coli*, 2: MRSA, 3: *P. aeruginosa*, 4: *S. pneumoniae*, For *P. aeruginosa*, *E. coli* and MRSA, gentamicin (Gentamicin Sandoz 40 mg/mL injection, Sandoz), for *S. pneumoniae*, imipenem (Imipenem/Cilastatin Kabi 500 mg/500 mg powder for solution for infusion; stock solution: 0.4 mg/mL) antibiotics were used.

In the case of anti-biofilm activity, peppermint EO was the least effective. Thyme EO proved to be the most effective. The most resistant pathogen was *P. aeruginosa*, since in this case we detected the lowest inhibition rate values. The inhibition rate values are illustrated in Figure 1.

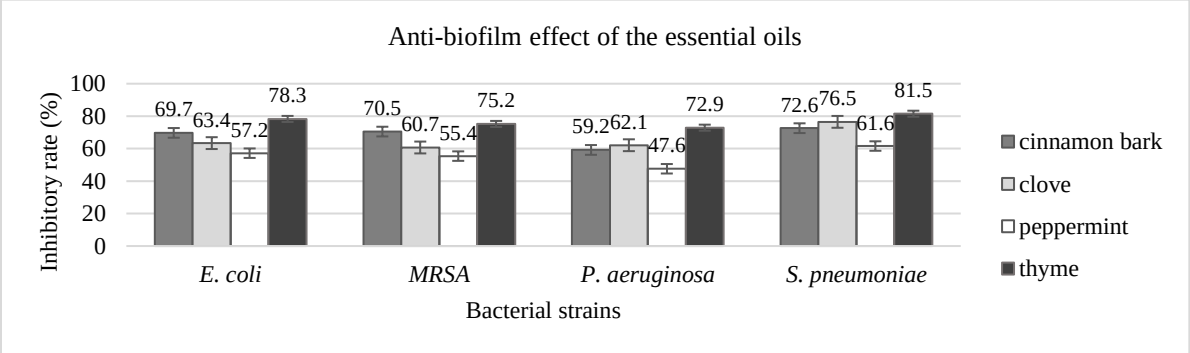

Figure 1. Antibiofilm effect of EOs. Inhibitory rate = (1 - S/C) ×100% (C and S were defined as the average absorbance of control and sample groups, respectively) [5].

By using MTT staining, we had the opportunity MTT assay is suitable for the determination of the viable bacterial cells in the biofilm. The most viable cells were detected after peppermint EO treatment. The results are summarised in Table 2.

Table 2. Percentage of viable cells, based on the MTT test carried out in six parallel measurements. Data are shown as means ± standard deviation

EOs	1	2	3	4
cinnamon bark	67.5±1.3	60.7±1.8	68.5±0.7	67.6±0.8
clove	58.2±0.2	63.5±0.6	77.5±1.2	59.2±0.7
peppermint	76.2±1.8	84.5±0.7	86.1±1.1	70.3±1.2
thyme	55.7±1.2	60.5±0.4	46.7±1.2	33.2±0.8

1: *E. coli*, 2: MRSA, 3: *P. aeruginosa*, 4: *S. pneumoniae*

The anti-QS experiment revealed that peppermint EO produced the largest inhibition zone in both undiluted and diluted form. This was followed by the EO of thyme and cinnamon bark. A similar trend was observed in the case of the main components, menthol produced the largest zone of inhibition, followed by thymol, *trans*-cinnamic aldehyde and eugenol. It is important to highlight that the activity of the EO is always more effective compared to the single component. Clove EO showed the weakest activity. In the case of biofilm tests, we observed that peppermint EO proved to be the least effective on the tested bacteria (Figure 1). On the other hand, the inhibitory effect of peppermint oil is associated with its anti-QS activity. We suppose that thyme, clove and cinnamon oils can inhibit the process of biofilm formation and influence its structure, the activity of peppermint EO manifests in its inhibitory effect on the QS mechanism.

Table 3. Inhibitory zones produced by EO samples and their main components in anti-QS assay

Samples	Concentrations (mg/mL)		
	2.5	10	undiluted
peppermint	8.32±0.5	23.12±0.7	39.28±0.6
menthol	-	4.2±0.3	-
cinnamon bark	5.12±0.6	18.3±0.4	33.39±0.5
cinnamic aldehyde	-	2.6±0.2	-
thyme	6.95±0.6	20.2±0.5	36.32±0.9
thymol	-	1.54±0.2	-
clove	2.21±0.3	15.97±0.2	26.47±0.3
eugenol	-	1.28±0.5	-

4. Discussion and Conclusions

Our biofilm inhibition results have been supported by other research groups, but this is the first study which compares the activity of the above-mentioned oils against nosocomial bacteria. Husain and his colleagues also described the inhibitory effect of peppermint EO [6], but we do not have data on the anti-QS effect of menthol. Previous studies have already described the anti-QS effect of eugenol [7], but this effect with clove oil has not been proven yet. Our study demonstrated the antibacterial, anti-biofilm and anti-QS activities of thyme, cinnamon bark, clove and peppermint EOs and their main components (thymol, cinnamic aldehyde, eugenol, and menthol) against some bacteria causing nosocomial infections. Thyme EO was the most effective oil in every test system. Peppermint oil showed the most significant result in anti-QS assay. In conclusion, our tested EOs may prevent the spread of bacteria used in our study, but they have different mode of action during antibacterial activity. Nowadays, when antibiotic-resistance is a worldwide problem, it is extremely important to study natural substances and their mode and points of action in/on bacterial cells.

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Conflict of Interest

The authors declare no conflicts of interest.

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Comparison of the free volatile compounds composition isolated from wild and cultivated species of the genus *Veronica* from moderate habitats

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Keywords: *Veronica*, essential oil, hydrosol, microwave-assisted water extraction, benzene acetaldehyde, *β*-ionone

Abstract

The aim of this study was to evaluate the similarities and differences in the chemical composition of free volatile compounds (FVCs) isolated from wild and cultivated *Veronica* species (Plantaginaceae): *V. hederifolia* L., *V. persica* Poir. nad *V. polita* Fr. The cultivated speedwell was grown from seeds collected in 2022. The isolation of FVCs was carried out by microwave-assisted extraction (MAE), and each extract obtained consists of two phases: essential oil (EO) and hydrosol (HY). The phytochemical composition of the isolates was determined by gas chromatography-mass spectrometry (GC-MS). Hexahydrofarnesyl acetone and phytol are common compounds in the EO composition of the studied species from natural habitat and cultivation. Other important common components in the composition of all EOs from samples collected in natural habitat and from cultivation are *β*-ionone in *V. hederifolia* and *V. persica* and benzene-acetaldehyde in the samples of cultivated plants in *V. polita*. In the composition of HYs, benzene-acetaldehyde and *β*-ionone were identified in all isolates, and in addition to these compounds, *(E)-β*-damascenone was identified in a significant percentage in the HYs of the species *V. hederifolia* and caryophyllene oxide in the HYs of the species *V. persica* and *V. polita*. The biological potential and compositional comparison of isolated volatile compounds from wild-collected and cultivated plants will be further explored to achieve a better understanding and utilization of the specialized plant metabolites of the genus *Veronica*.

1. Introduction

The species of the genus *Veronica* (family Plantaginaceae) are widespread in various habitats thanks to their adaptability to different living conditions [1]. The subject of this study are three species of the genus *Veronica* that thrive in a moderate habitat: *V. hederifolia* L. (ivy-leaved speedwel), *V. persica* Poir. (Persian speedwell) and *V. polita* Fr. (gray field speedwell). The plants of this genus are used worldwide in traditional medicine and nutrition [2-4], so it is important to investigate their specialized metabolites, which contain biological potential. Our previous research on *Veronica* species focused on specialized metabolites that form free volatile compounds (FVCs) in essential oils (EOs) and hydrosols (HYs) from wild growing species [5, 6]. The data from these studies were compared with FVCs from cultivated *Veronica* species. This comparison is important for the selection of extracts for further biological research, especially since these three *Veronica* species studied are known as medicinal plants [2-4].

2. Material and Methods

The isolation of FVCs from three *Veronica* species (30 g for each plant species) collected in its natural habitat and from cultivated plants was performed by microwave-assisted extraction (MAE, Milestone 'ETHOS X' device) with each extract obtained consisting of two phases: a lipophilic (essential oil) and an aqueous phase (hydrosol). Plant material was collected from March to July 2021 at different locations in Croatia [5]. The three cultivated *Veronica* species were grown from seeds collected in the previous year (2021). The phytochemical composition of the isolates was determined by gas chromatography-mass spectrometry (GC-MS). Chromatographic analyses were performed with a GC (model 3900; Varian Inc., Lake Forest, CA, USA) equipped with a flame ionization detector and a mass spectrometer (model 2100 T; Varian Inc., Lake Forest, CA, USA), retention indices (RIs) were determined relative to a series of n-alkanes (C8–C40) on capillary column VF5-ms (30 m × 0.25 mm i.d., coating thickness 0.25 μm, Palo Alto, CA, USA). Injection of HYs was carried out with a headspace injection needle and there was no split ratio (splitless mode).

3. Results

The compositions of FVCs isolated from *Veronica hederifolia*, *V. persica* and *V. polita*, consisting of two phases: EO and HY, are compared between plants collected in their natural habitat and those cultivated in spring 2023. The (EO) of *V. hederifolia* extracted in the natural habitat [3] and from cultivated plants consisted of hexahydrofarnesyl acetone and *β*-ionone as the main constituents. A special feature is the significant percentage of caryophyllene oxide detected in the cultivated species (Table 1). Hexahydrofarnesyl acetone and phytol are the major compounds in the EO composition of *V. persica* isolates derived from plants from natural habitats and cultivation, with *β*-ionone being the dominant compound in isolates from cultivated plants (Table 1). In the *V. polita* cultivar, the most common compound in the EO composition is phytol, followed by benzene acetaldehyde and hexahydrofarnesyl acetone. All these compounds were also identified in the EO of *V. polita* collected in natural habitat, with the exception that benzene acetaldehyde was identified in a very low percentage of less than 1% [5]. Benzene-acetaldehyde and *β*-ionone are common compounds in the hydrosol composition of all three *Veronica* species studied, from natural habitat [6] and cultivation (Table 2). The HY of wild and cultivated forms of *V. hederifolia* species is also dominated by *(E)-β*-damascenone and muurolol, which is particularly prevalent in the HY of cultivars (Table 2).

Table 1. Comparison of the main constituents (%) of the essential oil from the aerial parts of the species *Veronica* (Plantaginaceae) collected in the natural habitat and obtained by cultivation

Component	RI	*V hederifolia* Wild (%)	*V hederifolia* Cultivated (%)	*V. persica* Wild (%)	*V. persica* Cultivated (%)	*V. polita* Wild (%)	*V. polita* Cultivated (%)
Benzene acetaldehyde	1036					0.46	18.65
β-Ionone	1487	15.03	10.32	4.54	18.32		
Caryophyllene oxide	1581	0.51	10.52				
Hexahydrofarnesyl acetone	1839	59.15	43.45	18.47	15.58	10.82	18.32
Phytol	1942	14.58	3.07	23.71	10.27	19.88	25.48
Heptacosane	2700			14.28	1.23	15.13	1.71

RI - retention indices,were determined relative to a series of n-alkanes (C8–C40) on capillary column VF5-ms

Table 2 Comparison of the main constituents (%) of the hydrosol from the aerial parts of the species *Veronica* (Plantaginaceae) collected in the natural habitat and obtained by cultivation

Component	RI	*V. hederifolia* Wild (%)	*V. hederifolia* Cultivated (%)	*V. persica* Wild (%)	*V. persica* Cultivated (%)	*V. polita* Wild (%)	*V. polita* Cultivated (%)
Benzene acetaldehyde	1036	14.36	7.54	20.05	35.02	10.43	3.42
(E)-β-Damascenone	1388	23.86	16.89				
E-Caryophyllene	1424					11.74	11.56
β-Ionone	1487	10.14	6.62	16.49	5.78	19.23	10.86
Caryophyllene oxide	1581			10.17	20.01	14.17	14.46
Muurolol	1645	1.91	22.72				

RI - retention indices,were determined relative to a series of n-alkanes (C8–C40) on capillary column VF5-ms

4. Discussion and Conclusions

The main volatile substances identified in the wild and cultivated species *Veronica hederifolia*, *V. persica* and *V. polita* are very similar (Table 1, and 2), and it is clear that the cultivated species contains biologically active natural products. Considering that previous studies on biological activity have shown that FVCs consisted in EOs and HYs isolated from *Veronica* species collected in natural habitats have antioxidant activity [6], further research will focus on the overall biological potential of the natural compounds obtained from cultivated and wild species of the genus *Veronica*.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The essential oil of alpine Rhododendron honey

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Keywords: honey, *Apis mellifera*, Rhododendron, GC/MS, distillation

Abstract

Rhododendron honey, also known as "Almrauschhonig," is a unique type of honey found in the Alpine region. The source of this honey is collected by the bees from *Rhododendron ferrugineum*, *R. hirsutum*, and their hybrid *R.* x *intermedium*, which are naturally found in the Central European Alps. Honey samples of various varieties, including rhododendron honey, flower honey, honeydew honey, and mountain honey, were gathered from the Alpine area of Austria. Subsequently, the essential oil was extracted from these samples using distillation. The essential oil components were analysed using gas chromatography/mass spectrometry (GC-MS). The essential oil of alpine rose honey was found to include four distinct components that set it apart from the other samples. The compounds were 2-pentyl furan, *cis*-linalool oxide, decanal, and carvomenthenal. The flower honey samples exhibited three chemicals that enabled their differentiation from other honey samples, namely 6,10,14-trimethyl-2-pentadecanone, 9-eicosyne, and decyl hexadecyl ester carbonic acid. The honeydew honey exhibited a sole distinctive component, namely borneol, which set it apart from the other honey samples. Conversely, the mountain honey samples did not exhibit any notable components that could differentiate them from other types of honey. Nevertheless, they exhibited a comparable compound pattern to that of alpine rose honeys and honeydew honeys.

1. Introduction

Rhododendron honey, also known as "Almrauschhonig," is a unique type of honey found in the Alpine region. The source of this honey is collected by the bees from *Rhododendron ferrugineum*, *R. hirsutum*, and their hybrid *R.* x *intermedium*, which are naturally found in the Central European Alps. Honey samples of various varieties, including rhododendron honey, flower honey, honeydew honey, and mountain honey, were gathered from the Alpine area of Austria. Our assumption was that rhododendron honey possesses a unique essential oil composition.

2. Material and Methods

42 floral and honeydew honeys were purchased from beekeepers or specialised stores in Austria, all of which were sourced from the Austrian Alps. The samples were stored at ambient temperature in the dark until analysis. Certain honeys underwent crystallisation over the storing process and were subsequently liquefied in a water bath at 30°C prior the analysis. A total of 200g of honey were distilled with 400mL water for 3h in a modified Clevenger apparatus. The GC-MS system used in this study was an Agilent 7890A coupled to a 5957C VL MSD (Agilent Technologies, Santa Clara, USA). Separation was carried out on an HP-5MS column (30 m x 250 µm x 0,25µm). The flow rate remained constant at 2mL/min utilising He as carrier gas. The oven temperature started with initial temperature 40°C and increased at a rate of 5°C/min until 100°C. It then increased with 10°C/min until it reached a temperature of 320°C. The injection volume was 1µl with a split ratio of 1:2. The transfer line to the MSD was programmed to 280°C.

3. Results

The rhododendron honey's essential oil was discovered to include four constituents that significantly distinguished it from the other samples. The compounds identified were 2-pentyl furan, *cis*-linalool oxide, decanal, and carvomenthenal. The floral honey samples had three compounds that distinguished them from other honey samples: 6,10,14-trimethyl-2-pentadecanone, 9-eicosyne, and decyl hexadecyl ester carbonic acid. The honeydew honey displayed a single, unique component, specifically borneol, that distinguished it from the other honey samples. In contrast, the mountain honey samples did not possess any distinctive constituents that may distinguish them from other varieties of honey, they displayed a similar chemical composition to that of alpine rose honeys and honeydew honeys. When considering the whole essential profile in a multivariate appraoch, discriminant analysis distinguished all samples well (Figure 1).

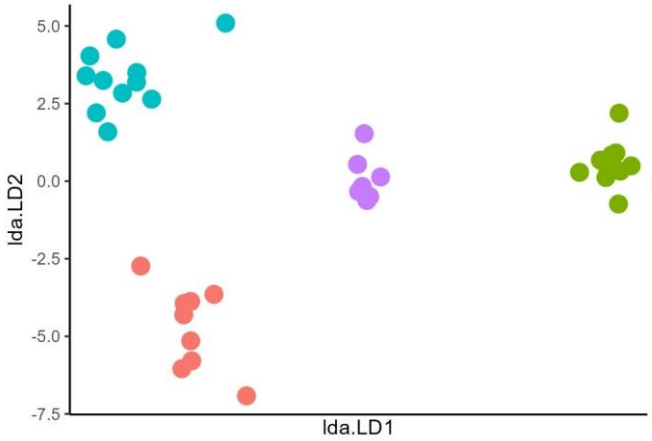

Figure 1 discriminant analysis of the essential oil composition of different Alpine honeys (red … rhododendron honeys, green … flower honeys, blue … honeydew honeys, lilac … mountain honeys).

4. Discussion and Conclusions

Distillation is conducted by subjecting the honey to high temperatures. The outcome is influenced by the interaction between the sugars and amino acids. Thermal artefacts are prone to happening, and delicate molecules are likely to undergo oxidation or decomposition while new ones arise (1). While certain chemicals are typically found in the scent of honeys, others, such as hotrienol, benefit from the elevated temperatures employed during distillation for extraction (2).

In summary, four components were found that may accurately differentiate alpine rose honey from other honey samples.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-44

Directed evolutionary engineering of the non-mevalonate pathway for efficient terpenoid production as aroma ingredients.

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Keywords: terpene, directed evolution, screening, metabolic engineering, non-mevalonate pathway

Abstract

1. Introduction

In Flavor and Fragrance industry, natural and biobased terpenes are now highly focused due to recent SDGs demands as well as natural market trend, while pertoleum based synthetic terpenes and its derivatives are not preffered. Especially for the flavor application as natural flavoring substances, natural terpenes by the isolation from essential oil are mainly used. However, there are potential issues for sustainable supply of natural terpenes for (1) the unstable supply of essential oils due to climate condition and cultivation capabilities; (2) the relatively low yield of terpene from essential oils; (3) the inefficiency of extracting terpenes from the corresponding plants. Therefore, terpene production using precision fermentation is the key for future Flavor and Fragrance industry[1].

It is known, all types of terpenes are biosynthesised dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) with five carbon units. DMAPP and IPP have two main biosynthetic pathways: the mevalonate (MEV) pathway and the non-mevalonate (MEP) pathway. The MEV pathway is well known in eukaryotes, such as plants, and archaea. The MEP pathway is found in bacteria and eubacteria in 1993.The MEP pathway is less productive of DMAPP than the MEV pathway due to feedback control and lack of substrate concentration. However, the MEP pathway is superior in that it has a higher carbon yield than the MEV pathway and can directly supply DMAPP[2]. In this study, we focused on the MEP pathway and investigated evolutionary engineering designs for the highly efficient production of terpenoid as aroma ingredients.

As a result, we found that in the microbial production of terpenes *via* the MEP pathway, the final enzyme in this pathway is the rate-limiting enzyme. Furthermore, the elimination of rate-limiting by the final enzyme found the next rate-limiting enzyme and identified room for further improvement of the MEP pathway.

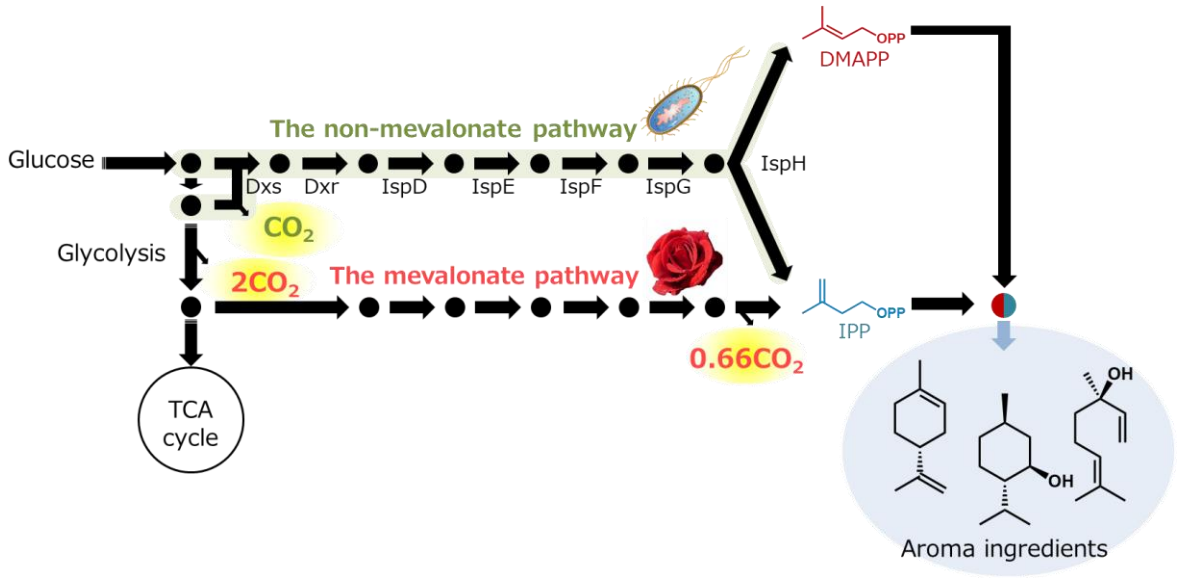

Figure 1 The non-mevalonate pathway used in this system.

2. Material and Methods

2-1. Construction of screening systems

When DMAPP and IPP are excessively depleted from the cells of microorganisms, the microorganisms stop growing. This is because DMAPP and IPP are also involved in sterols and ubiquinones, which are essential for microbial growth[3]. Therefore, geraniol synthase (GES) (*Ocimum basilicum*), which has a high consumption capacity of DMAPP and IPP, was overexpressed in *Escherichia coli* XL1-Blue to establish a state of over-depletion of precursors.In this system, as soon as DMAPP and IPP is biosynthesised, it is immediately converted into geraniol or isoprene, so no microbial growth is observed in <A> of Fig.2. On the other hand, if DMAPP and IPP can be biosynthesised beyond the conversion capacity of the GES, microorganisms will proliferate in <B> of Fig.2.

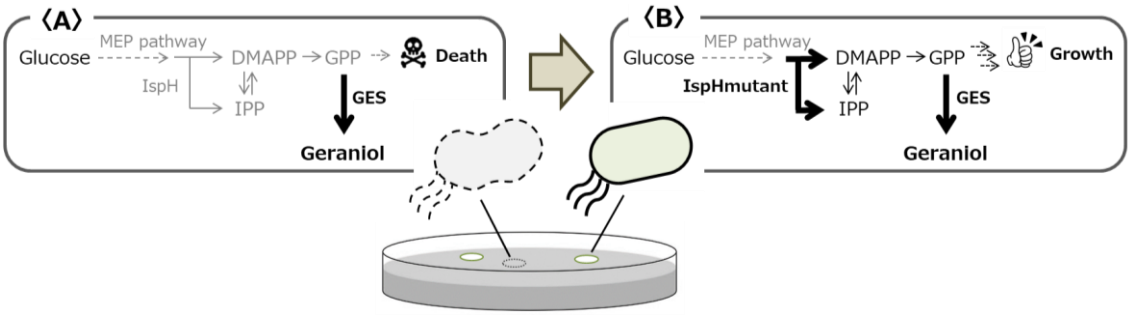

Figure 2 Highly efficient screening system using microbial growth as an indicator.

2-2. Assessment of terpene production in the MEP pathway.

The obtained MEP pathway mutant expression plasmids and terpene synthesis pathway plasmids were used to introduce and culture *Escherichia coli* XL1-Blue strains. After incubation, the cultures were extracted with dodecane and terpene production was assessed by GC-FID.

3. Results

3-1. Acquisition and product analysis of high-productivity IspH mutants.

GES-expressing strains were transformed with the IspH mutant library (library size: 4,650). Approximately 5,000 clones of IspH mutants were inoculated onto Luria–Bertani (LB) plates and incubated for colony formation while inducing GES expression. Plasmids were extracted from the formed colonies and the *ispH* gene sequence analysed. As a result, four unique IspH mutants (IspHmut1, 2, 12, 14) were identified. These IspH mutants were expected to have higher DMAPP/IPP-supplying activity than the unmutated IspH (IspHwt).

To test the actual DMAPP/IPP-supplying activity by the highly active IspH mutants, terpene (*α*-pinene) production was assessed. When the activity supplying DMAPP/IPP is improved, the production of *α*-pinene, which is formed by condensation of DMAPP and IPP one by one, should also be improved.

Pinene synthase (PS) (*Pinus taeda*)-expressing strains were transformed with IspH mutants and *α*-pinene production was assessed by GC-FID. Result in, IspHmut12 was very effective in increasing *α*-pinene production, producing 49-fold more *α*-pinene than the IspHwt. This result suggest that IspH may be a bottleneck enzyme in the MEP pathway.

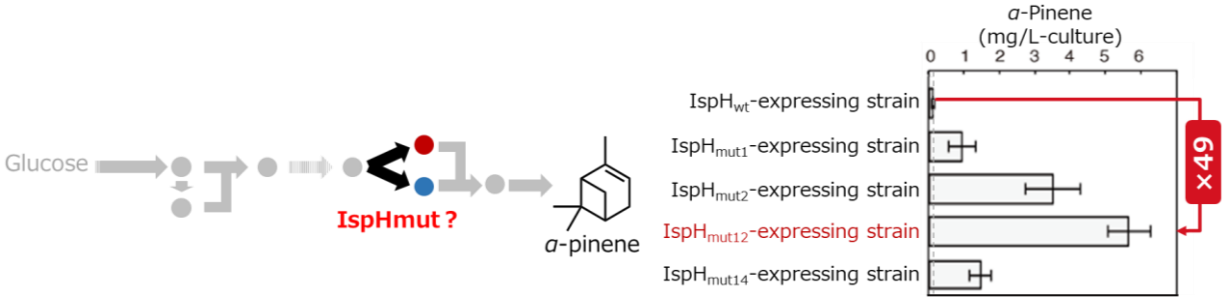

Figure 2 *α*-Pinene production of acquired IspH mutant-expressing strains.

Next, the MEP pathway was reconstructed using the obtained IspHmut12 and compared with the MEV pathway. Expression of the MEP pathway in *E. coli* XL1-Blue using IspHwt resulted in only 2.6-fold *α*-pinene production compared to the wild strain. On the other hand, expression of the MEP pathway in *E. coli* XL1 Blue using the obtained IspHmut12 resulted in a 171-fold increase in the one. The amount of α-pinene production using the MEP pathway is about half of that using MEV pathway.

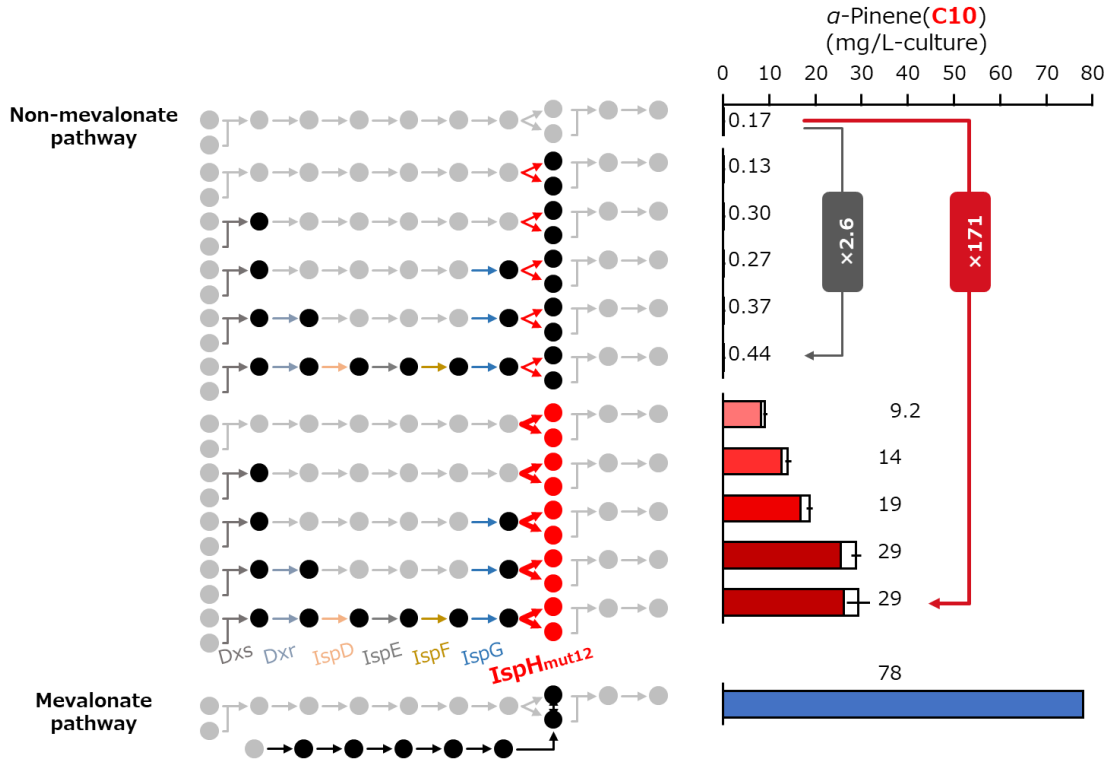

Figure 3 *α*-Pinene production of the MEP pathway-expressing strains with IspH mutant.

4. Discussion and Conclusions

In this study, an evolutionary design of the MEP pathway was conducted to increase the production of aroma ingredients. The results show that modification of IspH, the final enzyme in the MEP pathway, increases in production of terpen 171-fold. This is close to half the amount of α-pinene production using the MEV pathway. Further, the MEP pathway improvements might have the potential to far exceed the MEV pathway.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-45

Inhibitory effects of lavender (*Lavandula angustifolia Mill*.) essential oil and main component (linalool) against biofilm-forming bacteria causing respiratory diseases

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Keywords: respiratory infections, antibiotic resistance, biofilm, lavender, essential oil

Abstract

Bacteria causing respiratory infections are characterized by antibiotic resistance and their ability to form biofilms. These pathogens can colonize mucous membranes which cause respiratory infections in immunosuppressed patients. It is important to explore alternative solutions to fight against resistant bacteria in clinical enviroment. Our aim was to investigate lavender (*Lavandula angustifolia Mill*.) essential oil and its main component (linalool) against four bacteria which are causing respiratory diseases. *Pseudomonas aeruginosa* (ATCC27853), *Haemophilus influenzae* (ATCC49766), *Haemophilus parainfluenaze* (ATCC33392) and *Streptococcus pneumoniae* (DSM20566) were involved in our *in vitro* experiments. First the minimal inhibitory concentrations (MICs) were determined. Secondly biofilms were formed on 96-well microtiter plates, treated with half of the previously determined MIC concentration of lavender oil or linalool after 4, 6, 8, 12, and 24 hours of incubation phases. Then the biofilms were fixed, stained, and absorbance was measured at 590 nm to determine the inhibitory effect of the tested materials on biofilm-formation. Our results showed that the biofilm-formations of the four test bacteria were disrupted by the oil treatment. *P. aeruginosa* was the most resistant to the treatment while *S. pneumoniae* was the most sensitive. The increase in incubation time was accompanied by a decrease in inhibitory rates. Based on our results it can be concluded that both linalool and lavender essential oil were effective against biofilm-formating bacteria, and the time of the treatment is crucial. Our further goal is to investigate whether both have efflux-pump inhibition.

1. Introduction

Bacteria causing respiratory infections are one of the most hazardous nosocomial infections due to their prevalence and impact on patient health [1]. Pathogens with biofilm formation (e.g. *Pseudomonas aeruginosa, Haemophilus influenzae, H. parainfluenaze* and *Streptococcus pneumoniae*) cause significant challenges to healthcare facilities. Hospital-acquired respiratory infections can also spread easily among patients and healthcare workers, posing a threat to overall hospital safety. The other huge problem associated with biofilms is antibiotic resistance. According to the latest WHO data it continues to escalate, jeopardizing our ability to treat common infections [2]. The biggest problem is that according to irresponsible antibiotic use, the antibacterial agent penetrate the biofilm and it is only sufficient to induce resistance in the bacterial strain. It means that infections that were once easily treatable with antibiotics are becoming difficult to cure. This poses significant challenges for healthcare systems worldwide, leading to prolonged illness, higher healthcare costs, and increased mortality rates. Based on the above mentioned problem it is crucial to stop the spreading of these multiresistant and biofilm-forming pathogens. Essential oils have a rich history of use dating back centuries and are valued for their various therapeutic properties. Throughout the ages, essential oils have been used for their diverse benefits, including their antibacterial, antifungal, and anti-inflammatory properties [3]. Studies have shown promising results in areas such as stress reduction, pain management, and antimicrobal effects [4, 5]. Lavender essential oil is one of the most frequently tested oil. In our in vitro experiments we focused on it and its main component linalool. The question of our study was: does the main component have the same antimicrobal effect as the whole essential oil, and in which time of the biofilm formation is the best to make an intervention in the process.

2. Material and Methods

The examined lavender essential oil was distillated in our Institute in 2021, and the synthetic linalool was ordered from Sigma Aldrich Ltd. First the minimal inhibitory concentrations (MICs) were measured with microdilution method [6]. After setting the appropriate microbial count (10^5 CFU/ml), 100-100 µl of both the tested components (lavender oil and linalool) and the bacterial suspension were measured into the wells of the microplate. Both were dissolved in nutrient broth, linalool was dissolved in ethanol and the essential oil was dissolved in Tween40. In case of both test materials 5, 2.5, 1.25, and 0.625 mg/mL concentrations were used in the wells. Incubation (24 hours, 37°C) was followed; absorbance was measured at 600 nm (BMG Labtech, Bio-Tek Ltd.). A cell suspension nutrient solution without essential oil was the positive control, while a cell-free nutrient solution a negative control. Additional ethanol and Tween40 (solvent controls) and antibiotic control (cefixim) were also applied. Biofilms were formed on 96-well microtiter plates, and treated with half of the previously determined MIC concentration of linalool or lavender oil after 4, 6, 8, 12, and 24 hours of incubation phases. Inhibitory rates were calculated using the following formula [7]:

$$\text{Inhibitory rate} = (1 - M/K) \times 100\%.$$

M = the absorbance of the sample
K = the absorbance of the control

3. Results

The following minimal inhibitory concentration (MIC) values were calculated (*Table 1*). The data were determined in mg/mL in all cases.

Table 1. MIC values of lavender essential oil and linalool against our tested bacteria

	H. influenzae	*H. parainfluenzae*	*P. aeruginosa*	*S. pneumoniae*
linalool	0.3	0.4	**0.4**	**0.2**
linalyl-acetate	0.4	0.4	**0.5**	**0.2**

As the values show in Table 1, *P. aeruginosa* was the most resistant to the treatment while *S. pneumoniae* was the most sensitive. Figure 1 shows the inhibition of biofilm formation after linalool treatment.

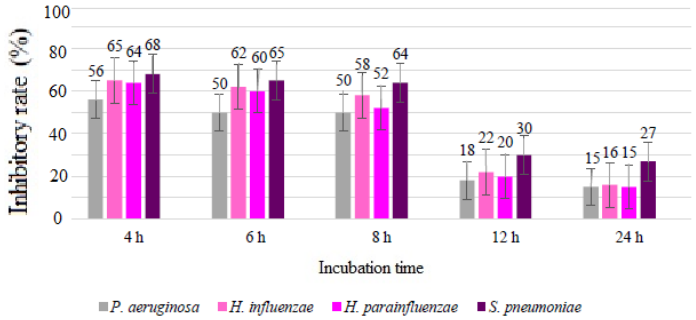

Figure 1. Biofilm-inhibition of linalool against our tested bacteria

Based on Figure 1 the linalool could reduce biofilm formation after 8 hours, but in the 12th and 24th hour of the experiment this component was less effective. With lavender essential oil the following results were obtained (Figure 2).

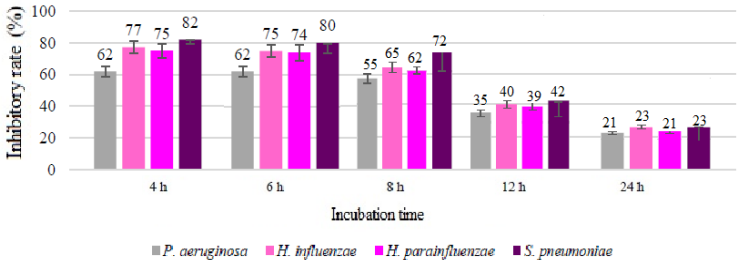

Figure 2. Biofilm-inhibition effect of lavender essential oil against our tested bacteria

Based on Figure 2 it can be concluded that lavender essential oil was more effective than linalool. Both were most effective at the time of 4 hour of incubation time.

4. Discussion and Conclusions

The spread of antibiotic resistance is becoming an increasingly serious problem. Its main cause is the excessive and inappropriate use of antibiotics. In order to stop the widespread resistance, it is necessary to investigate new plant-derived compounds to explore alternative options. According to literature data lavender essential oil has a board spectrum antimicrobial activity against bacterial strains and fungi [8]. Based on these facts we focused on lavender essential oil and the main component of the oil (linalool) to find out their effects against four, biofilm-forming bacteria causing respiratory diseases. According to Paweł K. et al. [9] linalool has antimicrobial effect against multidrug-resistant *Klebsiella pneumoniae.* Puvača et al. identified *in vitro* antibacterial effect of linalool against *E. coli, S. aureus, S. tyhpi*, and *C. koseri*, and found the same result [10]. As Badr et al. found lavender essential oil and its main componenst were effective against Gram-negative (*Salmonella typhimurium*) and Gram-positive (*Staphylococcus aureus*) bacteria, and a fungus (*Aspergillus flavus*) and a yeast (*Candida albicans*) [11]. We found same results, because linalool and lavender essential oil were effective against *Pseudomonas aeruginosa, Haemophilus influenzae, Haemophilus parainfluenaze* and *Streptococcus pneumoniae*. *P. aeruginosa* was the most resistant to the treatment while *S. pneumoniae* was the most sensitive. The importance of incubation time was found. It is a crucial information at which time the treatment is being used, as the activity of the components (linalool or lavender oil) decreases with the incubation time progress. Both were the most effective at the time of 4 hour incubation. We conclude that lavender essential oil and its main component linalool have antibacterial and anti-biofilm activity. Overall, our *in vitro* results may provide further information on the practical use of lavender essential oil.

ACKNOWLEDGEMENTS

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-46

Lavandula species and varieties: resources of essential oils and distillation by-products of different quality

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Keywords: lavender and lavandin varieties, total polyphenol content, antioxidant capacity, liquid residue, plant residue

Abstract

The main objective of the present study was to highlight heterogeneity of five lavander (*Lavandula angustifolia* Mill.) and four lavandin (*L. x intermedia* Emeric ex Loisel) cultivars, concerning content and composition of the essential oils (EO), total polyphenol content (TPC) and antioxidant capacity (AC) of distillation by-products (LR: liquid residues; PR: plant residues). Inflorescences of 6-year-old plants belonging to 5 *Lavandula angustifolia* (LA) varieties ('Hidcote', 'Munstead', 'Maillette', 'Budakalászi 80', 'Tihanyi') and to 4 *L. x intermedia* (LI) ('Grosso', 'Grappenhall', 'Super', 'Judit') were collected in full flowering in 2023. Dried original samples (OS) were hydro-distilled, then the essential oil (EO) samples were subjected to GC/MS analysis. As it was expected, significant differences found among species and cultivars in EO content, ranging between 0.68-4.70 ml/100g in LA, and 3.86-8.19 ml/100g in LI varieties. In the case of antioxidant capacity, data varied considerably not only by genotypes but also according to the extract types (OS, LR or PR). AC data were mostly outstanding in liquid residues, except for LA 'Hidcote' and LI 'Super', where the peak AC values (296.16 and 234.28 mg ASE g^{-1} DW) were detected in PRs. On the contrary, extract yields (EY) and total polyphenols (TPC) varied exclusively by extract types, irrespective of genotypes. Peak TPC values measured in liquid residues (0.902-1.507 mg GAE g^{-1} DW. Based on our results, we can conclude that *Lavandula* wastes have highly diverse quality. Low TPCs in distillation wastes suggest that non-phenolic compounds (e.g. triterpenes) may also contribute to the considerable antioxidant capacity detected.

1. Introduction

By-products of industrial steam distillation of lavander and lavandin have thoroughly investigated in the past decades to provide data on their active compound levels and to overview the possible areas of utilization [1, 2]. Distilled staws of *Lavandula* species were found to be mineral and carbon-rich plant residues which are readily available sources of valuable substances (terpenoids and phenolic compounds, including oleanolic and ursolic acids, herniarin, rosmarinic and chlorogenic acids, etc.) of industrial interest [3]. Antioxidant activity and total polyphenol content of lavandin waste after essential oil distillation were proven by using different extraction methods [1]. In vitro antifungal and antibacterial activities of residual water and solid waste of hydrodistillation of lavender were also found by Ciocarlan et al [2]. The main objective of the present study was to point out to the differences among five lavander (*Lavandula angustifolia* Mill.) and four lavandin (*L. x intermedia* Emeric ex Loisel) cultivars, concerning content and composition of the essential oils (EO) as well as total polyphenol content (TPC) and antioxidant capacity (AC) of distillation by-products (liquid and plant residues). According to our previous findings [4] on disctinct TPC and AC values of the same cultivar collection, we were aimed to get confirmation of the hypothesis on the quantitative and qualitative differences of their distillation waste products, either.

2. Material and Methods

Inflorescences of 6-year-old plants belonging to 5 *Lavandula angustifolia* Mill. (LA) varieties ('Hidcote', 'Munstead', 'Maillette', 'Budakalászi 80', 'Tihanyi') and to 4 *L. x intermedia* Emeric ex Loisel (LI) ('Grosso', 'Grappenhall', 'Super', 'Judit') were collected in full flowering in June and July, 2023, by 5 replications, in the *Lavandula* collection of the MAP experimental field of the Hungarian University of Agriculture and Life Sciences (Budapest, Hungary). Dried original samples (OS) were hydro-distilled by a Clevenger type apparatus for 2 h. The essential oil (EO) samples were subjected to GC/MS analysis using an Agilent Technologies 6890 N GC equipped with an Agilent Technologies MS 5975 inert mass selective detector. After the distillation procedure, we collected the liquid residue (LR) and the plant residue (PR) of each sample as by-products. Total phenolic content (TPC) and antioxidant capacity (AC) of all the original samples (OS), liquid (LR) and solid (PR) distillation waste products were then determined. Based on a modified method of Singleton and Rossi (1965), TPC assessment

results expressed as a gallic acid equivalent (mg GAE g^{-1} DW). The antioxidant capacity was determined by the FRAP assay according to Benzie and Strain (1996), where AC values of samples were calculated from a standard curve equation and expressed as ascorbic acid equivalent based on the dry weight (mg AAE g^{-1} DW). The proportion of the residual mass (RM) and the extract yields (EY) were also determined.

3. Results

As it was expected, significant differences found among species and cultivars in essential oil (EO) content, ranging between 0.68-4.70 ml/100g in LA, and 3.86-8.19 ml/100g in LI varieties (**Figure 1**). In the case of LI, 'Grosso' and 'Super', while in LA, 'Maillette' and 'Budakalászi 80' represented the highest EO values. After distillation, the proportional residual masses were also calculated, where data varied between in a quite narrow range of 51.24 % and 58.43%.

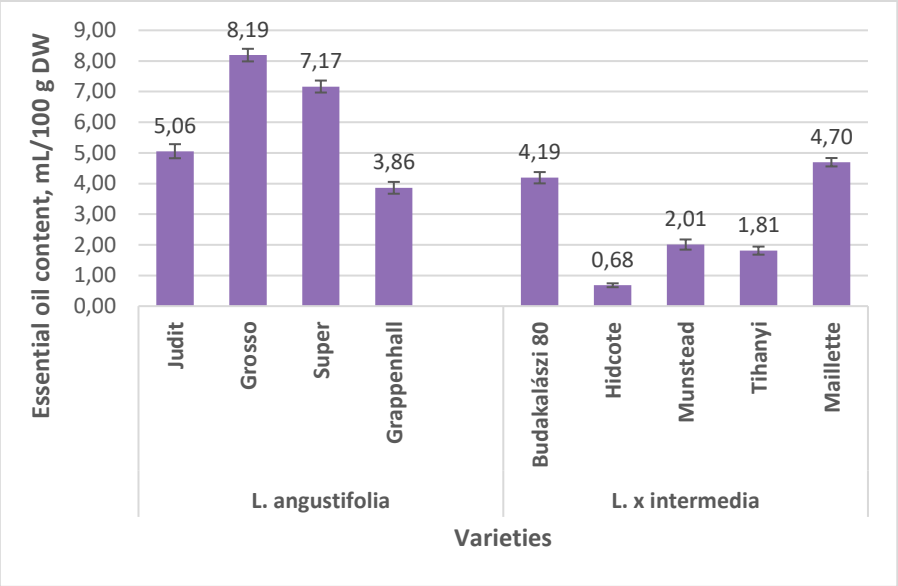

Figure 1 Essential oil content of varieties belonging to the 6-year-old experimental collection of *L. angustifolia* and L. *intermedia* (Budapest, 2023)

In general, the highest yields of extractable materials (EY) detected in the liquid residues (6.74-8.44 mg/mL) of *Lavandula* species and cultivars, followed by original samples (2.62-3.79 mg/mL) and the lowest amounts found in the plant residues (0.51-0.77 mg/mL). Significant differences of EY revealed only among the type of extracts (OS, LR or PR), while data belonging to each genotype of both species were similar within the same extract type. In most cases, the antioxidant capacity data were outstanding in the liquid residues of distillation, except for LA 'Hidcote' (296.16 mg ASE g^{-1} DW) and LI 'Super' (234.28 mg ASE g^{-1} DW), where the plant residues showed the highest AC values (**Figure 2**). The lowest ACs detected mainly in the original samples, however, considerable differences were determined not only among taxa but also in extract types, respectively.Total polyphenols of extract types varied in a general rule, irrespective of cultivars or species, with peak data of the liquid residues (0.902-1.507 mg GAE g^{-1} DW), lower ones in the original plant samples (0.461-0.714 mg GAE g^{-1} DW) and minimal values (<0.100 mg GAE g^{-1} DW) in plant residues (**Figure 3**).

4. Discussion and Conclusions

In our studies, we have found significant differences among varieties and species with respect to EO content and AC, while residual masses of distillation did not change considerably. Extract yields and TPCs varied mainly by extract types and peak values were found generally in liquid residues. We can conclude that both distillation by-products (LR, PR) still possess by considerable AC, while the highest TPC values found generally in liquid residues of *Lavandula* taxa. However, only antioxidant capacity values were comparable with data of the previous studies, our TPC values were generally lower than expected. This phenomenon can partly be attributed to the fact that various types of terpenoids and phenolic compounds coexist in *Lavandula* flowers which may also contribute to the considerable antioxidant capacity detected.

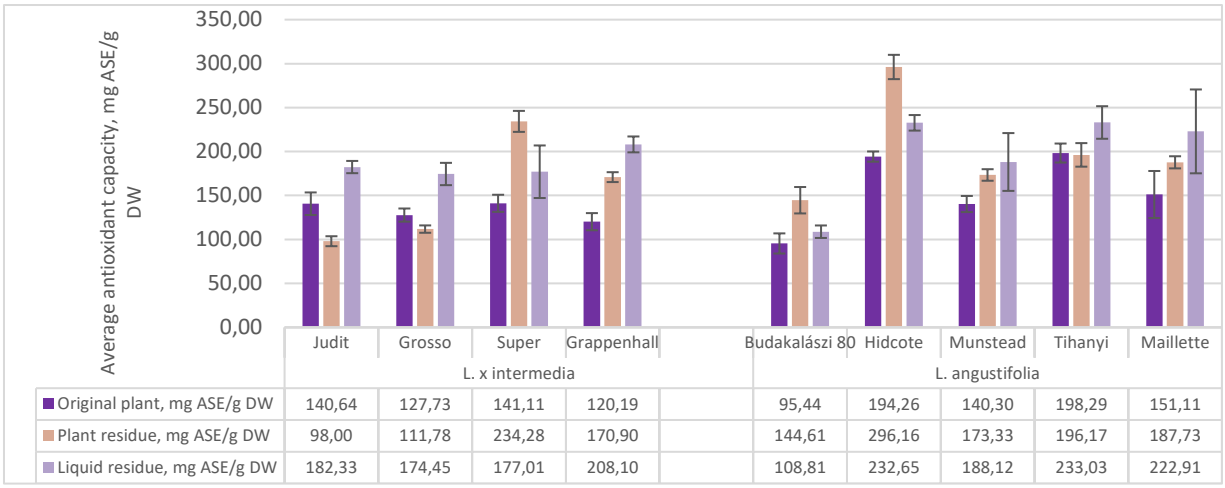

Figure 2 FRAP antioxidant capacity data (mg ASE g^{-1} DW) obtained in the original plant as well as the plant and liquid residues of hydro-distillation, belonging to the 6-year-old experimental collection of *L. angustifolia* and L. *intermedia* varieties (Budapest, 2023)

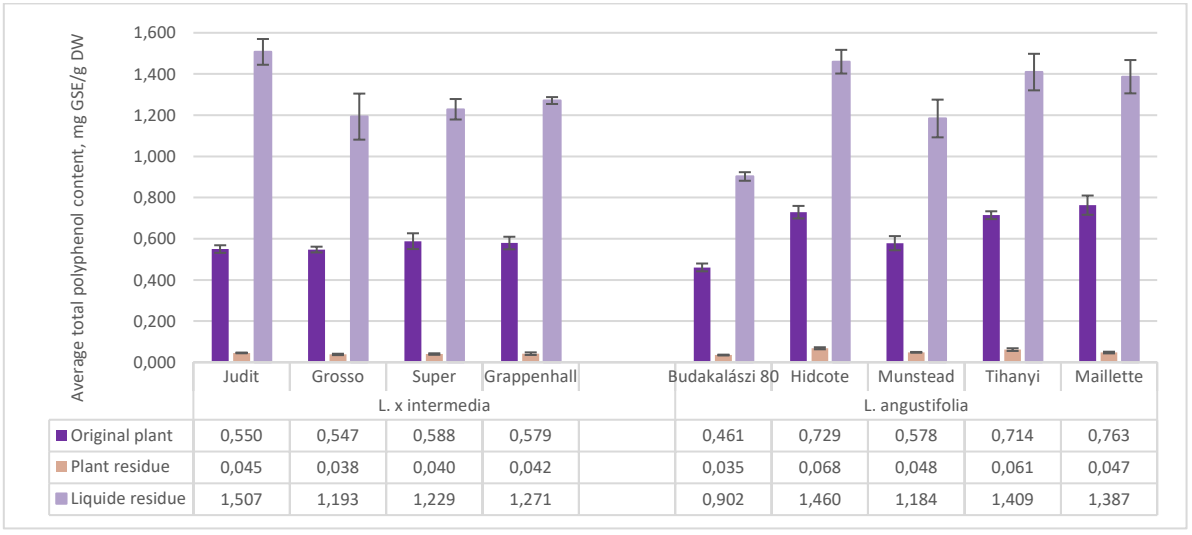

Figure 3 Total polyphenol content values (mg GSE g^{-1} DW) obtained in the original plant as well as the plant and liquid residues of hydro-distillation, belonging to the 6-year-old experimental collection of *L. angustifolia* and L. *intermedia* varieties (Budapest, 2023)

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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"Essential oil intervention: The Antimicrobial and Anti-Biofilm properties of clove oil and Eugenol against *vibrio cholerae* O1"

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Keywords: Antimicrobial Resistance, Biofilm Inhibition, *Vibrio cholerae* O1, Essential Oils, *Syzygium aromaticum* (Clove Oil)

Vibrio cholerae O1, a curved, gram-negative bacillus distinguished by a single polar flagellum, is categorized into serogroups based on somatic O antigens. This bacterium is the causative agent of cholera, a severe infectious gastroenteritis characterized by profuse diarrhoea and vomiting, which can be life-threatening. Cholera predominantly affects communities with poor sanitation and spreads through contaminated water and food (Morris, 2011). Throughout its life cycle, *V. cholerae* alternates between aquatic environments and human hosts, requiring it to adapt to a variety of external signals. Biofilms play a crucial role in this transition, as cells within a biofilm demonstrate significantly higher in vivo fitness and are better prepared for intestinal colonization compared to their planktonic counterparts. (Pombo et al., 2022) The extracellular matrix produced by biofilms protects the microbes, making biofilms highly resilient and difficult to eradicate. This resilience contributes to the major challenge of antimicrobial resistance associated with biofilms. Traditional antimicrobial strategies are increasingly compromised by the rise of antibiotic resistance, prompting the need to explore alternative therapeutic avenues. (Srinivasan et al., 2021) Essential oils, with their diverse chemical compositions and botanical origins, are emerging as promising candidates for combating *V. cholerae* infections. (Bassolé & Juliani, 2012) This study delves into the fascinating realm of microbial warfare, investigating the antimicrobial properties of *Syzygium aromaticum* (clove oil) and Eugenol (major compound found in clove) against *Vibrio cholerae* O1 during both planktonic growth and biofilm formation. Through a series of rigorous experiments—including zone of inhibition, broth microdilution, biofilm formation inhibition via crystal violet assay, visible test tube assay for biofilm formation inhibition and pre-formed biofilm eradication, planktonic time-kill assay, and membrane integrity assay by analysing protein and DNA concentration and fluorescent microscopy—we assessed the effectiveness of clove oil. Additionally, GC-MS analysis was performed to identify key antimicrobial compounds present in clove oil. The results demonstrated a strong capacity for biofilm formation inhibition and eradication. To further support these findings, real-time expression analysis of genes responsible for biofilm formation is ongoing, and scanning electron microscopy of biofilms is being conducted to analyse eradication. This research presents a novel therapeutic strategy using *Syzygium aromaticum* (clove oil) to combat *Vibrio cholerae* O1 infections. The captivating observations of this study unravel the dynamic interactions between these aromatic defenders and the formidable bacterial foe. The findings highlight the potential of essential oils as effective antimicrobial agents, paving the way for further studies and potential clinical applications.

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Comparative GC-MS study of essential oil constituents: headspace vs condensed phase methods with steam-distilled extracts

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Keywords: essential oils, gas chromatography-mass spectrometry, distillation, GC-MS, biological properties

Objective

Essential oils (EOs) are complex mixtures of volatile organic compounds extracted primarily through steam distillation from various parts of aromatic plants, such as leaves, flowers, bark, roots, and seeds. These EOs exhibit numerous biological activities, including analgesic, anti-inflammatory, immunomodulatory, antitumor, and antimicrobial properties. Notably, their antimicrobial properties help inhibit the growth of foodborne pathogens, enhancing food safety and preservation. Key active components in EOs include phenols like carvacrol and thymol, alcohols like linalool and menthol, and aldehydes like citral. These compounds are renowned for their antibacterial properties, acting through mechanisms such as disrupting cell membranes and inhibiting protein synthesis. The plants used in the study include *Mentha piperita*, *Thymus vulgaris*, *Lavandula stoechas*, *Origanum vulgare*, and *Eucalyptus cinerea*, which were provided by the horticultural company Meo Remo in Nettuno (RM). The essential oils were obtained through steam distillation of 5 kg of fresh material for each plant, including both flowers (or flowering tops) and leaves. A liquid-liquid extraction with diethyl ether was performed to isolate the oils.

Methods

This study focused on the comparative analysis of the chemical composition of five essential oils, specifically Mentha piperita (peppermint), Thymus vulgaris (thyme), Lavandula stoechas (lavender), Origanum vulgare (oregano), and Eucalyptus cinerea (eucalyptus). The chemical composition was analyzed in both the condensed phase and the volatile fraction. For the experiment, we used Agilent Technologies, model 5975, to obtain a detailed characterization, gas chromatography coupled with mass spectrometry (GC-MS) was employed. The chromatographic column used was an non-polar Agilent J&W HP5-MS (5% diphenyl, 95% dimethylpolysiloxane, 30 m x 0.25 mm x 0.25 μm film).

This technique allows for the separation, identification, and quantification of the various chemical components present in the EOs, providing an in-depth view of their properties.

Three different methodologies were adopted for the analysis:

- Analysis of EO solutions in cyclohexane: This methodology allows for the determination of the chemical composition of EOs in their liquid form, providing a complete overview of the substances present.
- Headspace (HS) analysis: This technique focuses exclusively on the volatile fraction of EOs, which consists of compounds that can easily evaporate at room temperature. Headspace analysis provides crucial information on the volatile components that contribute to the olfactory and biological properties of EOs.
- Headspace analysis with selective pre-concentration using solid-phase microextraction (SPME): Using a DVB-CAR-PDMS fiber, this advanced technique allows for the capture and concentration of trace volatile compounds that might not be detectable with the standard HS approach. This methodology enhances the sensitivity of the analysis and enables the identification of minor but potentially bioactive volatile compounds.

The antimicrobial activity of the EO samples was studied using broth microdilution tests on strains of carbapenemresistant Acinetobacter baumannii, methicillin-resistant Staphylococcus aureus, and Candida albicans. After determining the MIC, the MBC is assessed. The MBC is the minimum bactericidal concentration. The first clear test tube with the lowest essential oil concentration is selected. The MBC is performed using Muller Hinton agar plates (solid culture medium), followed by inoculating the clear tubes

Results

Through these complementary techniques, the study was able to provide a detailed and comparative characterization of the EOs, highlighting the differences in their chemical composition and, consequently, in their potential biological and industrial applications.

Conclusions

The integration of GC-MS and complementary analysis techniques provided a robust method for the detailed characterization of essential oils. The study successfully identified the chemical profiles of *Mentha piperita*, *Thymus vulgaris*, *Lavandula stoechas*, *Origanum vulgare*, and *Eucalyptus cinerea*, demonstrating the utility of these methods in ensuring the authenticity and quality of EOs. This approach can be applied to various EOs to support their use in food safety, medicinal applications, and other industries.

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Quality assessment and authentication of lavender essential oil

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The pleasant aroma of lavender (*Lavandula angustifolia*) essential oil, combined with its purported health benefits, makes it a popular choice for aromatherapy, cosmetic, flavor, and fragrance applications. The widespread use of lavender essential oil lends itself to potential adulteration. To assess the quality and authenticity of lavender essential oil, it is vital to also understand which factors influence the essential oil profile and which analytical techniques are optimal for analysis.

To investigate authentication of lavender essential oil[1], samples ($n = 41$) were procured directly from farmers/distillers and used as reference materials. These essential oils were analyzed to determine profiles and related data by GC, enantioselective GC, and GC/IRMS. Said analysis resulted in the identification of 43 authentic marker compounds, enantiomeric ranges for 15 compounds, and stable isotope ranges for four prominent compounds in authentic lavender essential oil. This dataset was used to assess the quality of commercially available lavender essential oil samples ($n = 12$) purchased from online retailers. Nine of the twelve (75%) commercial samples studied were adulterated, and 17 adulteration marker compounds were identified from these commercially available samples. These studies stress the importance of understanding factors contributing to natural variation and establish the utility and importance of using a multifaceted analytical approach to differentiate quality and determine authenticity of lavender essential oil.

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Evaluation of different spearmint populations

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Keywords: spearmint, essential oil, antibacterial

Abstract

Spearmint is a perennial plant belongs to the *Lamiacae* family and genus *Mentha*. It has both pharmaceutical and food industrial importance. Its essential oil is rich in compounds having antibacterial effect. It has several varieties, therefore this mint species shows a great essential oil variability. In this research, we have studied the essential oil amount, composition and in vitro antibacterial effect of six cultivated populations of *M. spicata*. Three populations (*M. spicata* J7, *M. spicata* B1, and *M. spicata* J11) exhibited a relatively high EO amount (3.2-3.7 ml/100 g d.w.) comparing to the rest (*M. spicata* B11, *M. spicata* B17, *M. spicata* B4) (1.5-1.6 ml/100 g d.w.). The main active compounds were limonene (12.5%), L-carvone (60.1%), 1,8-Cineole (18.2%), cis-dihydrocarvone (48.3%), trans-dihydrocarvone (33.1%), and in one case, surprisingly, carvacrol (25.7%). Among these compounds carvacrol has been found only few times before in this species, and this essential oil possessed a remarkable antibacterial effect.

1.Introduction

Spearmint or the so-called *M. spicata* is believed to be an ancient natural hybrid of *M. longifolia* and *M. suaveolens*, but it is not explicitly indicated in its botanical name. The synonym for this is *M. viridis*. It is commonly found in proximity to streams, lake coastlines, wet fields, and roadways throughout the temperate and Mediterranean regions of Eurasia and Northern Africa. Being a cultivated plant, it is one of the most economically significant species of the *Mentha* genus [1]. The variety that is widely recognized is '*crispata*'. The plant species mentioned is a perennial plant with rhizomes [2]. *Mentha spicata*, like other mint species, possesses a stem with four sides and leaves that grow in pairs on opposing sides of the stem. The plant has leaves that are of a medium shade of green, measuring 5-6 cm in length, and have either serrated or smooth edges. Additionally, it produces flowers that range in colour from pink to purple and are grouped in whorls. It contains essential oil and non-volatile phenolic compounds as well. However, the composition of its EO exhibits a high level of variation. The main active compounds in *M. spicata* are L-carvone, limonene, 1,8-Cineole. Many researchers have proven that *M. spicata* has several healing effects. It can be used as an antioxidant, antifungal, and antibacterial substance [3] [4].

Based on the importance of this plant species a collection of different originated spearmint populations has been cultivated in the experimental field of Hungarian University of Agriculture and Life Sciences. Our aim was to investigate their essential oil amount, composition, as well as their possible antibacterial effect tested on different bacteria lines.

2. Material and Methods

2.1. Plant materials and cultivation

Plantations of the examined mint populations have been maintained in the experimental field of the Department of Medicinal and Aromatic Plants, Hungarian University of Agriculture and Life Sciences, in Soroksár. Flowering stems of six different spearmint populations were collected in July, 2022. Natural drying in shade, protected place was applied.

2.2. Essential oil extraction

Only crumbled flowers and leaves (10 g) were used for hydrodistillation (according to the 7th Hungarian Pharmacopoeia) to define the essential oil amount expressed in ml/100 g dry weight. This measurement has been done in three replications. Water residue was removed by employing anhydrous sodium Sulphate. Subsequently, the extracts were filtered using a syringe filter and stored in tightly sealed vials within a refrigerator at a temperature of 4°C until the time of analysis.

2.3. Gas Chromatographic Mass Spectrometric analysis

The GC–MS analyses were carried out on each EO sample using an Agilent Technologies 6890N instrument equipped with HP–5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm) and an Agilent Technologies MS 5975 inert mass selective detector. The temperature program was the following: initial temperature 60°C, then increased by a rate of 3°C/min up to 240°C; the final temperature was maintained for 5 min. The carrier gas was helium (1 ml/min); injector and detector temperatures were 250°C. Split ratio: 30:1. 10 ul of EO has been diluted by n-hexane to 1 ml and from this; the injected quantity was 0.2 ul.

2.4. In vitro antibacterial analysis

The antimicrobial activity was conducted on five reference bacterial strains: *Escherichia coli* ATCC 25922, *Salmonella enterica* ATCC 13076, *Bacillus cereus* ATCC 10876 and *Staphylococcus aureus* ATCC 29213. The minimal inhibitory concentration (MIC) of the tested oils were performed using the broth microdilution method. Two-fold serial dilutions of the essential oils were performed in Muller-Hinton Broth (Sigma-Aldrich) supplemented with 1% DMSO to enhance the solubility of the oils. In this experiment the final concentration of oils ranged from 0.016% to 1%, v/v. IC50 (half maximal inhibitory content) and minimal bactericidal concentration (MBC) were also determined.

3. Results

3.1. Essential oil amount

According to our results great differences could be seen in the essential oil amounts of the analysed *M. spicata* populations. *M. spicata* J7, *M. spicata* B1 and *M. spicata* J11 contained the highest EO amount with average amounts of 3.7 ml/100g d.w., 3.5 ml/100g d.w. and 3.2 ml/100 g d.w. Lowest essential oil amounts were detected in *M. spicata* B4 and *M. spicata* B17 with average amounts of 1.5 ml/100g d.w. and 1.6 ml/100g d.w.

3.2. Essential oil composition

Upon analysis, it can be observed that the different spearmint varieties exhibited variations in the essential oil composition. Some compounds were found in higher ratios in certain species, while others were present in trace amounts or absent altogether. The data of the EO composition is presented in Table 1. The compounds with a relative abundance exceeding 0.2% are listed in the table.

To discuss the most abundant compounds in the spearmint, we will focus on the highest relative percentages or peak areas associated with each compound. As expected, limonene, 1,8-cineole, and L-carvone were the most dominant compounds with the highest value of limonene 12.5 % in *M. Spicata* B17 and 1,8-cineole was 18.2% in *M. Spicata* B4. Another most abundant compound was L-carvone 60.1% in *M. Spicata* B17. **Surprisingly, thymol and carvacrol were the main compounds in *M. spicata* B11 with the ratios of 14.0% and 25.7%, respectively.**

M. spicata J7 and J11 were rich in cis-and trans-dihydrocarvone with 37.3% and 48.3%.

3.3. In vitro antibacterial activity

Among the analysed spearmint essential oils *M. spicata* B11 (containing as main compounds carvacrol, 1,8-cineol, thymol) showed the strongest activity in each tested bacteria line (MIC: 0.03, IC50: 0.016-0.017, MBC: 0.06). Weakest results were produced by those lines containing cis-and trans-dihydrocarvone as main compounds (*M. spicata* J7 and J11) (MIC: 0.5-2, IC50: 0.1-0.6, MBC: 0.5-2).

Table 1. Essential oil composition (given as percentage %) of six *M. spicata* populations

E.O. compounds	*M. spicata* B1	*M. spicata* B11	*M. spicata* B17	*M. spicata* B4	*M. spicata* J11	*M. spicata* J7
Limonene	6.6	n.d.	12.5	5.9	4.7	4.0
L-carvone	51.7	0.1	60.1	n.d.	0.5	0.3
ß-Caryophyllene	1.6	3.4	7.3	2.6	5.0	4.3
1,8-Cineole	11.1	17.5	0.3	18.2	3.4	1.5
Cis-dihydrocarvone	0.3	0.2	0.7	n.d.	48.3	37.3
Trans-dihydrocarvone	n.d.	0.2	n.d.	n.d.	12	33.1
Germacrene-D	1.1	3.2	3.3	4.2	4.2	3.7
p-Cymene	0.1	11	n.d.	n.d.	n.d.	n.d.
Thymol	n.d.	14	n.d.	n.d.	n.d.	0.3
γ-Terpinene	0.4	6.1	n.d.	n.d.	n.d.	n.d.
Carvacrol	n.d.	25.7	n.d.	n.d.	n.d.	n.d.
Trans-sabinene-hydrate	10.8	1.1	n.d.	1.0	0.2	n.d.

n.d. – not detected

4. Discussion and Conclusions

The analysed spearmint populations showed great differences in all tested parameters. Population signed B1 was characterised by high essential oil amount and rather different essential oil composition than the other populations containing carvacrol, 1,8-cineol and thymol as main essential oil compounds. As we expected this oil showed the strongest in vitro antibacterial activity because of the high ratios of phenolic terpene compounds. Lowest activities were produced by populations containing cis-and trans-dihydrocarvone as main compounds.

To clarify the possible correlations between the essential oil compounds and the detectable in vitro antibacterial effect further experimental work is necessary in the future. Since carvacrol/thymol type spearmint description is rather scarce in the literature, analysis on this special population need to be continued.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Aroma characterization of commercial *Alpinia zerumbet* essential oils by fingerprinting analysis

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Keywords: *Alpinia zerumbet*, *Alpinia zerumbet* var. *excelsa*, Aroma characteristics, Fingerprinting analysis

Abstract

Alpinia zerumbet (AZ) is a perennial herb of the *Zingiberaceae* family that is widely distributed in tropical and subtropical regions of the world. The essential oil from the leaves, which has a characteristic aroma, is used as a raw material in perfumes and cosmetics. The essential oil of *A. zerumbet* has several biological and pharmacological properties. In Japan, essential oils of *A. zerumbet*, including closely related species, are sold; however, the composition of these essential oils varies greatly, and their fragrance properties are not stable, even among essential oils from the same manufacturer. This instability is due to several factors, including the low essential oil content of *A. zerumbet* leaves, significant differences in the composition of each plant, and contamination with closely related species. In this study, the aroma characteristics of commercially available *A. zerumbet* essential oils were analyzed in detail using fingerprinting combined with heat mapping and two-dimensional cluster analysis. Some of the essential oils of *A. zerumbet* contained key compounds γ-terpinene and terpinen-4-ol, both at concentrations above 10%, suggesting that a significant amount of *A. zerumbet* var. *excelsa* were either contaminated or misidentified as raw material species. Fingerprinting analysis combined with heat mapping and 2D cluster analysis proved to be an effective method for visualizing the differences between essential oil samples and extracting a group of compounds that characterize each species and essential oil sample.

1. Introduction

The species *Alpinia zerumbet* (Pers.) Burtt & Smith, belonging to the *Alpinia* spp. within the Zingiberaceae family, is widely distributed in the tropical and subtropical regions of the world, including India, Malaysia, Taiwan, Brazil, and Japan. In Japan, it is found from southern Kyushu to the Amami, Okinawa, Daito, and Sakishima Islands (Miyako and Yaeyama Islands). The essential oil extracted from its leaves, known for its characteristic fragrance, is used as a raw material in perfumes and cosmetics. The essential oil of *A. zerumbet* possesses various biological properties, including antibacterial, bactericidal, antifungal, diuretic, and antihypertensive effects, among other biological and pharmacological activities [1]. While extensive research has focused on the aromatic and functional properties of *A. zerumbet*, recent studies in Taiwan have reported five species of the *Alpinia* spp., with natural hybridization occurring between species within the genus. Liu et al. suggested that the effects of frequent interspecific hybridization on the phylogenetic reconstruction of the *Alpinia* spp. in Okinawa need further investigation [2]. In Japan, essential oils of *A. zerumbet*, including those from closely related species, are marketed by 22 different manufacturers. The compositions of these essential oils not only vary widely but also exhibit unstable fragrance characteristics, with significant variations in composition from one production batch to another, even among oils from the same manufacturer. This variability is primarily attributed to several factors, including the low essential oil content in *A. zerumbet* leaves, significant differences in the composition of constituents from each plant, and contamination with closely related species. In this study, the aroma characteristics of *A. zerumbet* essential oil sold in Japan were analyzed and evaluated in detail using fingerprinting techniques, coupled with heat mapping and two-dimensional cluster analysis.

2. Material and Methods

Twenty-six essential oil samples from *A. zerumbet*, including closely related species, were purchased from 22 manufacturers in Japan. Some samples contained essential oils produced by the same manufacturer in different production lots. The essential oils were analyzed using a GC/MS (QP-2010 Plus; Shimadzu, Kyoto, Japan) equipped with an auto-injector (AOC-20i; Shimadzu). The quantitative determination of essential oil components was performed based on peak area measurements. GC/MS analyses were performed using a DB-WAX column, 60 m in length, 0.32 mm ID, and 0.5 μm in thickness. The GC oven temperature program was set as follows: held at 40 °C for 3 min, increased at 5 °C/min to 165 °C, then increased at 10 °C/min to 220 °C, and held for 3 min.

The injector and detector temperatures were set at 250 °C, and the mass range was scanned from 30 to 600 amu. Control of the GC/MS system and data peak processing were carried out using Shimadzu GC/MS Solution software, version 4.3. Volatile components were identified by comparing their retention indices and mass fragmentation patterns with those of the MS libraries (NIST05 and FFNSC Library ver. 1.2; Shimadzu, Kyoto, Japan). Linear retention indices were determined for all constituents using a homologous series of *n*-alkanes (C8–C24) injected under identical chromatographic conditions. For each essential oil fingerprint, a heatmap was created from two-dimensional hierarchical cluster analysis using the Ward method, and the fingerprints were patterned by strain for the aroma constituent content. Heat mapping and 2D cluster analysis were performed using the Seaborn Library in Python.

3. Results and Discussion

Table 1 lists the essential oils of *A. zerumbet* (AZ) and its close relatives, *A. zerumbet var. excelsa* (AZe) and *A. formosana* (AF), that are commercially available. In previous studies, we have shown that the concentrations of certain key compounds can be used to discriminate between species. The concentrations of the key compounds β-pinene, γ-terpinene, and terpinen-4-ol are also shown. The concentration of β-pinene in AF-1, which is sold as the essential oil of AF, is low (<10%), and the concentrations of γ-terpinene and terpinen-4-ol are high (>10% in each case), suggesting that a considerable amount of AZe may be present in AF. Regarding the essential oil of AZ, the concentrations of γ-terpinene and terpinen-4-ol in AZ-1, AZ-2, and AZ-18 were high (all >10%), suggesting that a considerable amount of AZe was either contaminated or the species of the raw material was misidentified.

Table 1. Essential oils of commercially available *Alpinia zerumbet* and related species presented for analysis, and concentration of key compounds for species identification.

Sample*	Part	Scientific name description	Relative Conc. (%)**		
			β-Pinene	γ-Terpinene	Terpinen-4-ol
AF-1	Leaf, flower, stem, seed	*A. formosana*	5.17	**11.70**	**17.15**
AZ-1	Leaf	*A. zerumbet*	3.38	**13.56**	**11.26**
AZ-2	Unlisted	*A. zerumbet*	2.90	**14.14**	**10.43**
AZ-6	Leaf	*A. zerumbet*	3.20	**10.55**	3.28
AZ-9	Leaf	*A. zerumbet*	2.76	0.16	1.13
AZ-14	Unlisted	*A. zerumbet*	2.22	1.39	1.22
AZ-16	Leaf	*A. zerumbet*	3.54	0.46	0.42
AZ-17	Leaf	*A. zerumbet*	4.00	1.90	0.87
AZ-18	Leaf	*A. zerumbet*	3.03	**14.49**	**8.48**
AZ-20	Leaf	*A. zerumbet*	3.34	0.65	0.52
AZ-22	Leaf	*A. zerumbet*	5.16	0.31	0.37
AZ-Y	Leaf	*A. zerumbet*	1.87	0.48	1.05
NAZ-10	Unlisted	No scientific name listed	3.56	**15.36**	**10.96**
AZe-3	Leaf	*A. zerumbet var. excelsa*	3.64	**14.38**	**16.84**
AZe-4	Leaf	*A. zerumbet var. excelsa*	4.98	**14.10**	**14.40**
AZe-5	Leaf	*A. zerumbet var. excelsa*	4.98	**14.07**	**14.43**
AZe-7	Leaf	*A. zerumbet var. excelsa*	3.34	**13.32**	**16.70**
AZe-8	Unlisted	*A. uraiensis*	4.34	**11.19**	**16.28**
AZe-12	Leaf, flower, stem, seed	*A. zerumbet var. excelsa*	4.68	**10.97**	**17.10**
AZe-13	Unlisted	*A. zerumbet var. excelsa*	3.87	**13.96**	**17.12**
AZe-15	Unlisted	*A. uraiensis*	3.42	**13.98**	**16.95**
AZeAZ-19	Leaf	*A. zerumbet / A. zerumbet* var. *excelsa*	3.97	**11.29**	**18.16**
AZe-21	Leaf	*A. zerumbet var. excelsa*	2.98	0.00	**14.56**
AZe-K	Leaf	*A. zerumbet var. excelsa*	3.34	**12.62**	**20.18**
Aze-M	Leaf	*A. zerumbet var. excelsa*	1.94	**11.37**	**21.09**
AZe-Y	Leaf	*A. zerumbet var. excelsa*	3.42	**13.04**	**21.46**

*: All samples were produced in Okinawa and obtained by steam distillation.
**: γ-Terpinene concentrations above 10% and terpinen-4-ol concentrations above 5% are in bold

Fig. 1 shows the fingerprints of commercial essential oils obtained by heat mapping and 2D cluster analysis. Hierarchical cluster analysis classified the essential oil samples into two groups: AZ and AZe. The compound groups were classified into Component 1, which characterized AZe, and Components 2 and 3, which characterized AZ. As indicated by the concentrations of γ-terpinene and terpinen-4-ol in the component analysis, AF-1, AZ-1, AZ-2, AZ-4, and AZ-18 (red circle shown in Fig. 1) not only have high concentrations of these key compounds but also contain little of Components 2 and 3, which characterize AZ. Therefore, it is clear that although the scientific name is AZ, the aroma is characteristic of AZe. AZ-6, which contained high concentrations of γ-terpinene, not only contained Component 1 but also contained much of Components 2 and 3, which characterize AZ. The fingerprint pattern was similar to that of AZ-17 produced by the same manufacturer (blue circle shown

in Fig. 1), suggesting that AZ-6 was partially contaminated with AZe. In summary, fingerprinting analysis combined with heat mapping and 2D cluster analysis proved to be a very effective method to not only visualize differences between samples but also to extract groups of compounds that characterize each species and essential oil sample.

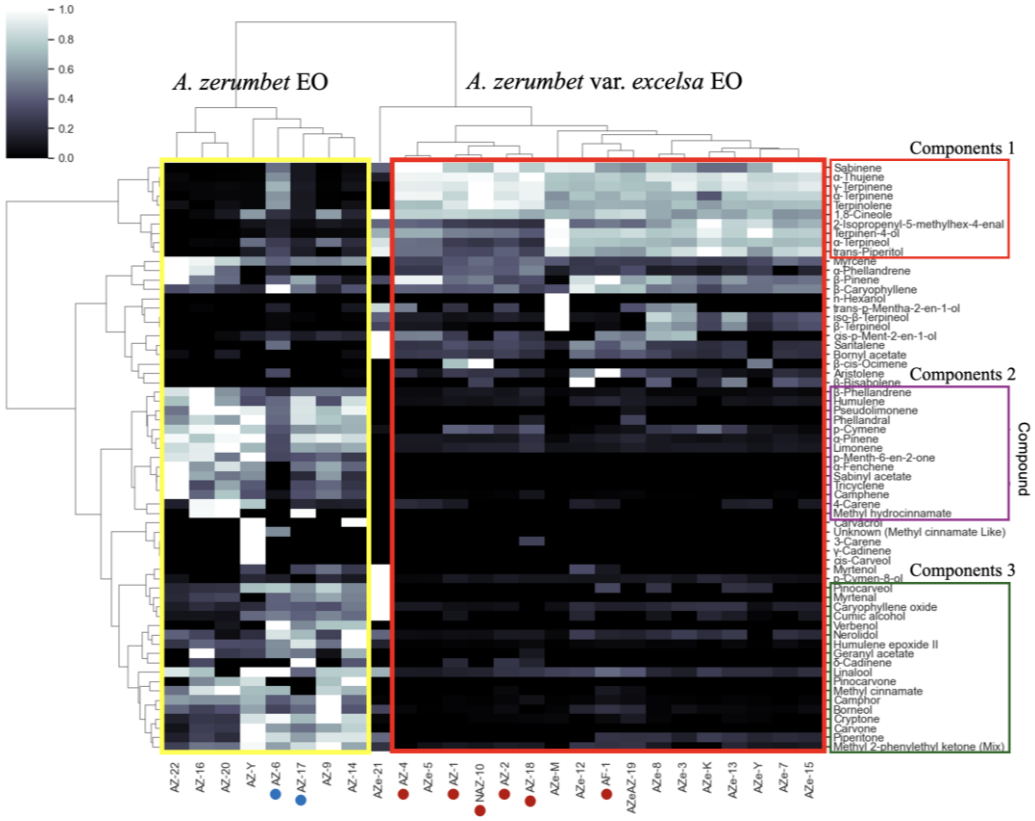

Figure 1 Fingerprints of commercial essential oils by heat mapping and 2D cluster analysis; blue dots indicate different lots from the same manufacturer, and red dots indicate a sample determined as an essential oil from a different species than the scientific name given.

4. Conclusions

In this study, the aroma characteristics of essential oils of *A. zerumbet* and related species sold in Japan were analyzed and evaluated in detail by fingerprint analysis combining heat mapping and two-dimensional cluster analysis. Key compound groups for species identification and sample fingerprinting suggested that commercial AZ essential oils were either contaminated with significant amounts of AZe or misidentified as the species of the source material. Fingerprinting analysis proved to be a very effective method to not only visualize differences between samples but also to extract groups of compounds that characterize each species and essential oil sample.

ACKNOWLEDGEMENTS

This study was supported in part by the FY2022 Okinawa Innovation Ecosystem Joint Research Promotion Project. We express our gratitude for the opportunity.

Conflict of Interest

The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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P-52

How effective are essential oils in inhibiting polymicrobial environments?

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Keywords: polymicrobial, resistance, ESKAPE, essential oils, *Origanum vulgare, Thymus vulgaris*

Abstract

Antimicrobial research on essential oils has mostly been focused on monomicrobial environments, however, recent research has highlighted the importance of polymicrobial infections, which involve multiple pathogens within one pathological infection. Polymicrobial ınfections are complex as different pathogens interact with each other, which in turn affects disease progression and response to therapy. This impacts on antimicrobial resistance. As essential oils have been shown to inhibit a broad range of pathogens, they may serve to inhibit polymicrobial environments, thus a study was designed to test this hypothesis. This study aimed to investigate the antimicrobial properties of six essential oils (*Origanum vulgare, Thymus vulgaris, Carum carvi, Matricaria recutita, Commiphora myrrha, Santalum austrocaledonicum*) against the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter aerogenes*) group of pathogens to determine how essential oils antibacterial efficacy changes once tested in a simulated polymicrobial environment consisting of different combinations of the selected bacteria. The minimum inhibitory concentration (MIC) was determined using the broth microdilution assay. Results of this study revealed that the polymicrobial environment had little effect on the antimicrobial activity of the essential oils ($p \geq 0.5$), whereas a significant shift in MIC value was observed for antibiotics when tested in the same environments. *Origanum vulgare* and *Thymus vulgaris* maintained the majority of the antimicrobial activity across the various polymicrobial environments, highlighting these two oils as noteworthy antimicrobials for further clinical studies. This study demonstrates how essential oils can withstand polymicrobial environments, making them prime candidates to combat antimicrobial resistance.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

P-53

Effect of *Alpinia zerumbet* essential oils on behavioral characteristics in ovariectomized mice

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Keywords: *Alpinia zerumbet*, Ovariectomized mice, Behavioral characteristics, Depressive state, Menopausal syndrome

Abstract

Alpinia zerumbet is an aromatic plant, distributed widely in Southeast Asia and Brazil, that has been extensively studied because its essential oil (EO) has a variety of biological functionalities. Ovariectomized mice, which are models for depressive state and menopausal syndrome, and sham-operated mice were automatically presented with two types of *A. zerumbet* leaf EO collected in Okinawa, Japan, while their behavior in cages was recorded. In the ovariectomized groups, a significant decrease in basal activity and a change in activity rhythm were observed compared to the sham groups. However, when comparing each EO group and the control group, a behavioral improvement was found in *A. zerumbet* EO groups. As a result of canonical discriminant analysis, the behavior of the ovariectomized group was characterized and plotted below the sham group due to a decrease in activity levels, frequency/time of access to water, and many behavioral characteristics. Meanwhile, the behaviors of *A. zerumbet* EO groups were shifted to the sham side because their behavioral peculiarities were improved by the presentation of each EO. Even though the result of the principal component analysis clearly separated behavioral characteristics of the ovariectomized groups and the sham groups, the characteristics of *A. zerumbet* EO group returns to normal class. The result of this study suggests that our *A. zerumbet* EOs have the potential to improve some symptoms of depression and menopausal syndrome.

1. Introduction

The aromatic plant *A. zerumbet* is distributed widely in tropical and sub-tropical regions such as Southeast Asia and Brazil. The EO has been reported to exhibit various biological activities, including antidepressant, anxiolytic activity and anti-inflammatory effects [1]. However, even among "*A. zerumbet*", the same species, the volatile compound composition and the yield of the EO are clearly different between individual plants [2]. Therefore, we believe that it is important to clarify the volatile compound composition of the *A. zerumbet* EO used in the study with reporting on the effects. On the other hand, we have been investigating that inhalation of *A. zerumbet* EO has the effect of increasing serum serotonin level and decreasing serum adrenaline and dopamine levels in urethane-anesthetized rats. Since a decrease in serum catecholamine levels reflects a reduction in sympathetic nervous activity, these results suggest that the EO might be expected to have a relaxing effect. We evaluated the effect of *A. zerumbet* EO in behavioral characteristics in ovariectomized mice, which are models of depression and menopause syndrome.

2. Material and Methods

The two types of EO, *Alpinia zerumbet* (Pers.) B. L. Burtt and R. M. Sm. (AZ) and *Alpinia zerumbet* (Pers.) B. L. Burtt and R. M. Sm. var. *excelsa* Funak and T. Y. Ito (AZe) were extracted using steam distillation from the leaves collected on Okinawa Island, Japan. The volatile compound compositions of each EO were analyzed by GC/MS (QP2010 Plus, column: DB-WAX 60 m × 0.32 mm i.d., 0.5 μm) and identified by comparing their retention indices and mass fragmentation patterns with MS libraries (NIST05 and FFNSC Library ver. 1.2; Shimadzu, Kyoto, Japan). Linear retention indices were determined for all constituents using a homologous series of *n*-alkanes (C8–C24) injected under the same chromatographic conditions as the samples. For recording stereotypical/repetitive behaviors in mice, the IntelliCage (TSE Systems) was used; it is the behavioral testing system that can evaluate spontaneous behaviors and cognitive abilities by fully automatically collecting the behaviors of a large number of mice 24 hours using RFID (radio frequency identification) and multiple sensors. Fifty-week-old C57BL/6J strain female mice were randomly allocated to ovariectomized mice and sham-operated mice. Behaviors of the ovariectomized groups and the sham groups were recorded, respectively, while they were automatically exposed

to each EO (10 µl, 3 times/overnight). The control groups were presented with water instead of EO. The test groups (n=8) in this study were the following six groups; control-sham group (C-S), control-ovariectomized group (C-OVX), AZEO-sham group (AZ-S), AZEO-ovariectomized group (AZ-OVX), AZeEO-sham group (AZe-S) and AZeEO-ovariectomized group (AZe-OVX). We analyzed basal activity, activity rhythm, and stereotypical/repetitive behavior collected for three weeks, and then, performed the canonical discriminant analysis and the principal component analysis using basal activity indexes.

3. Results

Although 1,8-cineole concentration was similar (Table 1), the main volatile compound components were quite different between AZEO and AZeEO as determined by GC/MS analysis. The main aroma components of AZEO were *p*-cymene, 1,8-cineole, *α*-pinene, and limonene whereas AZeEO contained terpinen-4-ol, 1,8-cineole, *γ*-terpinene, and sabinene.

Table 1. The top 10 volatile compound components of AZEO and AZeEO.

Compound	RI*	AXEO (%)	AZeEO (%)
α-Pinene	1026	10.48	(1.69)
α-Thujene	1029	(0.29)	3.87
Camphene	1068	5.18	(0.17)
β-Pinene	1113	(1.87)	3.46
Sabinene	1126	(0.34)	11.63
α-Terpinene	1186	(0.12)	3.66
Limonene	1203	10.10	1.89
β-Phellandrene	1213	3.79	-
1,8-Cineole	1217	15.50	18.42
γ-Terpinene	1253	(0.47)	13.19
p-Cymene	1279	19.25	7.00
Terpinolene	1293	(0.08)	1.74
Fenchone	1411	2.24	-
Camphor	1536	3.52	(0.08)
Terpinen-4-ol	1619	(1.04)	21.70
Humulene epoxide II	2070	2.94	(0.09)
Methyl cinnamate	2096	2.11	-

*Retention index values were determined relative to *n*-alkanes on the DB-WAX column., ( ): % of compounds detected but not in the top 10., -: <0.01%

Automatic presentation of each EO and water did not cause an aversion in either the sham groups (C-S, AZ-S, AZe-S) or the ovariectomized groups (C-OVX, AZ-OVX, AZe-OVX). Compared to each sham groups, the ovariectomized groups showed a decrease in basal activity and a change in basal activity rhythm; a change in the amplitude of the activity rhythm, and the included behavioral frequency. No significant effects on the basal activity and its rhythm were observed due to inhalation each EO (Figure 1). The stereotypical/repetitive behavior in the cage, including repeated access to the same corner, were observed in the ovariectomized groups compared to the respective sham groups. However, when comparing each EO group and the control group, a behavioral improvement was found in the AZ- and AZe-S or OVX groups (Figure 2).

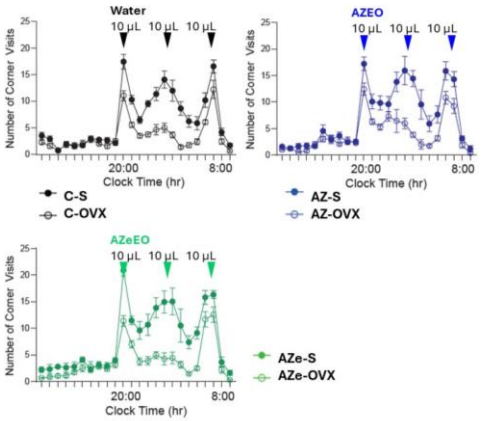

Figure 1. Basal activities of sham groups and ovariectomized groups after 2 weeks.

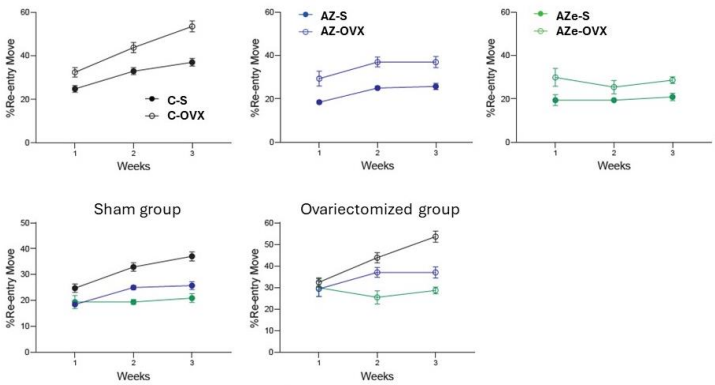

Figure 2. The stereotypical/repetitive behaviors. The above figure shows comparison between the sham and ovariectomized groups, and the below figure describes the effects of each EO compared to each control group.

As a result of the canonical discriminant analysis, the behavior of the ovariectomized group was characterized due to a decrease in activity levels, frequency/time of access to water, and many behavioral characteristics, so C-OVX was plotted below C-S. Meanwhile, the behaviors of AZ- and AZe-OVX were shifted to the C-S side because their behavioral peculiarity were improved by the exposure of each EO (Figure 3). Even though the result of the principal component analysis clearly separated the behavioral characteristics of ovariectomized groups and sham groups, the characteristics of AZ- and AZe-OVX return to normal class closer to that of the sham group (Figure 4).

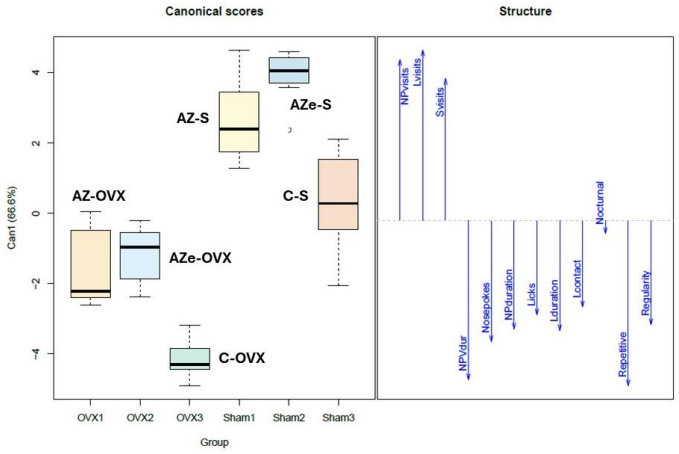

Figure 3. The canonical discriminant analysis of behavioral characteristics caused by ovariectomy and the effects of *Alpinia zerumbet* EOs on them.

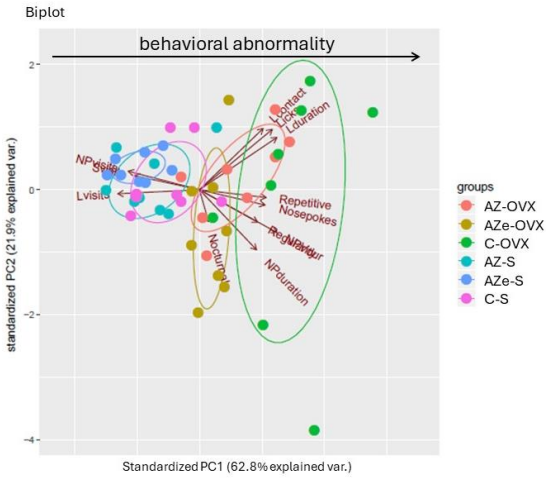

Figure 4. The principal component analysis of the behavioral characteristics of each group.

4. Discussion and Conclusions

It is important to understand that the compositions of the EO used in this experiment are just one example. Regarding ovariectomized mice and rats, they are used in experiments to improve symptoms of depressive state and menopausal syndrome. Anxiety-like behaviors are often observed in studies using the behavioral indicator of ovariectomized mice or rats [3]. In addition, we found that ovariectomized mice had a decrease in activity levels and more stereotypical/repetitive behaviors than sham groups in this experiment, so we evaluated the effect of AZEO and AZeEO on behavioral characteristics using the mice. By inhalation with automatic exposure, each EO used in this experiment suppressed and improved stereotypical/repetitive behavior in mice whose ovaries had been surgically removed, and the behavioral characteristics shifted to the sham group side in multifaceted indicators including activity levels and frequency/time of access to water. The result of this study suggests that our AZEO and AZeEO have the potential to improve some symptoms of depression and menopausal syndrome.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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P-54

Identification of 4,8-dimethyldeca-4,7,9-trienal compounds in the headspace aroma of blooming gardenia flower

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Keywords: *Gardenia jasminoides*, GC-MS-O, headspace aroma, 4,8-dimethyldeca-4,7,9-trienal, isolation

Abstract

Gardenia *(Gardenia jasminoides)* flower is known for its pleasant aroma, which attracts many people and inspires perfumers to create fragrances. In order to elucidate its aroma, gas chromatography-mass spectrometry-olfactometry (GC-MS-O) analysis of the headspace aroma of gardenia flowers was performed, and detected two odor-active unidentified compounds that smelled like calamus and muguet, respectively. These compounds were thought to be useful as fragrance materials, so we attempted to identify them.

Headspace aroma of gardenia flowers was collected on Tenax TA and then solvent-desorbed to create an aroma extract. Subsequent silica gel column chromatography and high-performance liquid chromatography (HPLC) fractionation successfully isolated the unidentified calamus-like aroma compound. The structure of the isolated compound was estimated through mass spectrometric analysis and proton nuclear magnetic resonance (^{1}H NMR) analysis, and the authentic compound was synthesized. By conducting multidimensional gas chromatography-mass spectrometry-olfactometry (MDGC-MS-O) measurements, the calamus-like aroma compound was identified as (4*E*,7*E*)-4,8-dimethyldeca-4,7,9-trienal.

Regarding the unidentified muguet-like aroma compound, its mass spectrum suggested it is an *EZ* isomer of calamus-like aroma compound. As a result of synthesizing *EZ* isomers and confirming their identity by MDGC-MS-O measurements, the muguet-like aroma compound was identified as **(**4*E*,7*Z*)-4,8-dimethyldeca-4,7,9-trienal.

Notably, these two 4,8-dimethyldeca-4,7,9-trienal compounds have not been reported to be detected in natural sources. Additional GC-MS-O analyses of headspace aromas from flowers of *Jasminum sambac*, *Hedychium coronarium*, *Lonicera japonica*, and *Michelia maudiae* also revealed the presence of these two compounds, suggesting their widespread occurrence in natural scents of flowers.

1. Introduction

In the fragrance industry, aromas inspired by the natural scents of flowers are developed and utilized in various products such as perfumes, shampoos, and fabric softeners, bringing delight to consumers. Gardenia flower, known for its pleasant aroma, has inspired the creation of numerous fragrance products that capture its scent. During a detailed analysis of gardenia flower's headspace aroma to develop a fragrance that more precisely replicate its scent, two odor-active unidentified aroma compounds were detected by gas chromatography-mass spectrometry-olfactometry (GC-MS-O) analysis : a calamus-like aroma compound and a muguet-like aroma compound. These compounds had the potential to greatly contribute to the headspace aroma of gardenia flower and were expected to be useful as new fragrance compounds, prompting further investigation for structural determination.

2. Material and Methods

The blooming flowers of gardenia, cultivated in the garden of T. Hasegawa Co., Ltd.'s R&D Center, were covered with sampling bags, and the volatile compounds inside the bags were trapped on Tenax TA using the dynamic headspace method. By passing diethyl ether through Tenax TA, the compounds were desorbed, and the desorbed liquid was subjected to atmospheric pressure distillation to remove the solvent. The concentrate was then analyzed using GC-MS-O to explore the compounds contributing to the flower's headspace aroma.

The unidentified calamus-like aroma compound was isolated using silica gel column chromatography and high-performance liquid chromatography (HPLC) with a fraction collector. For structural estimation, we conducted accurate mass measurements using gas chromatography-time-of-flight mass spectrometry (GC-TOFMS) and proton nuclear magnetic resonance (^{1}H NMR) analysis on the isolated compound. The authentic compound was synthesized based on the estimated structure, and multidimensional gas chromatography-mass

spectrometry-olfactometry (MDGC-MS-O) measurements were conducted on both the authentic compound and the isolated compound to confirm their identity.

Regarding the unidentified muguet-like aroma compound, its mass spectrum was similar to that of the calamus-like aroma compound. Considering this similarity, the muguet-like aroma compound was assumed to be an *EZ* isomer of calamus-like aroma compound. Consequently, we synthesized *EZ* isomers as authentic compounds and conducted MDGC-MS-O measurements on both the authentic compounds and the aroma concentrate.

Furthermore, to investigate whether these compounds are present in other flower's headspace aromas, GC-MS-O analyses of headspace aromas from blooming flowers of *Jasminum sambac*, *Hedychium coronarium*, *Lonicera japonica*, and *Michelia maudiae*, all cultivated in the garden of T. Hasegawa Co., Ltd.'s R&D Center, were performed using the same method as the headspace aroma of gardenia flowers was analyzed.

3. Results

GC-MS-O analysis of the aroma concentrate from gardenia flowers revealed several common floral compounds, including linalool, methyl benzoate, *γ*-decalactone and indole, were detected and contributed to the headspace aroma of gardenia flowers. However, alongside these well-known compounds, two odor-active unidentified aroma compounds were detected, yet their identification remained elusive based on mass spectra alone. These unidentified compounds exhibited aromas reminiscent of calamus and muguet, respectively.

To focus on the more abundant calamus-like aroma compound, we attempted isolation and identification. Headspace aroma from 40 gardenia flowers was collected, resulting in an aroma concentrate with a yield of 138 mg. Through silica gel column chromatography and HPLC fractionation, the calamus-like aroma compound was successfully isolated with a yield of 0.43 mg and a purity of 91%.

The isolated compound's mass spectrum, accurate mass measurements and ^{1}H NMR analysis led us to estimate its structure as (4*E*,7*E*)-4,8-dimethyldeca-4,7,9-trienal, and we synthesized it as the authentic compound. By conducting MDGC-MS-O measurements, which confirmed the match in the retention time (RT), mass spectrum and odor quality of the isolated compound and the authentic compound, calamus-like aroma compound was successfully identified as (4*E*,7*E*)-4,8-dimethyldeca-4,7,9-trienal. Similarly, for the unidentified muguet-like aroma compound, MDGC-MS-O measurements on the synthesized *EZ* isomers and the aroma concentrate confirmed the muguet-like aroma compound as (4*E*,7*Z*)-4,8-dimethyldeca-4,7,9-trienal.

GC-MS-O analyses of headspace aromas from flowers of *Jasminum sambac*, *Hedychium coronarium*, *Lonicera japonica*, and *Michelia maudiae* also detected both of these two 4,8-dimethyldeca-4,7,9-trienal compounds.

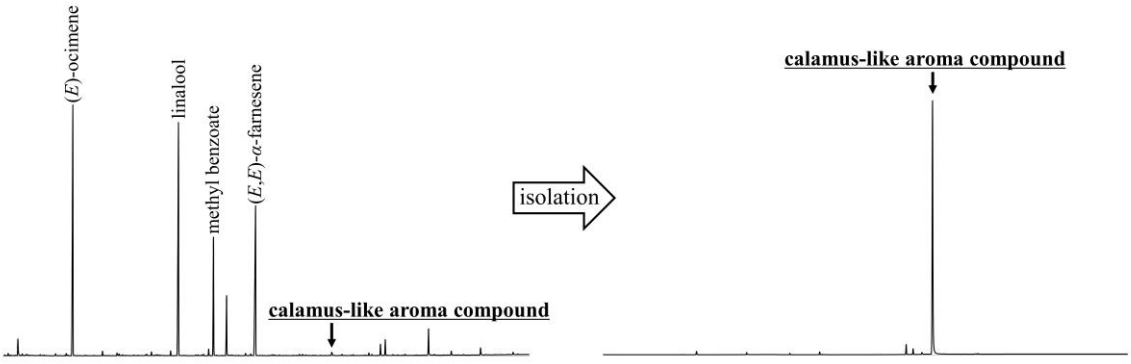
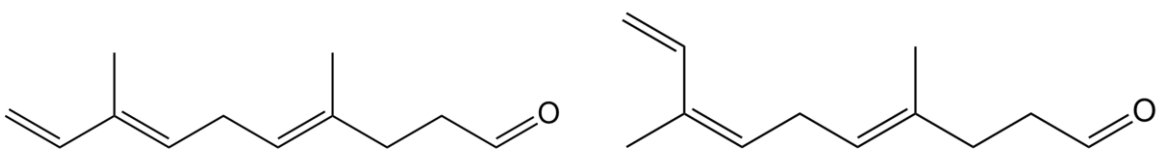


Figure 1 Gas chromatogram of headspace aroma concentrate from gardenia flowers before and after isolation.



(4*E*,7*E*)-4,8-dimethyldeca-4,7,9-trienal
odor : calamus-like

(4*E*,7*Z*)-4,8-dimethyldeca-4,7,9-trienal
odor : muguet-like

Figure 2 Identified two 4,8-dimethyldeca-4,7,9-trienal compounds.

4. Discussion and Conclusions

To the best of our knowledge, the two 4,8-dimethyldeca-4,7,9-trienal compounds identified in this study have not been reported to be detected in natural sources, including flowers. While there are suggestions that these compounds may be ozone oxidation products of α-farnesene, confirmation remains elusive [1]. However, it was found that α-farnesene was abundantly detected in the headspace aromas of the flowers where 4,8-dimethyldeca-4,7,9-trienal compounds were detected in this study, suggesting ozone oxidation of α-farnesene could be one of the pathways for their formation. In order to deepen our understanding of the conditions for the formation of these two compounds, GC-MS-O analyses of a solvent extract from gardenia flowers and the indoor and outdoor headspace aromas of cut flowers were conducted. As a result, these two compounds were not detected in the solvent extract, and it was found that the outdoor headspace aroma contains much more of these two compounds than the indoor headspace aroma. This result supports the possibility that ozone oxidation is one of the pathways for their formation.

Furthermore, not only gardenia flowers but also flowers of *Jasminum sambac*, *Hedychium coronarium*, *Lonicera japonica*, and *Michelia maudiae* were confirmed to contain 4,8-dimethyldeca-4,7,9-trienal compounds in their headspace aromas, suggesting the widespread occurrence of these compounds in natural scents of flowers. Consequently, these compounds demonstrate potential as valuable fragrance materials reminiscent of natural floral scents.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Study of the essential oil and conservation of the Andean aromatic species *Aloysia fiebrigii* (Hayek) Moldenke, from the Cusco region, Peru

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Keywords: fungicidal, sustainability, forest pathogenic fungi, seed germination, volatile compounds

Abstract

This work has three specific objectives: i) To know the fungicidal effect of the essential oil of Cedroncillo (*Aloysia fiebrigii*, from the sensitivity of 4 forest pathogenic fungi: (*Fomes fomentarius* (l.) Fr, *Trametes versicolor* (L. Fr.) Quél., *Ophiostoma ulmi* (Buism.), *Pycnoporus sanguineus* (L.) Murril) in vitro (LC2015). ii) Study the main components of the essential oil (volatile fraction) of Cedroncillo leaf and branches essential oil (SD) iii) Study the viability of germination and growth of seeds of the Andean aromatic species Cedroncillo from Perú.

Comparative studies highlighted significant similarities between Cedroncillo and lemon verbena essential oils. The fungicidal effect was statistically significant against four pathogenic fungi at specific concentrations.The germination and growth study of Cedroncillo seeds showed optimal results in Madrid, Spain, under specific conditions, suggesting its suitability for high mountain slopes with low rainfall.

This research proposes Cedroncillo as a solution to the current demand for new essential oils, aiming to contribute to scientific knowledge in the field and address the need for sustainable exploitation of this Andean aromatic species.

1. Introduction

Hispanic America's biodiversity has been recognized since the Enlightenment, with its rich flora and fauna driving economic and social interest. The 18th century saw numerous botanical expeditions to explore this biodiversity, including to the Cusco-Peru region, noted for both its archaeological significance and biodiversity [1], particularly the Cedroncillo (*Aloysia fiebrigii*) plant [2] . The Huancalle Peasant Community in Cusco hosts a small Cedroncillo forest within a subtropical low montane dry forest environment [3],. The area experiences average temperatures of 15.79°C and varying precipitation levels, with notable climatic and soil conditions conducive to Cedroncillo growth.

Rural communities utilize various native aromatic species for their essential oils, which have significant yet under-researched medicinal and aromatic properties. The verbenaceae family, including the genus Aloysia [ ], is of particular interest for its diversity and utility. Cedroncillo is closely related to lemon verbena[4] and is found in several Peruvian regions, where it serves medicinal purposes and is valuable in producing essential oils for natural fungicides, cosmetics, and aromatherapy.

2. Material and Methods

The plant material was collected in the lands of the Peasant Community of Huancalle, District of Taray, Province of Calca, Department of Cusco - Peru. Coordinates: Latitude: 13 ° 27 '17 "S; 71 ° 52' 36.7" W, estimated altitude of 3,750 m. The composition of the volatile secondary metabolites, present in the essential oil of Cedroncillo, which was obtained by steam distillation, was determined in the town of Pisac (Empresa Aroma Inka SRL).The characterization was carried out at the Madrid Institute of Agrarian Sciences - Higher Council for Scientific Research (CSIC). Comparisons were also made (Bibliography) between the concentrations of 13 components, between the essential oils of four species of Verbenaceae: *Aloysia fiebrigii*, *Aloysia triphylla*, *Lippia alba*, *Lantana camara*.

3. Results

i. The results obtained in the study of the chemical characterisation of the volatile fraction of the essential oil were obtained by the 'in situ' vapour dragging method, revealing the presence of 38 compounds with antifungal properties, such as Limonene (30.94%), Sabinene (24.42%), Citronella (10.98%) and 1.8 Cineol (7.39%), representing 73.73% of the total. Figure1.

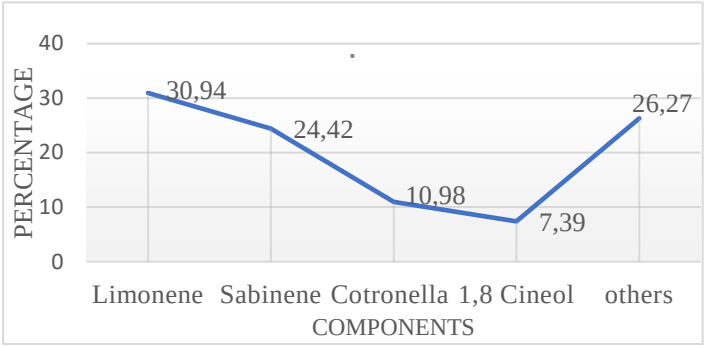

Figure 1 Main Components *Aloysia fiebrigii*

Table 1 Statistical comparison between 4 species of Verbenaceas

	Species (Plant)		N	Rango	Mediana
1	*Aloysia fiebrigii*		13	30,76	1,22
2	*Aloysia triphylla*		13	26,41	0,00
3	*Lippia alba*		13	15,25	0,00
4	*Lantana camara*		13	0,30	0,00

Confidence Interval (CI): 95%

Comparisons were also made (Bibliography) between the concentrations of 13 components, determining a greater statistically significant similarity between A. fiebrigii and A. triphylla. H (3) =18.735, p< 0.05. Similarly, a comparison of yields of the essential oil of Cedroncillo (0.08%) and lemon verbena (0.31%) was carried out in the laboratory (Bibliography), determining values close to those found in the experiment (0.11%) (0.28%), respectively. The results of the Mann-Whitney test indicate that, although there are differences in the yield of essential oil, these are not statistically significant, according to the significance values obtained. However, the small sample size is taken into account.

ii. Analysis of the fungicidal action of the essential oil revealed a statistically significant effect in inhibiting the growth of the forest pathogenic fungi studied, at concentrations of 1% and 5%.

Tabla 2 Games-Howell test on the growth of Fomes fomentarius, Trametes versicolor, Ophiostoma ulmi, and Pycnoporus sanguineus with unequal variances

Species	Concentration	Concentration	X	Decisión
Fomes f.	0%	1%	0,21	Significant
		5%	0,48	Significant
	1%	5%	0,27	Significant
Trametes v.	0%	1%	0,09	Significant
		5%	0,31	Significant
	1%	5%	0,22	Significant
Ophiostoma u.	0%	1%	0,42	Significant
		5%	0,51	Significant
	1%	5%	0,09*	**Not significant**
Pycnoporus s.	0%	1%	0,34	Significant
		5%	0,35	Significant
	1%	5%	0,02**	**Not significant**

*Note: p <,001; * = p=,30; ** = p=,94*

X: Diference in means

According to the one-factor ANOVA (Games Howell), the fungicidal effect of the essential oil of cedrongrass (Aloysia fiebrigii), at concentrations of 1% and 5% on the growth of the four forest pathogenic fungi: *Fomes f.*, *Trametes versicolor*, *Ophiostoma u*, *Pycnoporus s*. Likewise, the essential oil of Cedroncillo (1%) has a greater fungicidal effect on *Ophiostoma u.* and *Pycnoporus s.* and (5%) has a greater fungicidal effect on *Ophiostoma ulmi* and *Fomes f.*. Showing great potential as a fungicidal agent.

iii. The study of the viability of germination and growth of Cedroncillo seeds was carried out in the Municipality of Arganda del Rey (Madrid, Spain), with genetic material from the Andean region (Urubamba-Vilcanota Basin, Cusco, Peru). The experiment showed its best germination percentage (71.25 %), in the first trial, during the second week of July (Summer - Madrid 2015), using limestone soils, with freshly collected seed. Likewise, a germination speed coefficient of 74%, 1.88 % of pure seed and 437,636 seeds per kg were obtained. Thus, Cedroncillo is recommended on high mountain slopes, with low rainfall, as an aromatic species for industrial use, soil-conserving, forming small shrubs with evergreen foliage.

Tabla 3 Seed germination results – Trials 1 y 2.

Trial	1	2
Date:	15/05/2015	03/08/2015
Place (Madrid)	Madrid	Madrid
T°C average (Períod)	29,66	20,73
Períod (days)	15	15
Total seeds	80	80
Germinated seeds	57	26
% Germination (GP)	71.25	32.50
G.Speed Coefficient (CS)	74 %	15 %
Pure seed	1,88%	-------
Seeds /Kg.	437,636	--------
Waitint time (Storage)	98 días	148 días

4. Discussion and Conclusions

The main volatile components are limonene, sabinene, and citronella (66.4%). Aloysia febril and Aloysia trifila are the most closely related species. The studied essential oil is antifungal against the four forest pathogenic fungi. Preliminary germination tests show favorable results in Madrid in summer.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. José Luis de Pedro Sanz, for his invaluable guidance and unwavering support. I also extend my deep respect and thanks to the Qosqomaki Association of Cusco, the Municipality of Taray, the Óptima Association for Ecological Development, and Aromatic (Madrid), Aroma Inka SRL for their invaluable contributions and support.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ABSTRACTS AND BIOS OF EFEO IFEAT SESSION

Dr Jocelyn Kurtz

General introduction to the EFEO IFEAT Scientific Platform

Position: Vice President, Innovative Ingredients and Raw Materials Global Regulatory Affairs & Product Safety
Institution: Mane
EFEO IFEAT Scientific Platform Coordinator

Bio
Not available.

Abstract
The European Union has granted the essential oils industry a derogation regarding classification rules (CLP). On December 25, 2024, the European Parliament and the Council reached a provisional agreement. According to this agreement, plant extracts containing more than one constituent are now exempt from the mixture rule. Consequently, the overall properties of an essential oil can take precedence over the properties of individual ingredients when assessing hazards. Additionally, a review clause has been included, requiring the Commission to draft a scientific report within the next five years. If necessary, a new legislative proposal will follow.

How do we provide the expected scientific evidence? What approaches and methodologies do we use? How do we finance this project? This is what this conference is about!

Prof Sylvain Antoniotti

Multidimensional complexity of natural complex substances: the case of essential oils

Position: Research Director at CNRS and Director of the Institute for Innovation and Partnerships Flavour Fragrance Cosmetics at Université Côte d'Azur.
Institution: Université Côte d'Azur, CNRS

Bio

Dr. Sylvain Antoniotti is CNRS research director at the Chemical Institute of Nice, France, specializing in sustainable chemistry and applications for the perfume industry for 24 years. He has been partnering with many F&F companies locally in Grasse and globally on synthesis, biocatalysis and analysis R&D projects. He is the author of ca. a hundred of publications, book chapters and monographs, and has given dozens of conferences nationally and internationally.

Abstract

Essential oils are obtained from plants by physical processes such as distillation or cold pressure and contain typically dozens of molecules. This complexity of the chemical composition is expressed in different dimensions such as the number of individual molecules, their class, their proportion. Another aspect of the complexity stands in the global properties of the mixture, and the question whether the properties, and importantly the biological properties, are the linear combination of individual properties and proportions or are following different laws. Biological testing and evaluation thus require a detailed characterization of the chemical composition of essential oils in case by case approaches to secure a valid interpretation of biological assays. We will introduce the session with a few words on the complexity of essential oils and some examples of synergy, additivity or antagonism of their biological activity constituents to contribute to the conversation.

Dr Mathilde Hagège

Evaluation of the Endocrine Disruptor Risk on Essential Oils: Is Constituent Analysis Sufficient?

Position: Senior R/D Innovation Manager
Institution: Laboratoires Lés Nature, France

Bio
As a Research and Development Manager at Laboratoire Léa, I thrive on creating innovative products for the French market, especially for organic cosmetics and aromatherapy brands. My passion for aromatic and medicinal plants has been with me for more than 20 years, and I am delighted to be able to defend and advance this fascinating field. In addition, I am proud to be part of the ethics committee of Léa Nature, where I contribute to responsible and sustainable practices.

Abstract
As part of the revision of the CLP Regulation, the European Commission proposes to classify a multi-constituent substance on the basis of the hazard of one (or more) of its constituents, even if the same properties are not found in the substance itself. Essential oils would be primarily affected by this new rule, even though their "totum effect" does exist.

No one can ignore the omnipresence of the issue of endocrine disruptors in the news, nor the fact that essential oils often enter into the debates they generate. Mathilde Hagège will present the results of three years of reflection, tests and exchanges on this theme carried out by the company Léa Nature and the toxicology laboratory of the University of Paris Descartes.

Prof Marc Vocanson

Essential Oils & Skin Sensitization: Quenching effect: myth or reality?

Position: Head of Research - Epidermal Immunity and Allergy
Institution: Centre International de Recherche en Infectiologie (CIRI), Lyon, France

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Bio
Marc Vocanson is an i6mmunologist at the Centre International de Recherche en Infectiologie (CIRI) in Lyon. He holds a tenure track position as Research Scientist INSERM and is now leading the team « Epidermal immunity and Allergy » since 2021.

Abstract
I will be presenting our research into the pathophysiology of allergic contact dermatitis

(ACD), a common inflammatory skin disease in industrialized countries. I will also present our recent work on the development of new approaches for predicting the sensitizing properties of chemicals and for the molecular diagnosis of ACD.

Finally, I will talk about the “quenching effect”, a concept which studies the possibility that a chemical compound (other than a drug) can inhibit or reduce the allergenic activity of another, and which has been and still is much debated today.

Dr Peter Jenkinson

Genotoxicity, MOCS, and EOs

Position: Genetic Toxicologist
Institution: Consultancy for Environmental & Human Toxicology and Risk Assessment (CEHTRA SAS)

Bio

Peter Jenkinson gained his PhD in reproductive and genetic toxicology in 1987. He has ~30 years of practical experience in genetic toxicology and managed a Genetic Toxicology laboratory with a team of >30 at a UK CRO for ~27 years. For the past 14 years he has been a scientific consultant to the chemical industry and provides expert support to assist to monitor studies and manage difficult data. He is a fellow of the UK EMS.

Abstract

The MOCS principle of classification presents a risk to essential oils (EOs), especially for the genotoxicity endpoint. EOs contain multiple constituents, many with highly variable content in the EO, dependent on source, manufacturer, batch, etc. Under MOCS, if a constituent is classified as Muta 1A or 1B and is present in an EO ≥0.1% (w/w) in an EO, then the EO must carry the same classification as the constituent; for Muta 2 then the cutoff level is ≥1%. Currently, data on the substance itself takes precedence over a MOCS classification, however, it may not always be this way. The EU commission, Parliament, and Council agreed in December 2023 a 5-year derogation from MOCS classification for EOs but with a 5-year review of scientific evidence by the Commission. The five constituents of EOs currently classified are all Muta 2, so the applicable cutoff is 1%. This presentation examines estragole as an example and why the MOCS principle may not be applicable to EOs; it also offers some suggestions on what may be done to generate relevant data for the Commission review.

Dr Andreas Natsch

Metabolism matters – understanding positive test results and relevance of reproduction studies on isolated essential oil constituents

Position: Senior Research Fellow, Head of In vitro Molecular Screening
Institution: Givaudan

Bio

Andreas Natsch studied at the Swiss Federal Institute of Technology with a PhD in environmental microbiology. After postdoctoral studies in Madrid, he moved to the research department of Givaudan in 1998. Here he elucidated the biochemical mechanisms of body odor formation in order to design new deodorant ingredients. Since 2008, the research focus has shifted to the development of in vitro assays to study toxicological risks without using animal testing. The work of his Lab contributed to six recent OECD test guidelines for non-animal test methods. In the current position as Senior Research Fellow he leads the in vitro molecular screening group at Givaudan to evaluate risks and bioactivity of novel ingredients early in the development pipeline and to decipher the code of how individual odor receptors shape our scent perception.

Abstract

The set of metabolic enzymes in the liver have co-evolved with the diet of mammals over eons. Thus typical plant and essential oil constituents are rapidly oxidized by phase I reactions and further conjugated by phase II reactions to be easily excreted. However, current OECD guidelines require testing of chemicals up to 1000 mg/kg or to a toxic level although there is no scientific validation of this arbitrary number. Due to the efficient metabolism, many essential oil constituents have a low toxicity and can indeed be tested up to such high concentrations - yet for an increasing number of substances at a high dose effects on reproduction or development are observed. Often these are apparent at the same dose where compensatory effects on the liver (mostly hepatocellular hypertrophy) are observed: If a single ingredient is tested at such an excessive dose, the specific metabolic pathway for its excretion can get saturated, which may lead to effects specific to this high dose scenario, and which are not relevant to a dose where the metabolic capacity of the liver is within its linear range. More importantly, as essential oils are multicomponent substances which will be metabolized by multiple metabolic routes, such effects of a single constituent at an exaggerated dose may not be observed in tests on the mixture where a more balanced metabolism occurs. In this talk some general thoughts and observations on metabolism of essential oils and fragrance ingredients are shared and specific examples are discussed.

Dr Paul Thomas

An experimental definition and an in silico methodology to predict biodegradation of mixtures

Position: President & Expert in silico Ecotoxicologist
Institution: KREATiS

Bio
Paul THOMAS, a European Registered Toxicologist, has a PhD in aquatic ecotoxicology and more than 25 years' experience with industrial chemicals, agrochemicals and biocides. He was study director for 4 years, ATOFINA (now ARKEMA) where he practiced ecotoxicology and Risk Assessment for existing substances and then AkzoNobel (now Nouryon) where he was head of department, head of the ecotoxicology GLP and research laboratories and organiser of REACH services for the company for over 6 years. He joined a French consultancy in 2008 as director of the Lyon office specialising in REACH-related services and manager of the ecotoxicology team and contributed strongly to numerous successful registrations out of the >600 substances the consultancy registered since 2010 and he has worked in the area of predictive ecotoxicology since this time. In 2014, while retaining all his consultancy roles, Paul founded and is President of KREATiS in 2014.

Abstract
Not available.

Dr Sylvia Gimeno

Environmental Assessment of Natural Complex Substances' constituents

Position: Director, Ecotoxicology
Institution: dsm firmenich

Bio
Sylvia Gimeno has a PhD of ecotoxicology from the University of Utrecht in the Netherlands. Her research on the estrogenic effects in fish had been published in Nature. Other publications during her 25years career in the industry have been focused on animal alternatives approaches. She is now working at dsm-firmenich since 12 years, providing environmental support to the company for testing and registration strategies of perfume ingredients, including those of natural products.

Abstract
Not available.