

Medicinal and Aromatic Crops: Production, Phytochemistry, and Utilization

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**Medicinal and Aromatic
Crops: Production,
Phytochemistry, and
Utilization**

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Preface

In the later part of the 20th century the United States experienced a remarkable surge in public interest toward medicinal and aromatic crops and this trend continues. This consumer interest helped create a significant demand for plants with culinary and medicinal applications as the public discovers their benefits for a wide range of applications. Consequently, this consumer call has generated a huge demand on farmers, but has also provided opportunities for new agricultural crops to support both fresh and dry raw material markets. Processing raw materials for end use by consumers introduces even more variables at all levels from harvesting to final desired product. Maintaining quality and authenticity throughout this process has inspired farmers, processing facilities, and regulatory agencies to adopt new practices and new laws to maintain safety and quality. Maintaining this quality and authenticity is often made possible using analytical methods for quality control, which subsequently triggered a demand for both regulatory agencies and scientists throughout the world. For aforementioned reasons, it is imperative that we continue to explore related topics from the field to the final consumer product. This book touches on many of the issues currently being addressed by scientists working to produce the desired consumer product while maintaining authenticity and quality and environmental stewardship.

This volume was developed from a symposium entitled “Medicinal and Aromatic Crops: Production, Phytochemistry, & Utilization” that took place at the 249th ACS National Meeting & Exposition held on March 22-26, 2015 in Denver, Colorado, USA. The original symposium presentations addressed a wide range of issues pertaining to production, phytochemistry, and utilization of medicinal and aromatic crops, and we have attempted to maintain this platform throughout the book. The chapters provide discussions on regulatory and quality issues with the essential oil supply chain, utilization of essential oils for the prevention and treatment of human opportunistic fungal diseases, phytochemistry of *Schizandra chinensis* cultivated in Bulgaria, breeding of German chamomile, pesticides based on plant essential oils, screening of herbicides for weed control in medicinal and aromatic crops, chemical profile and bioactivity of essential oil fractions as a function of distillation time, essential oils as powerful antioxidants, effect of plant-derived oils and compounds on ruminant fermentation, controlled environment production of medicinal and aromatic plants, benefits of soil microorganisms on medicinal and aromatic plants, and discovery of new biopesticides from medicinal and aromatic plants. We hope that this collection of chapters prepared by an international group of experts will be a valuable resource for educators and investigators working with medicinal and aromatic crops as well as those drawn by curiosity wishing to improve upon their understanding of the current challenges facing the industry.

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Chapter 1

Overview of Medicinal and Aromatic Crops

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This book is based on the Symposium *Medicinal & Aromatic Crops: Production, Phytochemistry & Utilization* held on March 23, 2015 as part of the 249th American Chemical Society National Meeting & Exposition, March 22-26, 2015, Denver, Colorado. The Symposium was sponsored by the ACS Division of Agricultural and Food Chemistry.

The book summarizes some of the current knowledge on medicinal and aromatic plants (MAP), including the production and breeding of economically important crops and their products, beneficial effects of soil organisms on MAPs, the utilization of essential oils as pesticides and their potential to suppress human opportunistic fungal diseases, utilization of MAP and their products in animal production systems, regulatory and quality issues with essential oil supply chain among other subjects. The book is expected to serve a diverse audience such as teachers, chemists, food scientists, agronomists, and agroecologists in industry, government agencies, and in academia. The book will be a basis for an online course with the same title at Oregon State University and possibly other universities in the USA.

Medicinal and aromatic plants (MAP) and medicinal and aromatic crops (MAC) are high-value crops; the natural products obtained from these crops are low-volume high-value commodities that have numerous applications in various

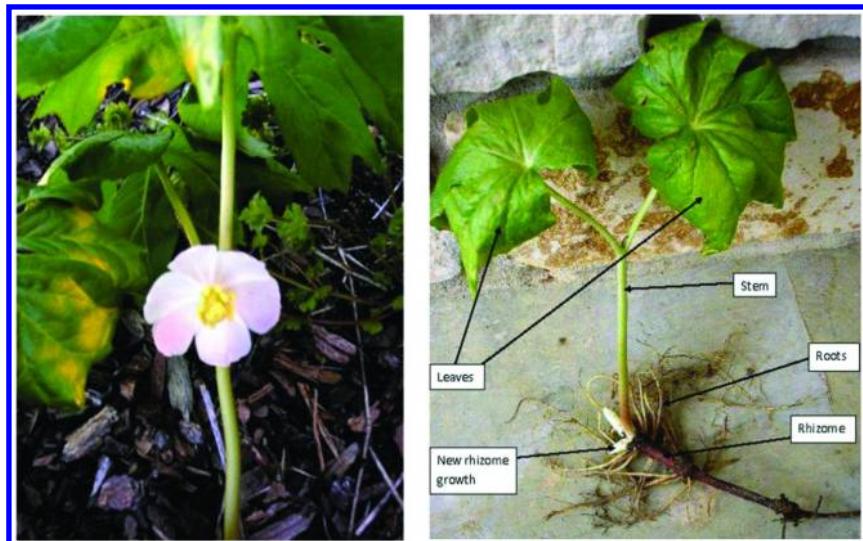
industries such as the food, beverage, food supplement, flavor and fragrance, perfumery and cosmetics, pharmaceutical, aromatherapy, and as ingredients in various consumer products. In addition, the plant biomass is used in the production of teas and medical applications in traditional and also modern medicines.

MAPs are important mainly because they contain plant secondary metabolites (such as essential oils, alkaloids, glycosides, saponins, tannins, vitamins, and other bioactives). Plant secondary metabolites are differentiated from plant primary metabolites of photosynthesis and respiration that are directly involved in growth and development of plants. The plant secondary metabolites are substances produced by pathways derived from primary metabolites pathways, that do not play a direct role in the growth and development of the plants but have roles associated with protection and defense mechanism. For example, it has been demonstrated that the essential oils may play significant role in attracting pollinators. Additionally, the essential oils are thought to protect plants from various bacterial, fungal, or viral infections, they can repulse various pests, including herbivore animals. Essential oils may also play a role in overcoming different types of abiotic stresses. Studies have shown that the aromatic compounds found in plants or extracted from them have physiological and psychological effect on humans and animals; they modify emotions and even behavior.

Some MAP are used as spices and culinary herbs; these plants contain mainly essential oils, and are used as tonic to digestive system, they modify appetite, influence digestive, and other systems and may facilitate nutrient uptake and utilization from various foods. Significant amount of MAP and their natural products have also demonstrated antimicrobial, antifungal, bactericidal, activity, and significant antioxidant capacity. In the past, MAPs and their natural products have been used as a source for various medicines, in the food and beverage production, and in aroma products. MAPs and MAP-derived extracts and compounds are still widely used not only in the traditional but also in modern medicine across the world. Generally, greater use of MAPs and their products have been reported for traditional societies such as India, China, in countries especially around Mediterranean, and in the developed and developing world in Africa, Asia, Europe, Australia, and North and South America.

A great number of synthetic aromatic compounds have been developed and used in the food, beverage, pharmaceutical industries, in perfumery and cosmetics industries. However, some natural products cannot be synthesized commercially. For example, podophyllotoxin, a lignan is a natural compound found in mayapple, Himalayan mayapple (*Podophyllum hexandrum* Royle), American mayapple (*Podophyllum peltatum* L.) (Figure 1) (1–6), in some junipers and other species (7–9), and is used as precursor in the semi-synthesis of commercially used cancer treating drugs etoposide and teniposide (10). The synthetic production of podophyllotoxin was found to present challenges and to be uneconomical. Other compounds were found to be easily synthesized; e.g. menthol (Figure 2), a major oil constituent in peppermint (Figure 3) and Japanese cornmint oil (11), has been produced synthetically. However, regulations in different countries limit

the utilization of synthetically produced compounds in a number of end products and applications.



*Figure 1. American mayapple (*Podophyllum peltatum L.*) is a medicinal and poisonous plant found in the Eastern part of the US (2–5). The mayapple rhizomes and leaves are toxic, they contain podophyllotoxin (3–5), which is used as precursor for anticancer drugs (10). The fruit is edible and used in the Appalachian region for preparation of jams, the plant is used as ornamental. Mayapple is endangered and protected species in the state of Florida. (Photo: V. D. Zheljazkov; courtesy of the author.)*

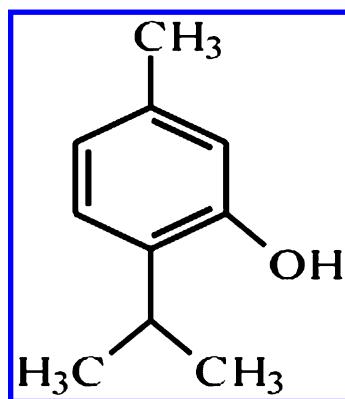


Figure 2. Menthol, the major constituent of the peppermint essential oil (11).



Figure 3. Peppermint (*Mentha x piperita L.*) from experiments in Mississippi (12).
(Photo: V. D. Zheljazkov; courtesy of the author.)

Therefore, currently there is a significant interest in plant natural products, because they have better compatibility with human and animal physiological systems, they may be significantly less expensive and more effective in some instances, degrade faster, contain fewer contaminants, and generally may have fewer side effects. Nowadays, consumers are more educated and health-conscious, and they will continue to drive the increased utilization of natural plant products. We hope the readers will find the information in this book useful and applicable in educational and other activities.



Figure 4. (left) Medicinal plants research plots: testing 38 different sweet basil (*Ocimum basilicum L.*) genotypes (13). Sweet basil is one of the most widely used culinary herbs, it is also used as medicinal, potherb, ornamental, and essential oil crop. (Photo: V. D. Zheljazkov; courtesy of the author.)



Figure 5. (right) Spearmint (*Mentha spicata L.*) transplants. Some plants (such as spearmint) are considered medicinal plants, culinary herbs, or essential oil plants because they possess all these properties. (Photo: V. D. Zheljazkov; courtesy of the author.)

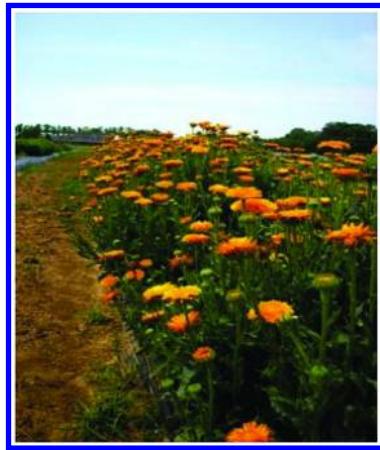


Figure 6. (left) Most MAPs have multiple utilizations: Pot marigold (*Calendula officinalis L.*) is used as medicinal plant, as edible potherb, ornamental, natural dye for fabrics foods and cosmetics and as essential oil crop. (Photo: V. D. Zheljazkov; courtesy of the author.)



Figure 7. (right) Research plots with sweet sageworth (*Artemisia annua L.*) in Mississippi (14, 15)). Sweet sageworth contains artemisinin, a sesquiterpene lactone, which is used in antimalarial drugs. Artemisinin has a worldwide demand of over 2 million doses, and is recommendation by the World Health Organization as the first line of defense against multi-drug-resistant *Plasmodium falciparum* malaria. Some derivatives of artemisinin are also studied as anticancer drugs. The plant also contains highly aromatic essential oil and is grown commercially in some European, Asian, and African countries. (Photo: V. D. Zheljazkov; courtesy of the author).

Introduction to the Terminology of MAPs and Their Products

- **Herb** is any green, growing plant that has a fleshy stem (not woody), this is the botanical definition. Herbs are usually aromatic plants that are not usually consumed as primary sources of nutrients, but often have very favorable nutrient profiles when consumed. Herbs are used for flavoring foods and beverages, herbs are mainly plants grown or coming from the temperate regions.
- **Culinary herb** is a plant, defined in a popular sense, as one that can be usually grown in temperate regions and is used in minor quantities to flavor food and beverages. Some common culinary herbs are shown in Figure 4 and Figure 5.
- **Potherb** is a leafy plant from temperate or tropical climates and used as a minor adjunct to salads. Potmarigold (Figure 6) is an example of a potherb.
- **Spice** is any of various aromatic pungent vegetable substances used to flavor foods or beverages. It usually designate plants and their products from the tropical regions. The American Spice Trade Association emphasizes commercial trade to designate both herbs and spices as spices. American Trade and Tariff regulations define spice as an aromatic

vegetable substance (including, seeds, leaves, stems, bark, roots, or other relevant plant parts) from which virtually none of the volatile oil or other flavoring principle has been removed.

- **Medicinal plant** is any of various plants used in the treatment of diseases or other afflictions of the body. Examples of medicinal plants are shown in Figure 6 and Figure 7.
- **Essential oil plant** (volatile oil plant) is a plant (Figures 8, 9, 10 and 11) from which an oil can be obtained by the use of distillation, expression, or solvents. Another definition is a plant with chemical constituents based on the six carbon ring structure of benzene (Figure 12).
- **Aromatic plant** - A plant with an aroma, something we can smell, has aromatic qualities (Figures 8, 9, 10 and 11). Not all aromatic plants are essential oil crops.
- **Poisonous plants** are higher plants that produce toxic effects when introduced into the human body. Examples include Jimson weed (Figure 13), Grecian foxglove, nightshade, water hemlock, and amanita mushrooms, these usually contain alkaloids.
- **Endangered species** are species of plants of which very few exist. Endangered species are usually protected by federal and state laws (Figure 14).
- **Plant extract** is a substance or chemical removed from plant, usually the essential oil.
- **Essential oil** is extract (oil) of a plant obtained by use of distillation, expression, or solvents. The essential oil is the portion of the plant that usually contains the bioactive constituents. The essential oils do not contain glycerol esters of fatty acids. Examples of essential oils include lavender oil, mint oil, rose oil, juniper oil.
- **Fixed oils** are different from essential oils, these are glycerol esters of fatty acids that are saponified by alkalis. Examples of fixed oils include olive oil, peanut oil, sesame oil, castor oil.
- **Resins** are solid or semisolid amorphous products of complex chemical nature.
- **Oleoresins** are resins and volatile oils in homogeneous mixtures. Oleoresins are the volatile and non-volatile portions of herb or spice. Some prepared oleoresins include paprika, ginger, and pepper.
- **Oleo-gum-resins** are oleoresins and gums in homogeneous mixtures, example is myrrh.
- **Balsams** are resinous mixtures that contain large proportions of benzoic acid, cinnamic acid, or both or esters of these acids.
- **Tincture** is a substance that colors, a dying compound, pharmacologically an alcoholic solution of a nonvolatile medicine.
- **Concretes** are water insoluble fractions (waxes, pigments) of plants remaining in retorts after distillation.
- **Absolute** (integral essence) represent the pure essence, the pure odoriferous extract of flowers. Also, absolute is an alcohol soluble extract of concrètes for use in perfume.

- **Principle** is the essential character, the foundation of the plant or the essential oil. It characterizes the substance, essential bioactive part of drug.
- **Decoctions** are water soluble extracts of plants (usually extracted by boiling plant material in water followed by straining or filtering, not commercially available in the U.S.A).
- **Infusion** is water soluble extract of plants (by steeping or soaking without boiling).
- **Herb Tea** is a drink prepared from herb plant or a tea with herb extract added.
- **Tisane** is a herbal infusion drunk as a beverage for mildly medicinal effect.
- **Tonic** is an extract of plant that energizes the body and/or mind.
- **Stimulant** is a plant or plant extract that energizes the body and/or mind.
- **Health foods** are Products of natural origin used by the laity in the self-treatment of disease states or less than optimal health conditions. Some foods with this designation are without therapeutic effect, some could be toxic.



*Figure 8. (left) Research plots with East Indian lemongrass [*Cymbopogon flexuosus* (Nees ex Steud.) J.F. Watson] in Mississippi (16). (Photo: V. D. Zheljazkov; courtesy of the author.)*



Figure 9. (right) Research plots with lemon grass (second plot from left) and palmarosa (*Cymbopogon martini* (Roxb.) Wats) in Mississippi (17). (Photo: V. D. Zheljazkov; courtesy of the author.)



Figure 10. (left) Blue giant hyssop (*Agastache foeniculum* (Pursh) Kuntze) is used as medicinal plant, as seasoning, ornamental, and essential oil crop. (Photo: V. D. Zheljazkov; courtesy of the author.)



*Figure 11. (right) Clary sage (*Salvia sclarea L.*) is commercially grown in Europe, the essential oil is extracted via steam distillation of fresh inflorescences. The oil is utilized in perfumes and cosmetics, and also as a muscatel flavoring for alcoholic beverages such as wines and liqueurs. Clary sage is also listed as class A noxious weed in Washington state. (Photo: V. D. Zheljazkov; courtesy of the author.)*

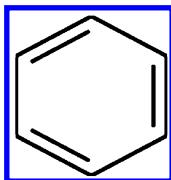


Figure 12. Benzene ring, a hydrocarbon and building block of essential oils.



*Figure 13. (left) Some plants can be considered medicinal plants, ornamentals, weeds, or poisonous plants. Jimson weed, syn. pricklyburr or thornapple, (*Datura innoxia* Mill.) is poisonous plant, it can be fatal if ingested by livestock or humans. The plant is commercially cultivated in some European countries for the production of the alkaloids atropine, scopolamine, and hyoscyamine, which are used in pharmaceutical drugs. Jimson weed is also used as ornamental.*

(Photo: V. D. Zheljazkov; courtesy of the author.)



*Figure 14. (right) Edelweiss (*Leontopodium alpinum*) production in the Swiss Alps at around 1,800 m asl. Edelweiss is a medicinal plant, extracts are used in sunscreens, natural remedies, and beverages. Edelweiss is protected species in several European and Asian countries. A number of medicinal plants throughout the world are endangered. Such plants are cultivated and the wild collection of these species is banned to conserve genetic resources. (Photo: V. D. Zheljazkov; courtesy of the author.)*

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Chapter 2

Pesticides Based on Plant Essential Oils: Phytochemical and Practical Considerations

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Although plant essential oils have long been recognized to possess insecticidal and/or insect repellent actions, commercialization of pesticides based on common plant essential oils dates back less than two decades. In large part commercialization of such pesticides in the U.S.A. was facilitated by exemption of certain oils from regulatory requirements, but other pesticides based on essential oils are beginning to reach the marketplace in the European Union, India and China. Unlike conventional pesticides based on synthetic chemicals, bioactivity of an essential oil in a pest insect cannot always be attributable to the major constituent(s); in some well-documented cases there is internal synergy among constituents of a particular essential oil, with putatively non (or less)-toxic constituents facilitating or enhancing the putatively toxic (active) principles. In addition to chemical considerations, there are a number of practical considerations in commercialization of pesticides based on plant essential oils. These include regulatory requirements and costs thereof (in some jurisdictions), commodity prices of oils used as active ingredients, ongoing availability of oils in large volumes, and chemical consistency of oils. Commercial challenges for essential oil-based pesticides include stability of active ingredient oils in storage and transport, residual action of active ingredients after application, and phytotoxicity on crop and ornamental plants. These considerations and challenges are addressed in this chapter.

Recent History of Essential Oils as Pesticides

Plant essential oils have a long history of use in the fragrance and flavor industries. More recently, the rise of their use in aromatherapy has expanded markets for essential oil producers. And while there has been documented traditional use of many plants and preparations thereof to repel insects and other pests, there have been hardly any commercially successful repellents (with the possible exception of citronella), let alone insecticides, based on plant essential oils prior to the late 1990s (1, 2).

Commercialization of such pesticides in the United States was greatly facilitated by recognition of a formerly little-regarded provision of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), namely “List 25b – Exempted Products”. Items found on the list, mostly food ingredients or food additives and all “generally regarded as safe” (GRAS), can be used as active ingredients in pesticide formulations and sold without scrutiny and approval by the Environmental Protection Agency (EPA), saving manufacturers months or years normally required for review by the regulatory authority, and the millions of dollars needed to generate toxicological and environmental data supporting the safe use of such products as pesticides. The list, the exact genesis of which remains obscure, includes half a dozen plant oils commonly used as flavoring agents in foods and beverages (3). It is these specific oils and their inclusion in List 25b that have been exploited to produce a wide range of EPA-exempt insecticides, fungicides and herbicides used in agriculture, professional pest control, and consumer (home and garden) markets. EcoSMART Technologies (USA) set the precedent for this movement with its introduction of insecticides for pest control professionals in 1998, followed by the introduction of products for the retail consumer market and agriculture in 2001. The company continues to be the market leader in essential oil-based pesticides to date.

As pesticides, essential oils enjoy a broad spectrum of action against arthropods, including pests on crops and ornamental plants, public health pests and disease vectors, and ectoparasites of farm and companion animals (4). Owing to their lack of persistence in the environment, they tend to be compatible with classical biocontrol agents and natural enemies, and generally safe for most nontarget organisms such as fish and wildlife. Their familiarity to most people and long history of safe use in humans is also a factor in their favor. Conversely, they are far less effective than most conventional pesticides, requiring higher rates of application, and their lack of environmental persistence can necessitate frequent reapplication in some contexts.

Essential Oils Used in Pesticides and Commercial Pesticides Containing Essential Oils

FIFRA List 25b includes a dozen plant oils, all of which, excluding soybean oil, are normally considered essential oils (Table 1). It also includes three specific compounds, eugenol from clove oil, geraniol from geranium oil, and 2-phenethyl propionate from peanut oil. Curiously missing from the list is orange oil, a large volume commodity (as a byproduct of the citrus industry) with a wide range of uses

as a cleaning agent and degreaser, and eucalyptus oil, widely used as a flavoring agent and in medicines. Both are used outside of the U.S. as insecticides (5).

Table 1. Plant Oils on FIFRA List 25b – Exempted Products

<i>Plant oil or derivative</i>	<i>Botanical Source(s)^a</i>	<i>Plant Family</i>
Cedar oil	<i>Juniperus</i> spp.	Cupressaceae
Cinnamon oil	<i>Cinnamomum</i> spp.	Lauraceae
Citronella oil	<i>Cymbopogon winterianus</i>	Poaceae
Clove oil, eugenol	<i>Syzygium aromaticum</i>	Myrtaceae
Garlic oil	<i>Allium sativum</i>	Amaryllidaceae
Geranium oil, geraniol	<i>Pelargonium graveolens</i>	Geraniaceae
Lemongrass oil	<i>Cymbopogon citratus</i>	Poaceae
Mint oil	<i>Mentha</i> spp.	Lamiaceae
Peppermint oil	<i>Mentha piperita</i>	Lamiaceae
2-phenethyl propionate	<i>Arachis hypogaea</i>	Fabaceae
Rosemary oil	<i>Rosmarinus officinalis</i>	Lamiaceae
Soybean oil	<i>Glycine max</i>	Fabaceae
Thyme oil	<i>Thymus vulgaris</i>	Lamiaceae

^a List 25b includes the common names shown in the lefthand column, but provides no strict botanical definitions for these. The botanical names shown in the middle column are representative but not inclusive. For some of these, multiple species are known by a single common name.

Agricultural insecticides developed by EcoSMART and currently sold under the Ecotrol® and Ecotek® brand names contain rosemary and peppermint oils and geraniol as active ingredients. Consumer insecticides sold under the EcoSMART® brand all include rosemary oil, with different products also containing various mixtures of peppermint, cinnamon, thyme and clove oils and 2-phenethyl propionate. Professional pest control products originally developed by EcoSMART but now sold under the Essentria™ brand are mostly based on rosemary and peppermint oils and 2-phenethyl propionate. TyraTech recently registered two insecticides in Canada containing thyme oil and wintergreen oil (methyl salicylate; from *Gaultheria* species) as active ingredients. Other minor brands in the U.S. use cedar or geranium oils as active ingredients.

An important exception to these EPA-exempt products is Requiem®, the first EPA-registered insecticide based on a plant essential oil. This product, containing an extract from a variety of wormwood (*Dysphania* [=*Chenopodium*] *ambrosioides* nr. *ambrosioides*) was first registered in 2011 by AgraQuest but is currently marketed by Bayer CropScience. It was approved for use by the European Commission in 2015.

In South Africa, Prev-AM® is an insecticide/fungicide containing 50% orange oil as an active ingredient; a similar product containing 60% orange oil is sold in France and the Netherlands under the same brand. In the European Union, orange oil is one of only two plant oils approved for use as insecticides, although clove, spearmint, citronella and rape seed oils (and geraniol) are approved for use as insect repellents. In China there are two registered insecticides containing eucalyptol (= 1,8-cineole, from *Eucalyptus globulus* and related species), five insecticides containing camphor (from camphor wood oil, *Cinnamomum camphora*), plus fungicides based on eugenol (from clove oil, *Syzygium aromaticum*) and carvacrol (from oregano oil, *Origanum sativum*). In India, eucalyptus leaf extract (rich in 1,8-cineole) is approved for use as an insecticide while in Australia an insecticide/miticide has recently been approved containing both eucalyptus and tea tree (*Melaleuca alternifolia*) oils as active ingredients. Garlic oil can be found as a major active ingredient in insecticides in the U.S., Mexico and Colombia (5).

Phytochemical Considerations: Relationships Between Chemistry of Essential Oils and Toxicity to Insects

Commercial botanical insecticides often state a single natural product as the active ingredient, when in fact most plant defensive chemistry consists of suites of natural products derived from a common biosynthetic pathway. Examples include azadirachtin from neem, *Azadirachta indica* (azadirachtin and a series of closely-related limonoid triterpenes), rotenone from *Derris* and *Lonchocarpus* species (rotenone and a series of related isoflavonoids), and pyrethrum from *Tanacetum cinerariaefolium* (pyrethrins and related esters of chrysanthemic acids) (6). Within a plant essential oil, the major constituents, monoterpenoids and sesquiterpenoids, can be more diverse and complex. Many plant essential oils comprise up to 80 or more such compounds. In some common essential oils a single constituent can dominate, for example eugenol in clove oil (typically $\geq 80\%$ by weight), cinnamaldehyde in cinnamon oil ($\geq 80\%$ by weight), or *d*-limonene in orange oil ($\geq 90\%$ by weight). In others, two constituents can dominate, for example menthol and menthone in peppermint oil (together, 65-85% by weight) or thymol and *p*-cymene in thyme oil (together 65-80% by weight). In yet other oils, e.g., rosemary oil, 3-5 constituents (or more) collectively make up the majority of the oil by weight. Major constituents of some common essential oils used in pesticides are shown in Table 2.

It should not be surprising that in the latter situations, attributing insect toxicity to one or more constituents of an essential oil is anything but straightforward. In an earlier study we attempted to correlate toxicity of 10 commercial rosemary oils to the cabbage looper (*Trichoplusia ni*) and the true armyworm (*Pseudaletia unipuncta*) with their chemical compositions (7). We observed slight but significant correlation between two minor constituents (*d*-limonene and α -terpineol) and toxicity to the cabbage looper, but no significant correlations between constituents and toxicity to the armyworm. These results,

and earlier results testing rosemary oil constituents against the twospotted spider mite (*Tetranychus urticae*) (8) suggested synergistic interactions among constituents in the oil with respect to toxicity. Other investigators have also reported this phenomenon (9–11). We later confirmed this ‘internal’ synergy among essential oil constituents in a study of the toxicity of the essential oil of *Litsea pungens* to the cabbage looper (12). More recently we observed in this same insect that most of the toxicity produced by topical administration of rosemary oil can be mimicked or reproduced by a simple binary mixture (in their proportions naturally occurring in the oil) of 1,8-cineole and camphor, two major constituents of the oil (13) Figure 1. Most recently we provided evidence for the mechanism underlying the synergistic interaction between these two compounds: 1,8-cineole facilitates the entry of camphor through the insect’s integument into the bloodstream, where the latter compound is more toxic than the former (14) Figure 2. It is conceivable that other examples of such synergy will be found to be a consequence of enhanced penetration of the putatively toxic constituents by other constituents that, when tested individually, appear to be themselves inactive.

Table 2. Major Constituents of Some Common Plant Essential Oils

<i>Plant essential oil</i>	<i>Major constituent(s) (% by weight)^a</i>
Rosemary	1,8-cineole (52), α -pinene (10), camphor (9), β -pinene (8)
Peppermint	Menthol (45), menthone (30)
Clove	Eugenol (80)
Cinnamon	Cinnamaldehyde (85)
Thyme	Thymol (58), <i>p</i> -cymene (20), γ -terpinene (8)
Lemongrass	Geranial (48), neral (38)
Eucalyptus	1,8-cineole (85)
Orange	<i>d</i> -limonene (98)

^a unpublished data, Berjé Inc. (Carteret, NJ, U.S.A.) and EcoSafe Natural Products Inc. (Saanichton, BC, Canada)

Exacerbating this complex relationship between chemical composition of essential oils and their toxicity to pests is natural chemical variation in composition of the oils that can be a consequence of genetic, geographic, seasonal, climatic or other influences. For example, seasonal variation in chemical content and composition of essential oils has been reported for rosemary grown in southern Spain (15) and basil (*Ocimum basilicum*) grown in Pakistan (16). Essential oil composition of clove oils varied with country of origin (17). Chemical composition of citronella (*Cymbopogon winterianus*) varied with time of harvest after transplantation (18), and with daytime temperature. Temperature was also found to be the strongest bioclimatic factor influencing chemical composition

of rosemary oil in the Balkan Peninsula (19). Even significant diurnal changes in chemical composition have been observed in the essential oil of *Ocimum gratissimum* growing in Benin (20).

Certain species of essential oil-producing plants exist as distinct chemotypes or races that can differ markedly in chemical composition (e.g., wormwood, *Artemisia absinthium*) (21). To a large extent this variation is managed or mitigated by major producers of plant essential oils who rely on well established supply chains starting with plant biomass that is propagated, grown and harvested consistently following good agricultural practices. Nonetheless essential oils as pesticides constitute a conundrum for regulatory authorities owing to their potential chemical variation, the likelihood that toxicity cannot be attributed to a single, major constituent, and the fact that – at least for use as pesticide active ingredients – there is no strict chemical definition for a particular essential oil. Even the botanical definition of an essential oil can be subject to question; the taxonomic boundaries of some of the most common essential oils remain unclear or controversial at best. Faced with this dilemma, a regulatory agency could either recognize a major (and presumably toxic) constituent of the essential oil as the active ingredient of a pesticide product, or alternatively, recognize the intact oil itself as the active ingredient, the lack of definition of the oil noted above notwithstanding.

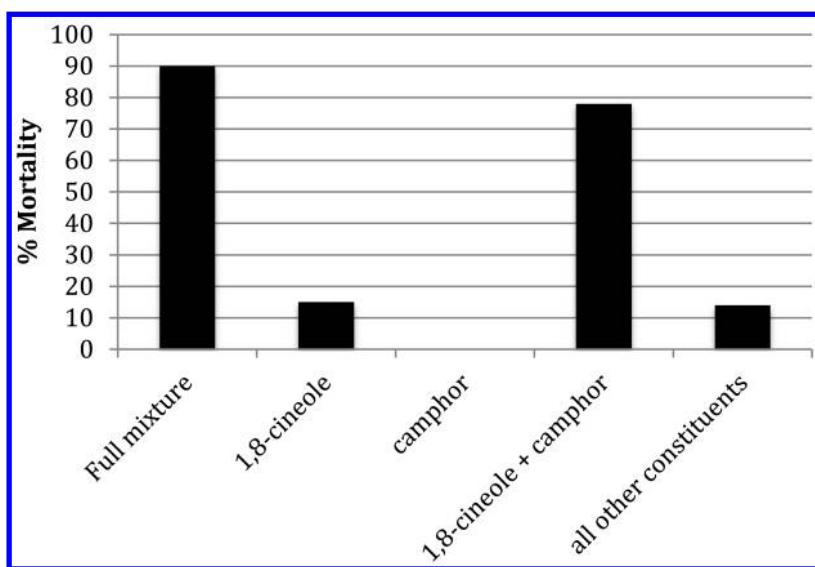
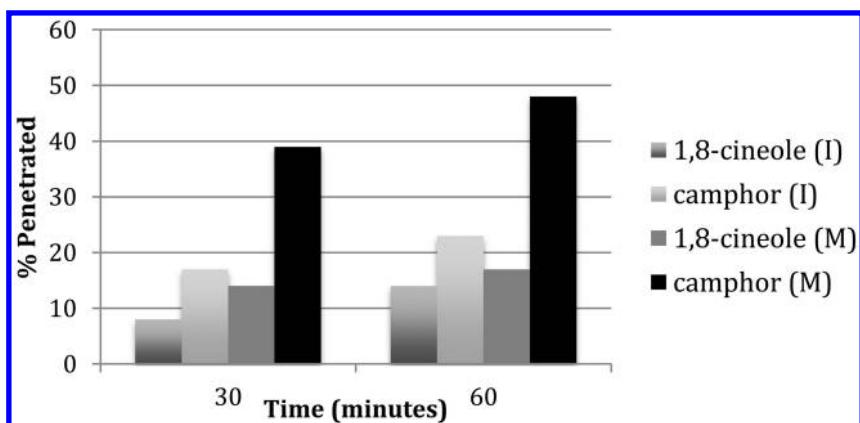


Figure 1. Toxicity of rosemary oil and its major constituents to 3rd instar cabbage looper (*Trichoplusia ni*) via topical administration. Based on data from reference (14).



*Figure 2. Penetration of topically applied 1,8-cineole and camphor, administered individually and as a binary mixture, in the cabbage looper, *Trichoplusia ni*. I = applied individually; M = applied as a mixture. Data from reference (14).*

Practical Considerations

Regulatory Concerns

In the developed world, regulatory approval remains the final, and often most difficult, barrier to overcome in commercializing a pesticide. Without such approval, no product can be sold and no revenue realized. The only obvious exception to this is the exempt active ingredient status afforded to certain essential oils in the USA as noted above (Table 1). In other jurisdictions, approval for essential oil-based pesticides has been problematic because pesticide regulatory guidelines developed historically to evaluate synthetic pesticides where there is a single active ingredient with no ambiguity. The EU is only now preparing to publish criteria for “low risk active substance” that some essential oils may meet, possibly clearing a path for approval of more pesticidal products based on such oils (22).

Regulatory approval is based on review of data on product chemistry, environmental fate, and toxicology to laboratory animals and nontarget organisms including fish, wildlife and pollinators. Some regulatory agencies require efficacy data while others do not. For essential oils used as flavoring agents in foods and beverages, as fragrances in consumer products and cosmetics, and medicinally in aromatherapy, product chemistry and laboratory animal toxicology are often well established and documented, although such data is usually considered proprietary and therefore not freely accessible. Less well documented is the environmental fate of plant essential oils, although published studies consistently indicate that these materials are minimally persistent in the environment owing to their volatility and consequential evaporative loss (23), except in closed containers

where fumigation is the goal. Fewer still are investigations of the effects of plant essential oils on nontarget organisms, although there is some evidence that they can be used with a margin of safety towards biocontrol agents and fish (24, 25). Essential oils have been evaluated and sometimes used to manage *Varroa devastator*, an ectoparasitic mite in honeybee colonies, but within the closed confines of a hive the margin of safety to bees is rather narrow (26).

Given the widespread use of essential oils in foods, beverages and consumer products, it is not surprising that most of the commonly used oils have minimal or low toxicity to lab animals, typically with rat, oral acute LD₅₀ values >2000 mg kg⁻¹. One notable exception is pennyroyal (from the mint *Mentha pulegium*) with a rat LD₅₀ of 400 mg kg⁻¹. The major constituent, pulegone, has a rat LD₅₀ of 470 mg kg⁻¹. Most importantly, there are reports that pennyroyal is a potential abortifacient in humans (27). Among the more common essential oils, carvacrol, a major constituent of oregano (*Origanum vulgare*) oil and a minor constituent of thyme (*Thymus vulgaris*) oil, is a strong corrosive and skin sensitizer. A more thorough review of the toxicology of essential oils can be found elsewhere (28).

Cost and Availability

The agrochemical business is extremely competitive. Even a pesticide with the least environmental and health impacts, or any other desirable traits, will not be successful in the marketplace if it is not cost competitive with other pesticide products available to the user. This is especially the case for pesticides used in agriculture or by pest control professionals, where volumes used are greater and costs of application are significant. What makes the material costs of certain plant essential oils low enough to be attractive for use in pesticides is the large scale international trade in those oils as commodities for use in the flavoring and fragrance industries. It is these latter, well established uses and supply chains that dictate prices; their use in pesticides thus far has been a minor alternative market for producers that has yet to grow large enough to influence prices.

Prices for the major essential oils tend to be relatively stable owing to these well developed supply chains. The greatest source of perturbations in price are occasional crop failures in a principal supply region as a result of abnormal weather events. On the other hand, some oils have seen a recent trend toward price reduction as production has moved to geographic regions where land and labor are less expensive, *viz.* China, India and Brazil. In contrast, the production of certain oils remains largely restricted to regions where the source crops were first successfully cultivated, for example clove and patchouli oils in Indonesia. Overall it must be said that posted prices for any particular essential oil can vary widely depending on the quality, source and volume of the oil in question.

Among the least expensive of essential oils is orange oil, in part because the biomass (fruit peels) used for extraction is effectively a waste product of another, very large, industry (orange juice), and in part because the oil can be obtained by cold pressing of the fruit peels rather than via hydrodistillation. Recent commodity prices for the most highly traded essential oils, including most of those use in pesticides, are listed elsewhere in this volume (29).

Commerical Challenges

Apart from the obvious issues of cost, availability and consistency, there are a number of challenges for those producing pesticides based on plant essential oils. The first of these is long term stability of a formulation containing essential oils, in storage and transport. Typically an agrochemical product is expected to remain stable for up to two years in storage; short term stability trials at elevated temperatures (e.g., 50°C for 90 days) attempt to simulate this. Though relatively stable, some oils are susceptible to oxidation in the absence of any antioxidants, even though certain oils themselves have been reported to have antioxidant properties. Given the volatility of many constituents of essential oils, the packaging material (bottles) used can be a factor in maintaining chemical consistency and stability of a product containing an essential oil, with the least porous and most chemically inert materials preferable.

Another challenge is persistence of an essential oil pesticide, i.e., how long the product remains biologically active against target pests after application to the target area. Often the direct insecticidal action of an essential oil-based pesticide is lost within hours of application, but field trials on horticultural crops suggest that a single application can provide crop protection for up to three weeks (1). Presumably this is because residual concentrations of the oils that are too low to kill insects are sufficient to deter/repel pests for a much longer period of time. Laboratory studies on the behavioral effects of essential oils in insects provide some evidence for this, but direct observations of behavioral effects on pests in the field are lacking. Nonetheless it is desirable to extend the residual life of essential oil-based pesticides, at least in outdoor contexts where efficacy is clearly not based on fumigant activity. Recent advances in pesticide formulation, especially the use of microencapsulation and nanoformulation, appear to provide avenues to solve this problem, possibly extending the residual activity of essential oil-based pesticides from hours to days (or even weeks) in the field (30).

A particular challenge for the use of some essential oil-based pesticides in agriculture or for home and garden use is phytotoxicity when application is made directly onto plants. Almost any oil, whether from a natural source or from petrochemicals, can be phytotoxic if applied at concentrations exceeding 2% (as an aqueous emulsion), and in some cases at a concentration as low as 1%. Most of the essential oil-based pesticides in current use are recommended to be used at concentrations in the 0.5-1.0% range, depending on the crop and pest. At higher concentrations (e.g. $\geq 5\%$), clove oil is sufficiently phytotoxic to be used as an herbicide (31). As the margin of error for certain plants can be quite small, empirical testing of any product on the intended crop under realistic conditions is highly recommended. Formulation is again an avenue to mitigate or eliminate phytotoxicity where the risk to a target crop is considerable.

Finally, in agricultural applications it is becoming common practice to tank mix different pesticides, or to use tanks and spray equipment sequentially for different pesticides with limited cleaning. For these reasons it is important to establish the chemical compatibility of an essential oil-based pesticide with other products that growers also use or have a high probability of use in tank mixtures.

Future Sources

Research interest in the insecticidal and repellent effects of plant essential oils has grown dramatically since 2000. By 2011, 23% of all published papers on botanical insecticides focused on essential oils, representing almost 5% of all published papers on insecticides (32). Between 2004 and 2014, over 3,600 papers were published on essential oils associated with insects and arthropods, according to the Web of Science. The vast majority of these reported on the screening and bioactivity of relatively exotic essential oils to insects, so the ‘discovery’ end of the R&D spectrum is well represented in the literature. Unfortunately there is a relative dearth of papers toward the ‘development’ end of the spectrum, although to be fair, a high proportion of the research effort in that regard is expected to be protected information (i.e., intellectual property) that might only reach the scientific literature after patent protection has been secured. But it is easy to observe that only a handful of essential oil-based pesticides have seen successful commercialization, relative to the expansive scientific literature devoted to the area.

That being said, and noting the practical considerations and challenges described above, what are the prospects for the introduction of new pesticides based on plant essential oils not currently in wide use in other industries? As of this writing, even the most successful essential oil-based pesticides occupy only a paper-thin share of the global insecticide market, although all indications are that they are at a very earlier stage in that market and are poised for faster market growth over the next decade than conventional pest management products. Perhaps if these predictions have any accuracy, essential oil-based pesticides will become significant participants in the rapidly expanding biopesticide market and in so doing make the prospects for the introduction of pesticides based on exotic oils more economically attractive.

One such example that has been subject to an extensive research effort in recent years is the essential oil from wormwood, *Artemisia absinthium* (Asteraceae) growing in Spain. This aromatic plant with a long history of medicinal use is also the botanical source of absinthe, a bitter liqueur banned for many years owing to its human toxicity, attributable to its major constituents, α- and β-thujone. Thujone-rich oils have also been demonstrated to be insecticidal. Although commercial samples of wormwood oil are often rich in thujones, there are numerous chemotypes of the species, some of which lack these compounds entirely (21). A series of thorough investigations of domesticated plants collected from two locations in Spain and grown under both field conditions and controlled conditions (in a growth chamber, greenhouse and in aeroponic culture) have documented chemical variations in their essential oils (33). The major constituents of essential oils produced from these cultivated plants, which lack thujones, are *cis*-epoxycimene, chrysanthenol and chrysanthenyl acetate. Though less active than thujones as antifeedants to the green peach aphid (*Myzus persicae*), bird cherry oat aphid (*Rhopalosiphum padi*) and the cotton leafworm (*Spodoptera littoralis*), oils containing the above noted compounds are effective antifeedants. Interestingly, bioactivity to insects was not well correlated to concentrations of any of the three compounds, providing another example of internal synergy

amongst constituents of an essential oil. These oils and extracts also have valuable bioactivity against the human parasites causing leishmaniasis and trypanosomiasis (33, 34). Rather striking quantitative year-to-year differences (up to 10-fold for individual constituents) in oil composition were found in cultivated plants from the two populations, and these in turn differed from plants grown under controlled conditions and from wild conspecifics (33, 34). However, supercritical fluid extraction of these plants gave much higher yields of the three compounds relative to hydrodistillation or organic solvent extraction, and the SF extracts were significantly more bioactive against all three pests (11, 35). Thujone-free cultivated *A. absinthium* appears to have good potential for the production of essential oils and extracts that can be formulated as biopesticides and for use as antiparasitic agents, but production practices will require standardization to achieve the consistency seen in other essential oils produced commercially over many decades (29).

Prospectus and Conclusions

As pesticides, plant essential oils should continue to make inroads into the marketplace, especially as the arsenal of conventional pesticide products becomes increasingly constrained, and consumers become ever more discerning about pesticide residues in the food supply, the workplace and the outdoor environment. They should find increasing use in organic production of high-value fruits and vegetables, and with increasing global urbanization, in professional pest control, consumer products and for vector control. Jurisdictions where pesticides are highly regulated may in the near future take a more favorable view of products based on essential oils, in particular those oils with a long history of human use and safety. Economics of production, driven by much larger markets (the flavor, fragrance and aromatherapy industries), will continue to dictate which essential oils are likely to be formulated as pesticides. It will indeed be interesting to see if a botanical source like wormwood can overcome the barriers and meet the challenges to become a new crop based on extracts and oils whose main purpose are for use as biopesticides and medicinal products.

Apart from purely descriptive biology and toxicology, our understanding of the pesticidal properties of essential oils and their constituents in insects, fungi and plants (as herbicides) remains somewhat rudimentary. As such, there is tremendous scope for selecting and tailoring plants to produce essential oils with enhanced efficacy as pesticides, for blending essential oils and/or constituents to achieve higher pesticidal efficacy or a broader spectrum of action, and in formulation to prolong residual action where desirable.

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Chapter 3

Regulatory and Quality Issues with the Essential Oils Supply Chain

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Essential oils have been used for centuries in ethnobotany and ethnobiology. Today, they are common ingredients in food, dietary supplements, personal care products, and aromatherapy. They are produced from aromatic plant material most often grown or collected by small farmers and low wage laborers from many different corners of the world. The loosely regulated supply chain tends to be long and opaque. Smuggling, adulteration, and contamination are commonly encountered biomass problems that affect finished good quality and integrity. Local governments and international organizations publish recommendations for Good Agricultural Practices (GAP) to resolve some of the supply chain issues, but adherence is voluntary. Since the U.S. and many other countries enforce good manufacturing practices (GMP) and quality standards on finished goods, companies must take meticulous steps to assure their plant biomass is clean and unadulterated.

Aromatic Plant Production

Cultivation and Wild Harvesting

Aromatic plants are produced by a variety of different methods around the globe. Many are grown on small farms where livestock are still used for plowing and crops are hand harvested, though a majority of aromatic plants are still wild-crafted (*1*). Wild crafting has lower production costs, but can deliver highly variable plant material with negative social and biodiversity impacts. Low

wage laborers or small farmers collect plant material from forests, pastureland, roadsides, and fallow agricultural land. Their main objective is to earn wages to provide for their family, often not considering proper land management or sustainable harvest. Unscientific and illegal practices are all too common, which can lead to overharvesting and possibly extinction. In rural areas, there is usually an ample supply of willing agricultural laborers. Contractors that employ the laborers act as middleman, selling the plant biomass to wholesalers. Although most wild crafted material enters the market from developing countries, a surprising amount of wild crafting is performed in developed countries (1). In Europe and North America, certain specialty crops are produced on larger farms using conventional farming methods such as lavender, peppermint, and citrus. Some herb and spice companies own and operate their own farms, achieving vertical integration of their supply chain. Companies that require high quality essential oils for drugs or high-end fragrances may contract farms to cultivate their desired plant material on a large scale (1). This type of production requires sophisticated crop management and therefore bears higher production costs.

Putting economic factors aside, there are a few other reasons why a majority of aromatic plants are still wild crafted. First, some forms of harvest are inherently more sustainable than others (2). For example, harvesting tree fruit like citrus does not typically have the same impact on plant populations like the harvest of roots or wood. Still, certain slow growing tree species that only produce a small number of fruits could be susceptible to over harvest. Some plants like *Artemisia* and *Eucalyptus* spp. are highly adaptable and resilient, growing in a wide range of habitats with large populations. The impact of wild harvesting is minimal in these types of species, especially if demand is low. There are cultural beliefs that wild plants have more medicinal power than cultivated plants (2). This notion is partially supported by science. Wild plants commonly contain higher levels of secondary metabolites produced in response to stressful conditions such as pests, pathogens, herbivory, and competition with other plants (3). Many of these factors are controlled during cultivation and so the plants may accumulate less stress compounds. Some wild plants grow more slowly, accumulating high levels of secondary metabolites over time (2). They can produce larger roots and thicker bark, as in the case of white sandalwood (*Santalum album* L.). Some plants are not amenable to large-scale cultivation; they only grow well in the wild. But, given enough demand and limited wild supply, wild populations will become scarce, and domestication may be necessary to avoid collapse of the market and the wild population (2). Investments in plant breeding and biotechnology can create cultivars with improved traits such as ease of cultivation and higher levels of essential oils. Ideally, local farmers would be involved in the breeding programs and eventual commercial cultivation. In many cases, sustainable wild crafting is the best option to maintain biodiversity in wild populations (2). Domestication does not preserve genetic diversity since a narrow range of improved genetic material is chosen to be grown the world over. Successful domestication and widespread cultivation of a crop will eventually eliminate the demand for wild harvested material (2). When this happens, local collectors lose incentive to preserve the wild populations since it is no longer part of their livelihood, potentially leading to further loss of biodiversity.

Crops and Countries

Essential oils have been used for centuries in ethnobotany, ethnobiology, ethnomedicine, agriculture, cosmetics, food, beverages, religious ceremonies, and rituals. Many of the traditional uses still exist today. While there are several thousand aromatic plant species that produce essential oils, less than 100 species are used for significant commercial essential oil production (4). Most are from the families Lamiaceae, Asteraceae, Rutaceae, Apiaceae, and Zingiberaceae. Orange, cornmint, cineole-type eucalyptus, citronella, peppermint, and lemon are globally the most highly traded essential oils by volume ((4, 5); see Table 1). Based on U.S. import prices of essential oils and oleoresins (6), sandalwood and rose are the two most expensive essential oils, fetching thousands of U.S. dollars per kilo (Table 1). Pure rose oil is valued around \$10,000 USD/kg, whereas a heavily manipulated product may only be worth \$500 USD/kg. East Indian sandalwood oil prices have held steady around \$2500-\$2900 USD/kg since 2011 (6).

Essential oil crops are produced in countries around the world, but assessing essential oil market data is not simple. Trade in botanical raw materials and bulk essential oils are assessed separately, each with their own Harmonized Commodity Description and Coding System number, generally referred to as “HS Code”. In some instances, a general term will be used to describe a commodity with various levels of quality or various plant parts. For example, cassia and “true” cinnamon oils from leaves and bark are often reported together, despite the fact they come from different species and the leaf extract is of lower quality/price than the bark extract. The term “clove oil” often includes clove bud, stem, and leaf oils, three oils of different values. Sometimes HS codes combine two unrelated species, e.g. HS code 33012911 includes terpenic oils of clove, niaouli, and ylang-ylang. Another misleading aspect of the market is the practice of importing and re-exporting the same plant material or essential oils. This is a common practice in France. For example, U.S. trade data shows nearly all of ylang ylang and cananga oils are imported from France (Table 1), but France is importing the oils from Comoros, Mayotte, and Madagascar, the leading suppliers (8). In the same regard, the U.S. exports a significant amount of licorice root, yet has no domestic commercial licorice root cultivation (8).

Based on export value, China, Madagascar, Indonesia, and India are the dominant herb and spice producers (1). India, U.S.A., France, UK, and Brazil were the top essential oil exporters in 2014 (5). Table 1 provides a summary of U.S. essential oil imports in 2013 showing the major producing countries as well as import prices and volumes. Current pricing information is available from the International Trade Centre’s monthly market reports.

Table 1. U.S. Imports of Essential Oils and Oleoresins (2013)

<i>Esential Oil</i>	<i>Major U.S. Suppliers</i>	<i>Price (USD/kg)^a</i>	<i>Imports (t)^a</i>
Orange oil	Brazil, U.S.A.	\$5-16	^b 12445.6
Lemon oil	Israel, Brazil, Belize	\$35-63	^b 3706.4
Cornmint oil	India	\$22	2957.7
Lime oil	Mexico	\$26	1623.4
Sandalwood	Australia, India	\$2500-2900	^c 1000.0
Eucalyptus oil	China, Brazil, India	\$11-15	858.3
Paprika oleoresins	India	\$17	755.0
Spearmint oil	Canada, India, China	\$30-43	706.0
Clove oil	Indonesia	\$15-30	577.5
Peppermint oil	India	\$25	537.3
Lavender oil	France	\$30	496.0
Cassia oil	China	\$40	456.3
Black pepper oleoresins	India	\$37	427.0
Citronella oil	Indonesia, China	\$15-18	298.9
Patchouli oil	Indonesia	\$40-60	270.9
Nutmeg oil	Indonesia	\$102	263.0
Anise oil	China	\$17	173.1
Cedarwood oil	China	\$7	135.9
Lemongrass oil	India, Guatemala	\$18-72	94.3
Rosemary oil	Spain, France, Tunisia	\$33-61	88.0
Bergamot oil	Italy	\$70	74.5
Petitgrain bitter orange oil	Paraguay	\$32	41.4
Geranium oil	Egypt, China, France	\$100-130	33.6
Ylang ylang oil	France	\$108	23.4
Vetiver oil	Haiti	\$139	17.1
Rose attar oil	France, Bulgaria	\$300-\$6435	12.1
Cinnamon oil	Sri Lanka	\$14	5.6

^a U.S. import data collected from (6) ^b UN COMTRADE 2014 data (5) ^c Estimation based on March 2015 import data (7).

Supply Chain Distribution Channels: Problems and Solutions

The essential oil supply chain tends to be long and opaque, involving many middlemen (9). Typically, local farmers or low wage laborers are the primary plant collectors or producers. They sell their plant materials to a local contractor or farm cooperative. From there, the plant biomass can follow several different channels. The contractor or cooperative may sell the aggregate plant material to larger wholesalers, who sell it to an international biomass importer/exporter. Then the product development company (e.g. U.S. flavor and fragrance manufacturer) purchases the biomass and distills the essential oil themselves. Alternatively, the plant biomass may go to an in-country processor to be cut and sorted or distilled into essential oil before it is sold to the international import/export company. In some cases, a finished goods company has a contract with the local producers and purchases plant biomass directly, bypassing the middlemen. If traceability is important, companies become vertically integrated, owning and operating the farms, distillation operation, and distribution channels. Unfortunately, when purchasing plant biomass or bulk essential oils on the open market, tracing the exact origin of the plant biomass is often very difficult. The more times biomass changes hands, and is repackaged, the more opportunity there is for adulteration and contamination. Smuggling and illegal harvest may be difficult to identify, especially when purchasing bulk oils or large lots of biomass from an unknown source. Federal and local governments, international organizations, biomass importers, and end-users are all working to bring more transparency to the industry with the goal of producing safe, high quality essential oils while preserving biodiversity.

Smuggling and Overharvest of Aromatic Plants

Indian Sandalwood

East Indian white sandalwood oil from *Santalum album* has a long history of smuggling and illegal harvest. *S. album* is native to the Western Ghats mountains in southwestern India. White sandalwood is valued for its essential oil, which contains around 90% santalols (10). The essential oil is steam distilled from chipped and powdered heartwood. It has been used for thousands of years in folk medicine and religious ceremonies and is highly valued as a fragrance (10). The Mysore region in the Indian state of Karnataka has historically been the center of sandalwood oil production. Four sandalwood species are on the IUCN Red List of Threatened Species, *S. album*, *S. fernandezianum* Phil. (considered extinct), *S. haleakalae* Hillebr., and *S. macgregorii* F.Muell (11), mainly due to overharvest for essential oil and wood for fine furniture. The export of the timber is illegal in India (12, 13), but domestic production of essential oil continues in the Mysore region. Despite the export ban, smuggling is extensive. India's most notorious bandit, Koose Muniswamy Veerappan, began his unlawful career as an ivory smuggler, forming a large gang in the 1960's (14). Eventually he became involved in sandalwood smuggling. To stop the decimation of sandalwood stands, the

Indian government banned the export of sandalwood and laid claim to any trees over a certain girth in the state of Karnataka. The Indian government eventually killed Veerappan in a shoot-out in 2004 (14). But smuggling continues, largely in response to the high prices of the essential oil and the scarcity of the raw material. Today, there are few wild stands of sandalwood remaining, so smugglers resort to cutting trees under cover of darkness from government and university properties. Another tactic is smuggling trees to remote distillation stills to covertly produce the essential oils (15). The oils are then smuggled to domestic or foreign markets. Overexploitation of sandalwood is not limited to India; native stands have been diminished in many areas including Australia, Indonesia, Hawaii, Papua New Guinea, New Caledonia, Vanuatu, and Tanzania (16).

In response to the skyrocketing prices of sandalwood (currently around \$2500–\$2900 USD/kg, Table 1) and the disappearing stands of this natural resource, plantations of *S. album* and other sandalwood species with lower quality essential oils (e.g. African sandalwood, *Osyris lanceolata* Hochst. & Steud.; Australian sandalwood, *S. spicatum* (R.Br.) A.DC.; and New Caledonia sandalwood, *S. austrocaledonicum* Vieill.) have been created. In western Australia, large stands of *S. album* and *S. spicatum* are coming into production. It is yet to be seen if these plantations will satisfy the global demand for sandalwood oil or reduce the illegal harvest of endangered trees (16).

Sassafras Oil

Another troubling example of essential oil smuggling is sassafras oil harvested primarily from members of Lauraceae *Cinnamomum camphora* (L.) J.Presl. and *Ocotea pretiosa* (Vell.) Rohwer. True sassafras oil was once produced from the North American species *Sassafras albidum* (Nutt.) Nees (17). It was the original flavoring for root beer, but due to concerns over carcinogenicity, the U.S. Food and Drug Administration (FDA) banned the ingredient in 1960 under 21CFR189.180 (18). Since the ban, sassafras oil is no longer produced commercially in North America (17). Today, sassafras oil is primarily used for the isolation of safrole. Safrole is a phenylpropanoid that is valued as the precursor for heliotropin (piperonal) and piperonal butoxide (PBO) (17). Heliotropin is used in the flavor and fragrance industries, with a similar flavor/fragrance profile as vanilla or cherry. PBO is used as a synergist in the manufacture of pyrethrum insecticides, i.e. it enhances the toxicity of pyrethrins. But neither of these industries is responsible for the smuggling of safrole-rich oil and overharvest of *C. camphora* trees in areas like southwestern Cambodia. Safrole is also a precursor for the illicit psychoactive drug 3,4-methylenedioxy-methamphetamine, also known as MDMA, ecstasy, or Molly (19). Structural similarities between safrole and MDMA are quite apparent (Figure 1).

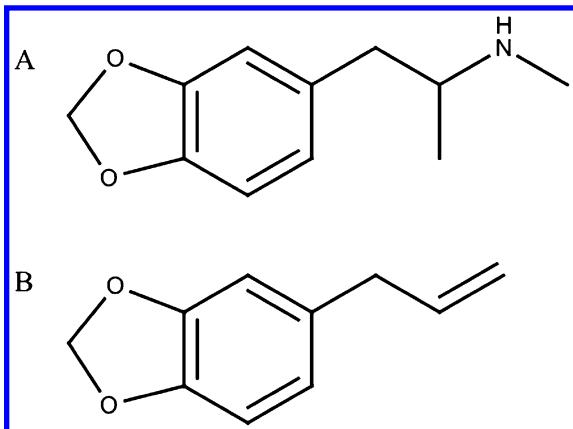


Figure 1. Structure of 3,4-methylenedioxymethamphetamine (MDMA; A) and safrole (B).

MDMA manufacture and abuse has been a problem around the globe since the late 1980's (20). Most of the precursors come from suppliers in Southeast Asia (20, 21). The Cambodian tree Mreas Prov Phnom, most likely *Cinnamomum porrectum* (Roxb.) Kosterm., Lauraceae (syn. *C. parthenoxylon*) (22), produces high yields of essential oil that contains 90% safrole (23). One UNODC publication claims the species is *Dysoxylum loureiri* [sic], Meliaceae (24). However, the Cambodian name for *Dysoxylum loureirii* is marah-prao and this species is not known for safrole-rich essential oil. *C. porrectum* is native to remote forests in Vietnam and the Cardamom Mountain area in southwestern Cambodia, including the Phnom Samkos Wildlife Sanctuary (23, 25). Smugglers set up stills in the forest to produce large amounts of SRO for the black market. In addition to overharvesting the threatened trees, smugglers have also been accused of deforestation to obtain firewood to fuel their stills; poaching local rare animals including tigers, pangolins, peacocks, pythons and wild cats; and polluting the Mekong River with their distillation run-off (23). In 2007, the Cambodian government made harvest of the Mreas Prov Phnom trees, production of SRO, and import or export of SRO illegal (23). Collectively, the UN Office on Drugs and Crime (UNODC), the Cambodian government, NGO Fauna and Flora International, and the Australian Federal Police raided clandestine production facilities and seized tons of SRO from 2007-2009. According to a UN report on ecstasy, the intense crack-down on SRO production had the desired effect. Safrole became so scarce, illicit drug manufacturers had to find other precursors and change the formula for ecstasy (20). The Cambodian National Anti-Drug Commission insists SRO production is over in their country; a recent aerial survey of the Phnom Samkos Wildlife Sanctuary did not reveal any apparent stills (25). But conservation groups speculate Mreas Prov Phnom trees are now smuggled out of the Cardamom Mountain region to produce SRO elsewhere (25).

Table 2. Essential Oil Crops Listed As Threatened on CITES Appendix (29) and/or the UN Red List (II)

Essential oil	Species	Habitat/Origin	CITES Appx.	UN Red List Status
Rosewood oil	<i>Aniba rosaeodora</i> Ducke	South America	II	No
Agarwood oil	<i>Aquilaria malaccensis</i> Lam. and other tree spp.	South and Southeast Asia	II	Vulnerable; some spp. critically endangered
Calamus oil	<i>Acorus calamus</i> L.	Widespread temperate regions	No	Least concern, local extinctions
Atlas cedarwood oil	<i>Cedrus atlantica</i> (Endl.) Manetti ex Carrière	Morocco, Algeria	No	Endangered, declining populations
Kenyan cedarwood oil	<i>Juniperus procera</i> Hochst. ex Endl.	NE, E and S tropical Africa	No	Least concern, declining populations
Cedrela oil	<i>Cedrela odorata</i> L.	Tropical Central and South America	III	Vulnerable, large specimens scarce, especially in Amazonia
Cinnamomum oils	<i>Cinnamomum</i> spp.	Southeast Asia	No	Vulnerable to critically endangered, depending on the spp.
Costus oil	<i>Saussurea costus</i> (Falc.) Lipsch. (syn. <i>S. lappa</i>)	Western Himalayas	I	No
Elemi oil, resinoid	<i>Canarium luzonicum</i> (Blume) A.Gray	Endemic to Philippines	No	Vulnerable
Hinoki wood oil	<i>Chamaecyparis obtusa</i> (Siebold & Zucc.) Endl.	Japan, Taiwan	No	Near threatened in Japan; vulnerable in Taiwan
Melanje cedarwood oil	<i>Widdringtonia whytei</i> Rendle	Mt. Mulanje in Malawi	No	Critically endangered
Mountain tobacco oil	<i>Arnica montana</i> L.	Europe	No	Least concern, regionally extinct in Hungary and parts of Ukraine

<i>Essential oil</i>	<i>Species</i>	<i>Habitat/Origin</i>	<i>CITES Appx.</i>	<i>UN Red List Status</i>
Orchid oils	<i>Orchidaceae spp.</i> (excluding vanilla)	Tropical distribution	II	Over 600 species listed, not all produce essential oils
Siam wood oil	<i>Fokienia hodginsii</i> A. Henry & H. H. Thomas	China, Lao PDR, Viet Nam	No	Vulnerable
Spikenard oil	<i>Nardostachys jatamansi</i> (D.Don) DC. (syn. <i>N. grandiflora</i>)	Bhutan, China, India, Nepal	II	No

Table 3. Essential Oil Plants Vulnerable to Overharvest Not Listed in CITES or UN Red List (30)

<i>Essential oil</i>	<i>Species</i>	<i>Habitat/Origin</i>	<i>Reason</i>
Amyris oil	<i>Amyris balsamifera</i> L.	Haiti	Collected from deadwood, but supply declining (30)
Anise scented myrtle oil	<i>Syzygium anisatum</i> (Vickery) Craven & Biffen	Australian rainforest	Pressure from myrtle rust, limited habitat (31)
Buchu oils	<i>Agathosma betulina</i> (P.J.Bergius) Pillans and <i>A. crenulata</i> (L.) Pillans	Endemic to South Africa	Red List of South African Plants: populations declining from essential oil harvest (32)
Indian wintergreen oil	<i>Gaultheria fragrantissima</i> Wall.	Himalayan Indo China	Depletion of wild populations (33)
Ginger-lily oil, Kapur kachari oil	<i>Hedychium coronarium</i> J. Koenig, <i>Hedychium spicatum</i> Sm.	India tropical forests	Habitat destruction, overharvest for medicine (34)
Inula racemosa oil	<i>Inula racemosa</i> Hook.f.	Northwestern Himalayas	Habitat destruction, overharvest for medicine (35)
Incense juniper	<i>Juniperus thurifera</i> L.	Western Mediterranean mountains	Livestock grazing, wood harvest, desertification (36)
Havozo bark oil	<i>Ravensara</i> spp.	Madagascar	Destructive overharvest for essential oils (37)

Other Overexploited Species

The aforementioned high profile cases are not the only examples of smuggling and overharvesting in the essential oils industry. There have been reports of rosewood smuggling from Madagascar (26) and agarwood smuggling throughout south and southeast Asia (27, 28). Species that are vulnerable to overharvest for commercial essential oil trade are listed in either the Convention on International Trade in Endangered Species (CITES) Appendices (29) or the IUCN Red List of Endangered Species (11) (Table 2). Commercial exploitation of plants appearing in CITES Appendix I is prohibited. Appendix II plant trade is restricted and plants appearing in Appendix III are locally threatened. Simultaneous pressure from habitat destruction, herbivory, fire, drought, disease, war, etc. further endanger the future of many of these species. In addition to this list, there are reports that other essential oil species are vulnerable to overharvest, but do not appear on the Red List or CITES Appendix (Table 3) (30).

Essential Oil Adulteration

In the current market for essential oils, buyers demand low prices in the face of dwindling supply and scarcity. Producers and distributors are under intense pressure to provide authentic, high quality essential oils, but below market price. Adulteration is one way producers can meet market demand while retaining a profit. There are several organizations that set quality and identification standards for essential oils, as a means to detect adulterated products. These include national pharmacopoeias (e.g. United States Pharmacopoeia, British Pharmacopoeia), trade organizations (e.g. Scientific Committee of the Essential Oil Association, American Herbal Products Association), and independent organizations like the International Organization for Standardization (ISO).

There are many different ways an essential oil can be adulterated. Starting at the beginning of the supply chain, substandard plant material could be mixed in or substituted for the original plant material (38). The substandard plant could be a closely related species similar morphologically and chemically or only morphologically to the original plant. For example, because of high demand for Ayurvedic medicine from *Nardostachys jatamansi* (D.Don) DC. (Valerianaceae), it is adulterated with *Valeriana jatamansi* Jones and *Selinum vaginatum* C.B. Clarke (39). A trained eye can differentiate these three species on a morphological basis, even when dried and ground, and their chemical composition is quite different (39). Sometimes pre-extracted plant material is passed off for the original, or other plant parts are mixed in with the aromatic material, e.g. woody stems mixed with leaves. These types of adulterations are more difficult to identify after the plant material has been ground. Many times, adulteration of the starting material is a case of careless misidentification during the collection process rather than a deliberate attempt at deception. There could also be confusion with common plant names that vary region to region (38). With the increase in certified plant material, another practice is to substitute conventionally grown plants for plants that were certified organic, fair trade, etc.

It is also common practice to adulterate the essential oils after distillation. There are many ways this can be accomplished. One of the simplest ways to adulterate essential oils is to cut it with a cheap vegetable or mineral oil or solvent like alcohols or glycols. Addition of 10% vegetable or mineral oil can be difficult to detect even when using standard gas chromatography (GC) identification methods, however they could be easily identified by a 90% alcohol solubility test (40). Addition of cheaper or lower quality essential oils is another common technique. For example, ylang ylang oil is produced from freshly harvested flowers of the *Cananga odorata* (Lam.) Hook.f. & Thomson tree. Grades are determined by density and aroma profile. As prices and demand have risen, quality decreased and adulteration increased. Other essential oils are added to increase volume and density, fractions of ylang ylang oils are remixed, and sometimes the oils are stored for a long time or heated to increase density (41). Cinnamon bark oil from *Cinnamomum verum* J. Presl (syn. *C. zeylanicum*) known as “true” or Ceylon cinnamon bark oil, is also frequently adulterated (42). In Ceylon, a mixture of bark and leaves may be distilled and sold as pure bark extract. Adding leaves increases the specific gravity and eugenol content, which

is easy to detect. Sometimes *Cassia* spp. bark oil is added instead. This also increases the specific gravity and cinnamic aldehyde content, which should be lower than 75% (ideally lower than 60%) in *C. verum* bark oils (42). Mixing in cheaper essential oils is frequently encountered in the citrus oil industry. The most common targets for adulteration are the high priced, low market availability oils like bergamot, Sicilian lemon, and bitter orange (43). Grapefruit oil, sweet orange oil or their terpene fractions have been added to bitter orange and Sicilian lemon oils. Grapefruit coumarins auraptene and epoxyauraptene can be detected by HPLC in the non-volatile fraction when an oil has been adulterated with grapefruit oil (43). Sweet orange and Sicilian lemon oil adulteration can be detected using GC to analyze the terpene profile. The δ-3-carene/camphene and δ-3-carene/terpinolene ratios are important markers of illegal additions to bitter orange oil (44). Reconstituted bergamot oils are often added to natural bergamot oil, but analysis of the linalool enantomer ratio by GC can expose the adulteration (45).

Another method to adulterate essential oils is addition of cheap synthetics that are identical to compounds found in the essential oil. For example, synthetic citronellal can be added to eucalyptus (*Eucalyptus* spp.) oil. Citral can be added to lemongrass (*Cymbopogon* spp.), aromatic litsea (*Litsea cubeba* (Lour.) Pers.), and lemon myrtle (*Backhousia citriodora* F.Muell.) oils. A combination of citral and citronellal can be added to lemon balm (*Melissa officinalis* L.), lemon verbena (*Aloysia citriodora* Palau, syn. *Lippia citriodora*), and lemongrass oils (46).

Contamination of Aromatic Plant Biomass and Essential Oils

There are several ways essential oils and their plant biomass can be contaminated including heavy metals, toxic residues, and microbes. Heavy metal contamination typically occurs when aromatic plants absorb metals from contaminated soil or irrigation water. Heavy metals are an ill-defined group, but most often refer to industrial contaminants such as Pb, Cd, Hg, As, Cr, Cu, Se, Ni, Ag, and Zn. Less commonly encountered metals include Cs, Co, Mn, Mo, Sr, and U (47). Specific sources of heavy metals can include sludge, municipal waste, pesticides, fertilizers, industrial emissions, mining run-off, and smelting operations (48). These sources are typically beyond the control of the local farmer and heavy metal remediation can be costly. Options include excavation of the top soil, stabilization of the heavy metals in the soil, or phytoremediation. If soils are contaminated with lead, addition of phosphate fertilizer can immobilize the lead by forming insoluble lead pyromorphite (49). Indian mustard (*Brassica juncea* (L.) Czern.) is a plant commonly used for phytoremediation since it readily takes up heavy metals (50).

Pesticide residues are limited under U.S. Environmental Protection Agency (EPA) regulations and enforced by the Food and Drug Administration (FDA) and United States Department of Agriculture (USDA). Pesticide tolerance limits are also set forth by other organizations such as the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). The United States Pharmacopoeia (51) has published methods for detection of organochloride, organophosphorus, and pyrethroid pesticides.

Good Agricultural Practices (GAP) allow for the responsible use of pesticides, leaving minimal residues. But, GAP recommendations are not always followed and sometimes a crop becomes contaminated from incidental pesticide drift from neighboring fields or highly contaminated irrigation water. Fumigants (ethylene oxide, phosphine, methyl bromine, sulfur dioxide) applied post harvest for microbial and insect control can also leave appreciable residues (52).

From 2008-2009, 87 people in the U.S. contracted *Salmonella* Rissen poisoning (53). The Rissen serotype was uncommon in the U.S., but it is fairly common in Southeast Asia. Cases were first identified in California and Nevada; most had exposure to Asian restaurants or had Asian surnames. Ground white pepper used in several Asian restaurants was eventually identified as the source. The FDA initiated an investigation into the prevalence of *Salmonella* contamination in herbs and spices and found globally fourteen outbreaks of illness were attributed to contaminated spices from 1973-2010 (54, 55). From FY2007-FY2009, 6.6% of sampled spice imports were positive for *Salmonella* (55). This is 1.9 times higher than the contamination rate for other FDA regulated food shipments during the same time period. Their study revealed over 80 serotypes; 6.8% demonstrated antimicrobial resistance. The study also revealed a considerable amount of filth in spices, most frequently insect parts, whole insects, and animal fur with an average prevalence of 12%. This value is 1.8 times higher than contamination rate for other FDA regulated food shipments during the same time period. The authors noted the presence of “stored product pests” indicates poor handling, storage, or cleaning of the spices.

Botanical raw materials carry a large number of naturally occurring bacteria and fungi that inhabit the root and shoot surfaces. This microflora is made up of a range of organisms, with aerobic spore forming bacteria as the dominant species (52). Mycotoxins including aflatoxins, ochratoxins, fumonisins and tricothecenescan are class I carcinogens that pose a significant health risk. They are excreted by fungi and are sometimes detected on botanical raw materials and plant extracts (56). Moldy plant materials pose a high risk of mycotoxin contamination. A large proliferation of microbes may indicate poor post harvest handling practices, including failure to control moisture levels during transport and storage. The presence of pathogenic organisms such as *E. coli* and *Salmonella* is all too common in the industry, as noted above, however Good Manufacturing Practices (GMP) recommendations set strict tolerance limits for these organisms, especially in finished products. Interpreting GMP recommendations and regulations can be difficult, especially when dealing with international suppliers and processors, since rules vary from region to region. In some cases GMP tolerance limits are recommendations that can be followed voluntarily by the industry but in other cases the GMP recommendations are legal requirements. In the U.S., the intended use of plant biomass (food, dietary supplement, cosmetic, etc.) dictates its regulatory status. Therefore, while biomass entering the U.S. may be turned away for phytosanitary reasons (visibly filthy or contaminated materials), there is no requirement to meet tolerance limits for microbial contamination. Similarly, producers of aromatic biomass in the U.S. are encouraged to follow the USDA and FDA guidelines to minimize microbial food safety hazards (57), but they are not legally bound to do so.

To confuse matter even more, different organizations set forth different microbial contamination limits that vary by orders of magnitude. So, depending on which set of standards are followed, biomass that passes microbial testing at one company may fail at another. The following table (Table 4) lists some of the microbial limits published by regulatory authorities for raw, pre-processed botanical materials intended for the dietary supplement industry.

Table 4. Microbial Tolerance Limits (Colony Forming units/g or mL) for Botanical Raw Materials Intended for the Dietary Supplement Industry (58)

Test	WHO ^a	USP ^b	EP ^c	NSF/ANSI ^d
TAMC	100,000	100,000	10,000,000	10,000,000
TYMC	100,000	1000	100,000	100,000
Enterobacteria	1000	1000	10,000	10,000
<i>E. coli</i>	10000	Absent in 10g	1000	100
<i>Salmonella</i>	Absent in 10g	Absent in 10g	Absent in 25g	Absent in 10g

TAMC=Total Aerobic Microbial Count; TYMC= Total combined Yeast and Mold Count ^a World Health Organization. Specific monographs may set different limits. ^b United States Pharmacopoeia. ^c European Pharmacopoeia (59). ^d National Science Foundation International Standard/American National Standard for Dietary Supplements.

Some of the disagreement may come from different philosophies regarding the microbiome and different conclusions of risk assessments. According to the European Pharmacopoeia 8.0 (59), “It is recognized that for some herbal medicinal products and extracts used in their preparation the criteria given above for TAMC, TYMC and bile-tolerant gram-negative bacteria [enterobacteria] cannot be met because of the typical level of microbial contamination. Less stringent acceptance criteria may be applied on the basis of a risk assessment that takes account of qualitative and quantitative characterization of the microbial contamination and the intended use of the herbal medicinal product or extract.” Health Canada makes a similar statement regarding setting microbial tolerance limits so low that no biomass can pass without sterilization measures. They compared published ranges of microbial contamination for untreated botanicals with the limits set forth by AHPA, ANSI, USP, and WHO and found more than half of the samples would be rejected without sterilization treatment (60). Sterilization treatments include dry heat, steam, irradiation, and fumigation. The EU prohibits fumigation with ethylene oxide (61) and many EU members do not allow aromatic herbs to be treated with radiation, so they must be labeled “irradiated” (62). Each method has benefits and drawbacks, but they all carry the risk that the active compounds will be degraded or residues will affect product quality. This loss of quality may be unnecessary when the goal is to meet stringent microbial plate count limits as compared to elimination of pathogenic bacteria.

Good Agricultural and Manufacturing Practices

Many different organizations have published GAP recommendations including local and federal governments, universities, non-profits, and international organizations like FAO and WHO. The WHO published guidelines specifically for growing and collecting medicinal plants (63). Some important topics include selection of botanical material and cultivation methods, selection of medicinal plants for wild collection, harvest and collection techniques, post harvest handling (sorting, processing, drying, etc.), packaging, storing, and quality assurance. The guide covers legal issues like collection on private lands and collecting endangered species and personnel issues like proper hygiene and health. Several methods suggested in the WHO guide would have a direct impact on one or more of the supply chain problems affecting the industry (Table 5).

The WHO has also published a set of guidelines for the good manufacturing of herbal medicines (64). These guidelines include the production of essential oils. Many of these practices overlap with the GAP recommendations (e.g. proper handling of plant material). Indeed one of the first recommendations is to only use plant material grown or collected in accordance with GAP. An emphasis is placed on maintaining sanitary conditions throughout the facility and keeping excellent records. All manufacturing procedures should be validated and written in clear language. Workers must be properly trained and qualified and provided with appropriate materials to perform their job. Detailed records must be maintained to show that all steps of the manufacturing process were correctly followed. Finished products must be properly stored and a system must be in place to quickly handle recalls or customer complaints. They must meet stability standards and contain the published level of active ingredient, in the case of standardized herbal drugs (65). As mentioned previously, several organizations publish limits for contamination including microbial, pesticides, and heavy metals.

There are a few GMP requirements that warrant further consideration, as they are sometimes challenging to implement. Distillation water quality can be difficult to ensure, especially when performed in a field distillery with tap water. According to EU GMP (66), water testing should be performed to ensure the water meets quality standards. A second point of debate is the practice of blending sub-par batches with higher quality batches to meet standards. A variation on this method is to further distill or fractionate an oil that contains undesirable components. These practices are currently allowed according to EU GMP.

There are numerous hurdles to achieving widespread compliance with GAP/GMP guidelines. One general issue is lack of funds and resources to change the status quo. Most producers or collectors of aromatic herbs do not have the financial ability to upgrade their facilities or equipment. And they lack the power to change major environmental problems like polluted soils and waters. Fortunately there have been efforts to build partnerships between large botanical suppliers, government organizations, NGO's, and primary producers to reform the system. The following are a few examples of these partnerships.

Table 5. GAP Recommendations (63) and Their Potential Impact on Aromatic Plant Supply Chain Issues

<i>Recommendation</i>	<i>Smuggling</i>	<i>Overharvest</i>	<i>Adulteration</i>	<i>Heavy Metals</i>	<i>Pesticides</i>	<i>Microbial</i>	<i>Filth</i>
Verify identity of all plant species grown or collected	X	X	X				
Obtain permits to collect wild plant material	X	X					
Use least-destructive collection methods		X					
Select site free from soil or water pollution				X	X	X	
Do not fertilize with human waste or uncomposted manure						X	
Use clean irrigation water				X	X	X	
Keep foreign matter out of plant material during harvest			X				X
Discard decomposed plant material						X	
Use clean equipment						X	X
Process plant material quickly, protecting from decomposition, insects, and animals						X	X
Dry plant material raised off the ground or on a clean tarp						X	X
Maintain clean storage and handling facilities						X	X
Properly label and document all material	X	X	X				
Workers should maintain health and hygiene standards						X	X

In response to diminishing quality and adulteration of ylang ylang oil, perfumer Givaudan developed partnerships with ylang ylang producers in Moheli, Comores (67). They purchased new distillation equipment and children's school supplies. Through education, the farmers reduced wood consumption for distillation fuel, learned the ideal harvest time for the flowers, and the importance of distilling the flowers in less than 2 hours post harvest (67).

Beraca, a large Brazilian supplier of botanical ingredients, works with many suppliers throughout Brazil. They are committed to biodiversity, creating partnerships with local communities to find sustainable ways to produce botanical raw materials. In Coopemaflima on the island of Marajo Para, Beraca promoted collection of andiroba (*Carapa guianensis* Aubl.) seeds that washed downriver and were deposited on their beaches (67). The seeds are a valuable source of oil, but the community treated them nuisance and had been cutting down the trees to rid the beaches of this "waste" (67). Beraca helped the community organize a collection cooperative, get organic certification, and helped them learn to extract the oil. Beraca now purchases around 500 tons of seeds per year for use in the cosmetics industry.

The island nation of Vanuatu has been working with landowners to maintain sustainable sandalwood (*Santalum austrocaledonicum* Vieill.) production. Sandalwood regulation started in 1995 after several years moratorium on sandalwood export (68). The Ni-Vanuatu Forestry Department issues licenses for an annual sale of 80 tons of sandalwood; 11 annual buyers compete for 30 tons and 2 long term buyers consume 50 tons (68). More than 70% of harvested trees came from plantations in 2013. Research grants from the Australian government have funded an oil analysis survey to determine the best phenotypes. The Vanuatu Department of Forestry (DoF) and local nurseries provide sandalwood saplings to farmers for replanting. But the DoF struggles to find financial support for these programs and to maintain adequate personnel for monitoring harvest and enforcing the laws.

American herb and spice supplier HQ Organics works with farmers in Peru and Egypt to gain and maintain organic certification (69). They have worked with Peruvian farmers to reclaim sandy fields for growing chili peppers, ginger, and other spice crops. In Egypt, farmers near the Nile were having difficulty maintaining their organic certification (70). The highly polluted Nile river contains organic pesticides (71). The farmers use this water to irrigate their crops and believed this was the source of their pesticide contamination. Along with the support of other suppliers and NGO's, some farmers moved away from the Nile and switched to well water for irrigation (72).

These are just a few examples of how industry, governments, and non-profit organizations are intervening to improve the integrity of the herb and spice supply chain. It is in suppliers' best interest to develop a secure supply of high quality plant biomass for their essential oils. The alternative is the continued risk of marketing contaminated or sub par products, which could lead to government fines and consumer backlash. Some organizations still caution consumers regarding "sustainable" essential oils, especially considering the challenges of truly sustainable production of essential oils from trees with long lifespans like sandalwood, rosewood, and cedarwood atlas (30). Many times the establishment

of plantations comes at the expense of native habitat. When a monoculture is grown, which is typically the case, it does nothing to maintain genetic diversity of the species. So despite the best efforts of corporations and governments to sustainably manage essential oil crops, for some the demand will always outstrip the supply, making sustainable production unattainable.

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Chapter 4

Controlled Environment Production of Medicinal and Aromatic Plants

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Controlled environment (CE) technology enables the production of plants and their products inside structures such as greenhouses, growth chambers, and indoor plant factories. Growth conditions are managed to optimize the concentration of high value phytochemicals, maximize yields, and minimize microbial and insect contamination on a year round basis. CE technology removes the geographical constraints to production by enabling environmental (temperature, photoperiod, light quality, CO₂) and cultural (rooting media, nutrient composition, irrigation) factors to be managed and replicated anywhere in the world. CE technology has potential to increase availability, improve quality, and reduce over-harvesting pressures of medicinal and aromatic plants (MAPs) supplying the commercial market. Although CE is widely used for the production of vegetables and ornamental species, there is limited published data on growth, production, and chemistry of MAPs in CE. This article provides an overview of research conducted on production of MAPs in CE, provides examples of potential CE's to increase yield and quality, and suggests areas for future development.

Introduction

It's estimated that up to 80% of the world's population use traditional herbal medicine as first, and often only, source of medicine. MAPs are botanical raw materials where their primary use is for therapeutic, aromatic and culinary purposes. They are components of medicinal production, additives to foods, and medicinal products (1). They are available as fresh or dried material, processed to essential oils, or processed to form extracts (2). These raw materials are used in a large number of products as constituents in prescription and over the counter drugs (3).

Although difficult to establish with any level of precision, it's been estimated that the global market value was \$32.9 billion in 2013, up from \$19.5 billion in 2009, an impressive 11.0% annual growth rate (4). Retail sales of herbal supplements in the US were estimated to be at least \$6.4 billion in 2014, with an average growth rate of >4% per annum over the previous decade (5). Although thousands of plant species have some medicinal use, far fewer (<400) have established international trade markets, and a minority of these species account for the bulk of sales (6).

With increased demand for MAPs, issues of reliable product availability and concern on quality of the product with respect to constituents and composition are increasing (7). The number of incidents involving adulteration and contamination are on the rise, posing health risks to consumers, liability issues for producers, and regulatory issues for the industry (8).

Opportunities for CE and MAPs

The demand for MAPs threatens the availability of raw material, creating the need to develop sustainable collection practices from the wild (3), improve and expand cultivation techniques (9–11), and use biotechnology to increase the availability of plant material (12–14) and bioactive products (15, 16). CE production can play a direct or supporting role in all these areas (12, 17).

Although there is limited information on CE production for the majority of MAPs of commerce, there is growing literature on greenhouse production of species with both ornamental and medicinal application such as *Achillea millefolium*, *Artemisia vulgaris*, *Calendula officinalis*, *Capsicum* sp., *Echinacea* sp., *Inula helenium*, *Matricaria recutita*, *Salvia* sp., *Stellaria media*, *Tagete* sp., *Tanacetum parthenium*, *Taraxacum officinale*, and *Valeriana officinalis* (18, 19).

Background on Controlled Environments

Controlled environment agriculture is the production of plants and their products inside structures, such as greenhouses, growth chambers and growth facilities. By using CE, temperature, relative humidity, nutrients and water can be optimized using environmental and control technology to increase yield and consistency in an efficient and sustainable manner. The ability to monitor and control the environment with CE technology removes climatic and geographic

barriers for production and enables year-round supply of plant material and product.

CE has particular advantage over field production of MAPs since the elevated CO₂ typically increases photosynthetic rate and yield (20–23). Similarly, higher quantities of light result in increased photosynthetic rates, and yields, as well (24). Both light and CO₂ concentration affect carbon partitioning and subsequent phytochemical content in the plant.

There is compelling evidence that CO₂ enrichment increases the total biomass produced by the plant, and generally increases the concentration of secondary metabolites produced. The effect of CO₂ concentration on plant physiology is species, and indeed cultivar, specific and with light intensity, temperature, nutrition and other environmental factors not well understood (25–28).

The following sections will summarize a cross-section of available literature to highlight potential to increase the quality and quantity of MAPs in CE.

Carbon Dioxide Enrichment and MAPs

Enriching the CE atmosphere with CO₂ is often used to increase yield, reduce harvest time, and enhance quality of ornamental, vegetable and fruit crops (26). While most of the research has focused on high value ornamental and horticultural species, there is growing evidence supporting the CO₂ enhancement of biomass production of MAPs including *Datura stramonium* (29), *Panax ginseng* (30), *Papaver setigerum* (31), *Echinacea* sp (32), *Podophyllum hexandrum* (33), *Hypericum perforatum* L. (34, 35), *Digitalis lanata* (36, 37), *Hymenocallis littoralis* Jacq. Salish (38), *Labisia nigra* L. (39), *Taxus baccata* (32) and *Zingiber officinale* (40).

In addition to increase in biomass, the concentration bioactive compounds also increases in these species. Table 1 provides an overview of effect of elevated CO₂ on concentration of bioactive compounds in MAPs (29–40). The increase in bioactive compounds is consistent with increases reported in horticultural and agronomic species such as *Brassica oleracea* va. *Italic Plench* (41) and *Glycine max* (L.) Merr. (42, 43).

Stutte *et al.* (44), grew *Scutellaria lateriflora* L., and *S. barbata* under fluorescent lamps in a controlled environment chamber at three CO₂ concentrations (400, 1200, and 3000 ppm). They reported more rapid flowering and significant increases in flavonoid concentration in response to elevated CO₂ in both species. There was a 2.4X increase in total flavonoid concentration from 400 to 1200 ppm, and a 4.9X increase over ambient CO₂ at 3000 ppm in *S. lateriflora*. There was a similar response in total biomass observed with *S. barbata*. When the combined effects of CO₂ enrichment on biomass and flavonoid concentration are taken together, this translates to a 13.7 fold increase in net production of bioactive compounds by increasing concentration from ambient (400 ppm) to 3000 ppm.

Similarly, Idso *et al.* (28) reported that increasing CO₂ from 400 to 700 ppm resulted in 48% increase in above ground and 56% increase in below ground (bulb) biomass and a mean increase of 12% in concentration of bioactive constituents, effectively increasing the amount of bioactive constituent per bulb by 75%.

Table 1. Summary of Selected Medicinal and Aromatic Plants That Have Shown Increases in Concentration of Bioactive Secondary Metabolites in Response to CO₂ Enrichment of the Atmosphere

<i>Species</i>	<i>Concentration (ppm)</i>	<i>Response</i>	<i>Reference</i>
<i>Brassica oleracea va. Italic Plench</i>	450, 750	Increase glycosinolates	(41)
<i>Datura stramonium</i>	294, 378, 690	Increase scopolamine	(29)
<i>Digitalis lanata</i>	350, 1000	Increase digoxin-momo-digitoxoside digoxin digitoxin	(37)
<i>Echinacea sp</i>	350, 500, 700	Increase in caftaric acid and total phenols in root	(32)
<i>Glycine max (L.) Merr.</i>	400, 700	Increase isoflavonoid concentration	(42)
	360, 650	Increase in daidzein, genistein, glycitein, diadzin, genistin, glycitin, 6"-O-acetyl daidzin, 6"-O-acetyl genistin, 6"-O-acetyl glycitin, 6"-O-malonyl daidzin, 6"-O-malonyl genistin ,-O-malonyl glycitin	(43)
<i>Hymenocallis littoralis Jacq. Salisb</i>	400, 700	Increase bulb biomass, increase in 7-deoxynarciclasine , 7-deoxy- <i>trans</i> -dihydronarciclasine, pancratistatin, trans-dihydronarciclasine, narciclasine.	(30)
<i>Hypericum perforatum L.</i>	360, 1000	Increase hypericin and pseudohypericin concentration	(35)
	500, 100, 1500	Increase concentration of hypericin, pseudohypericin and hyperforin	(34)
	350, 500, 750	Increase total flavonoids	(32)
<i>Labisia nigra L.</i>	400, 800, 1200	Increase total flavonoids, total phenolics, antioxidant capacity	(39)
<i>Nicotiana tabacum</i>	294, 378, 690	Reduce nicotine	(29)

Continued on next page.

Table 1. (Continued). Summary of Selected Medicinal and Aromatic Plants That Have Shown Increases in Concentration of Bioactive Secondary Metabolites in Response to CO₂ Enrichment of the Atmosphere

<i>Species</i>	<i>Concentration (ppm)</i>	<i>Response</i>	<i>Reference</i>
<i>Panax ginseng</i>	1, 2.5 and 5%	Increase total phenolics, total flavonoids	(30)
<i>Papaver setigerum</i>	300, 400, 500, 600	Increase in total alkaloid content	(31)
<i>Scutellaria barbata</i>	400, 1200, 3000	Increase concentration of scutellarein, baicain, apigenin	(44)
<i>Scutellaria lateriflora</i>	400, 1200, 3000	Increase concentration of baicalin, baicalein, wogonin and chrysanthemic acid	(44)
<i>Taxus bacatta</i>	350, 500, 750	Increase taxol	(32)
<i>Zingiber officinale</i>	400, 800	Increase total phenols, total flavonoids, antioxidant potential	(40)

Stutte (unpublished) found that increasing CO₂ concentration from 400 to 1200 ppm resulted in a 106% increase in shoot length, 64% increase in dry mass, and 12.8 % increase in anti-oxidant potential of *Prunella vulgaris* L. grown for 54 d under triphosphor fluorescent lamps at 300 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR) on 18h light/ 6 h dark photoperiod (19.4 Mol m⁻² d⁻¹ PAR). This was equivalent to a 69% increase in antioxidant potential (Table 2).

Table 2. Effect of Elevated CO₂ on Shoot Length, Fresh Mass, Dry Mass, And Anti-Oxidant Potential of 54 Day Old *Prunella vulgaris* Grown under Controlled Environment Conditions¹

<i>CO₂ (ppm)</i>	<i>Shoot Length (mm)</i>	<i>Fresh Mass (g)</i>	<i>Dry Mass (g)</i>	<i>ORAC (μmol TE/g FM)</i>	<i>ORAC (μmol TE/plant)</i>
400	71.8	25.8	3.26	20.6	529
1200	148.3	35.8	5.63	23.1	895
Significance ²	***	***	***	**	***

¹ Plant were grown at 23°C, 65% RH, and 300 μmol m⁻² s⁻¹ PAR on a 16h light/8h dark photoperiod under triphosphor fluorescent lamps. ² Tukey t-test, significant at P≥0.01=**, P≥ P.001=***.

Light Quantity and Quality in CE

Light quantity and quality both had significant impacts on growth and quality of MAPs. Increasing the quantity of light by increasing intensity or duration generally results in an increase in plant biomass (24) of horticultural and agronomic species (45).

The University of Wisconsin and the Wisconsin Center for Space Automation and Robotics evaluated the use of LEDs for plant growth in the late 1980s, and patents were awarded for this application in 1991 (45). Work at Kennedy Space Center (KSC) in Florida indicated that lettuce, wheat, spinach, and radish plants would grow and complete their life cycles under red light alone, but growth and development were significantly better when a small amount of blue light was added to the red (47). Since that initial work in the late 1980, early 1990's a substantial body of literature has developed demonstrating the potential of LEDs as supplemental and sole-source lighting in horticultural applications (46–50).

There is strong evidence suggesting that total yield of biomass increases with increasing quantity of light reaching the canopy. The quantity can be increased through either increasing the intensity or duration of a lighting cycling. The specific light response curves are dependent upon the species and cultivar. The effect of light intensity on yield and composition has been reported for a number of medicinal plants, including *Glycyrrhiza uralensis* (51), *Hypericum perforatum* (29, 52, 53), *Rhodiola sachalinensis* (54), *Tabernaemontana pachysiphon* (55), *Tanacetum parthenium* (27), and *Zingiber officinale* (56).

Figure 1 illustrates the effect of light intensity on size and flower number of *Tagetes patella* grown under cool white fluorescent lamps on 16 hr light/ 8 hr dark cycle at 22°C, 60% RH and elevated (1000 ppm) CO₂ for 79 days in a controlled environment chamber. Doubling the light intensity from 300 to 600 μmol m⁻² s⁻¹ resulted in a 58% increase in total plant dry mass, with a 117% increase in dry mass partitioned to the flowers/calyx. There was an 18% increase in lutein concentration/g in flowers/calyx resulting in an effective 2.6X increase in lutein content per plant (Stutte, unpublished).

In addition to using LEDs to increase yield, there is a growing literature demonstrating the use of LEDs to increase the concentration and composition of secondary metabolites (i.e. polyphenolics and glucosinolates) for many species, among which are included commercially valuable crops such as strawberry (*Fragaria vesca*) (57), tomato (*Solanum lycopersicum*) (58, 59), kale (*Brassica oleracea* L. var. *acephala*) (60), salad greens (e.g., *Lactuca sativa*) (61, 62), and microgreens (e.g., *Brassica oleracea*) (63).

There is also growing evidence that quality and composition of medicinal and aromatic plants can be managed through spectral quality. Stutte et al. (61), reported that addition of blue (440 nm) light affects not only morphology, but concentration of anthocyanin in *Lactuca sativa* L. cv. Outredgeous (Figure 2) well. Addition of blue light during final week of development increased anthocyanin content four fold over controls.



Figure 1. *Tagetes patula* grown for 79 days in CE chamber at 22°C, 60% RH and 16 hr light/ 8 hr dark photoperiod at 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (17.28 M $\text{m}^{-2} \text{d}^{-1}$) (left) or 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR (34.56 M $\text{m}^{-2} \text{d}^{-1}$) (right) from high pressure sodium lamps at 1000 ppm CO₂.



Figure 2. Twenty-eight day old *Lactuca sativa* cv. *Outredgeous* grown under either red (660 nm) (left) or blue/red (440/660 nm) (right) LEDs at 280 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR on 16h light/ 8 hr dark photoperiod at 23°C at ambient (365 ppm) CO₂. [Reproduced with permission from reference (50). Copyright 2015, Amer. Soc. Hort. Sci.].

Samuoline *et al.* (64), reported that light intensity and quality affected the concentration of phenolics, anthocyanins and ascorbic acid in sprouts of *Amaranthus cruentus*, *Ocimum basilicum*, *Brassica oleracea* cultivars, *Brassica juncea*, *Atriplex hortensis* L., *Borago officinalis*, *Beta vulgaris*, *Petroselinum crispum* and *Pisum sativum*. The responses were species specific, highlighting the need to optimize spectral quality and quantity for each species being considered.

Similarly, Tarakonaov *et al.* (65) found that altering the spectral composition with blue and red LEDs had varying effects on chlorophyll a/b, carotenoids and anthocyanin content and concentration of *Brassica juncea*, *Lactuca sativa*, *Ocimum gratissimum*, *Coleus blumei* and *Tagetes patula*. Park *et al.* (66) used narrow spectra LEDs to treat *Panax ginseng* Mayer roots with different wavelengths of light (380, 450, 470 or 660 nm) and found that blue wavelengths (450 and 470 nm) significantly increased the production of ginsenosides in the root tissue.

Nishimura *et al.* (67) found that growing *H. perforatum* under red, blue or white fluorescent lights in a controlled environment chamber at 27/24°C thermoperiod, and 16 hr photoperiod at 1000 $\mu\text{mol mol}^{-1}$ CO₂ at 250 $\mu\text{mol m}^{-2}\text{ s}^{-1}$ PAR increased total biomass, as well as concentration of hypericin, hypeicin, and pseudohypericin. Plants grown under red (600-700 nm) light had higher biomass than those grown under blue (400-500 nm) or white (400-700 nm), but spectral quality did not significantly affect the concentration of bioactive constituents. Doubling the light intensity from 250 $\mu\text{mol m}^{-2}\text{ s}^{-1}$ PAR (14.4 Mol m⁻² d⁻¹) to 500 $\mu\text{mol m}^{-2}\text{ s}^{-1}$ PAR (28.8 Mol m⁻² d⁻¹) increased the biomass per plant for each light level approximately 2 fold (range 1.5-2.6X). Although biomass of plants grown at 250 $\mu\text{mol m}^{-2}\text{ s}^{-1}$ PAR was lower, the concentration of hypericin, hyperorin and pseudohypericin was at least 2 fold higher, with greatest effect under red light (3.1-3.5X higher).

Solar light contains both UV-A (320-400 nm) and UV-B (280-320 nm) wavelengths that plants are adapted to, so indoor agriculture scenarios providing electrical sources of sole source lighting, especially of the narrow-spectrum type, may encounter situations in which produce quality and/or appearance may reflect a lack of UV radiation.

It has been hypothesized that the production of secondary products is reduced when UV-B is removed from the light spectra of plants grown in the greenhouse or under electric lamps. The effects of UV-B on plant production of secondary metabolites has been the subject of recent reviews (68, 69).

The addition of UV-B to the spectra has been shown to increase the production of hyperforin, pseudohypericin and hypercin in *Hypericum perforatum* (70), essential oil content and composition of *Nepata cataria* L., *Melissa officinalis* L. and *Salvia officinalis* L. (69), anthocyanin, total phenolics, anti-oxidant capacity and rosmarinic acid content of *Ocimum basilicum* L. (71, 72), melatonin concentration in *Glycyrrhiza uralensis* roots (73), brachycerine concentration of *Psychotria brachyceras* (74), and terpene content of *Mentha x peperita* L. (75).

Brechner *et al.* (70) found that a single 40 minute exposure of UV-B to 55 day old *H. perforatum* grown at 400 $\mu\text{mol m}^{-2}\text{ s}^{-1}$ PAR increased the concentration of hypericin, hyperorin and pseudohypericin from 2.5 to 3.7 fold within 24 hours. There was no additional benefit from additional or longer exposures. The effect of

UV-B treatment during flowering, the highest period of production of bioactives, is not known.

Constraints To Use of CE for MAPs

Although CE technology is widely used to insure quality and consistency of production of vegetable and ornamental crops, limited published information exists on the use of CE to produce MAPs. This is probably not surprising, given that MAPs requiring long harvest cycles, (e.g. *Ginseng panex*, *Hydrastis canadensis*, *Actaea racemosa*), large volumes (e.g. *Humulus lupulus*) or both (e.g. *Uncaria tomentosa*) are not economically viable for production.

Bioactives derived from byproducts of commercial horticulture (e.g. *Allium sativum*, *Vaccinium macrocarpon*, *Vaccinium microcarpum*, *Vitis vinifera* seed, *Citrus* sp. oil), agronomic (e.g. *Glycine max*) and forage (e.g. *Trifolium pratense*, *Medicago sativa*) crops are readily and cheaply available making it difficult to justify the capital investment and operating cost for CE production. Similarly CE production of bioactives derived from trees (e.g. *Crataegus monogyna*, *Ginkgo biloba*, *Serenoa repens*) is generally not economical due to long life cycle and large size.

However, there is significant opportunity to use CE technology for the production of uniform, high quality transplants for commercial cultivation. This has potential to significantly reduce time to harvest, reduce harvest pressure on endangered populations and increase profitability for the grower.

Summary

Controlled environment technology has a role in addressing several issues facing the medicinal and aromatic plant industries. Controlled environments and biotechnology have application in the propagation of threatened and endangered species to reestablish and preserve them in their native range. Availability of high quality, certified plant material for cultivation enables the commercial production of high value plant material to meet the increasing market demand. By providing a consistent supply of cultivated material to the market, the harvest pressures on wild populations should be reduced.

The risk of accidental and incidental contamination and adulteration of plant material grown under CE is inherently lower than that of plant material harvested from either wild or cultivated populations. The conditions are known, and opportunities for introduction of unknown or undesirable species is limited. The use of CE would conceivably be a key good manufacturing practice for production of standards to determine purity of product on the market.

The photoregulation of secondary metabolism through active management of spectral quality has been demonstrated to significantly enhance concentration of anthocyanins, glycosinolates, phenolics, flavonoids and other secondary metabolites. The increasing availability and reduced cost of LEDs for lighting holds great promise for enabling the consistent production of plant material with ‘custom’ biochemical constituents.

The yield of MAP per unit area can be greatly increased using CE technology through use of CO₂ enrichment. By leveraging faster life cycle, higher yield, and year round production that can be achieved with CE, there are significant opportunities to achieve high quality, consistent production (Table 3). For example, assuming that the average yield from a 90 day seed to harvest cycle of hypothetical medicinal plant is '1', and assuming that CO₂ enrichment to 1000 ppm will reduce seed to harvest cycle from 90 to 60 days and double plant size, the yield per m² is increased a minimum of 12 fold!

Table 3. Hypothetical Comparison of Productivity Per Unit Area of Medicinal Crop Grown under Either Field or Controlled Environment (CE) Conditions. This Assumes Year Round Production on a Single Layer under Elevated CO₂ Conditions.

<i>Variable</i>	<i>Field</i>	<i>CE</i>
Growth Cycle (days)	90	60
Harvest(no/yr)	1	6
Yield/(unit/m ²)	1	2
Total (unit/m²/yr)	1	12

CE technology and LED lighting also enables vertical production of plants in vertical farms or plant factories (76). If a typical production of 5 layers of plants is assumed the productivity will increase 60 fold/m²/year!

It is clear that the productivity and composition are affected by the growth environment, and that active management of these factors, specifically light quantity, quality and CO₂ concentration can have significant beneficial effects on the phytochemical composition. When targeting growth of a medicinal plant in CE in order to increase phytochemical production, the decision on what conditions to use will involve trade-offs on production of target compound versus potential changes in phytochemical profile, increases in productivity versus operating costs. However, from a technical perspective, there are significant opportunities to increase the yield of high quality MAPs with consistent phytochemical profiles. The diversity, high value, and unique properties of medicinally active plants make them promising candidates for production in CE.

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Chapter 5

Rapid Growth of High Quality Goldenseal Plants in Controlled Environment Growth Chambers

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Goldenseal, *Hydrastis canadensis* (L.), is a slow growing perennial forb, native to the Eastern United States, and is listed by CITES due to habitat loss and destructive harvesting for alkaloids found in roots and rhizomes. Since it takes 4 to 6 years for the plant to reach a harvestable size, controlled environments have been tested to determine if cultivation could be shortened without a loss in product quality. Two year old seedlings of goldenseal were grown in climate controlled growth chambers with two media, each with and without perlite supplements, at 3 CO₂ concentrations (380, 880, 1380 ppm) for 15 weeks. Fresh mass was harvested, dried, extracted, and the measured berberine and hydrastine concentrations were compared with extracts of forest cultivated goldenseal. Concentrations of both alkaloids, and the bioactivity of plant extracts were near identical when chamber grown and forest field plants were compared. Plants in forest field cultivation double in mass every 2 to 3 years. During 15 weeks in the chamber plants doubled in mass, and with a coarse medium they more than tripled in mass (this was reduced by additional perlite). Most of the plant mass (about 75%) was in the roots and rhizomes. Plants with the greatest root mass produced the

lowest concentrations of hydrastine and berberine. Leaf growth was reduced by the additional perlite, but the concentration of hydrastine was increased by additional perlite. Roots and rhizomes accumulated more alkaloids than leaves, and root mass was more important than concentration in determining yield. Therefore, plants with the greatest amount of alkaloid came from the coarse mix without perlite. By using controlled growth environments it was possible in a few months to accomplish what can be achieved after several years of growth under forest cultivation and to generate plants with greater or equal quality to forest cultivation.

Introduction

Goldenseal is native to the deciduous forests of Eastern North America and has been an item of commercial trade since the 1700's when the Native Americans first showed this species to European settlers. Traditionally used for infections, digestive disorders, sore mouth, and wound healing, modern medical research supports a wide range of potential uses in humans including diarrhea, mitigation of metabolic syndrome (diabetes, obesity, high cholesterol, and cardiovascular disease), multi-drug resistant bacterial infections, and Alzheimer's Disease (1).

Goldenseal was widely used as a medicine by the European settlers of North America and by the mid-1880s concern was expressed for its survival in the wild due to over collection. By 1908, more than 300,000 pounds were reportedly wild collected annually prompting the USDA to urge farmers to initiate cultivation of the plant (2). Goldenseal was a mainstream medicine in the 19th and early 20th century, until the advent of fungus based antibiotics in the 1940s. Demand for goldenseal waned during the 1950s and 60s with a dramatic resurgence beginning in the 1970s and 80s when consumers began searching for natural alternatives to synthetic medicines. By 1997, almost 100 years of USDA calls (2-4) for cultivation of the plant had gone largely unheeded while the market was again consuming more than 350,000 pounds of goldenseal root/rhizome per year (~60 million plants) with less than 2 % (~6,000 pounds) coming from cultivated sources (4).

Goldenseal is sparsely distributed across USDA cold hardiness zones 4-8 from Vermont to Georgia to Arkansas to Minnesota. Across its broad range, goldenseal is found in mature hardwood cove forests, characterized by a well formed organic soil profile that prevents desiccation in well-drained soils that are not subjected to periodic flooding. The greatest occurrences were found along forest paths and bright light intrusions (5) as 95% of leaf growth occurs in early spring prior to canopy closing of the deciduous forest (6). Bright sunlight and cool temperature characterize the short vegetative growing season. Flowering plants lose shoot and root mass during senescence and subterranean rhizome growth and rhizome size is well correlated to the initial size of the leaf. Plants started from seed usually flower the third or fourth year. Each plant can produce a single, green raspberry like fruit which turns red and ripens in July (7).

Cultivation has not been well-developed because of the plant's slow growth and the long period to maturity. Under woodland conditions, goldenseal spends most of the calendar year in sub-optimal conditions and does not actively grow. Natural forest canopies or some form of artificial shade are necessary to produce goldenseal in cultivation (8). With the use of polypropylene shade structures, the best foliar growth was with 80% shade, the greatest rhizome mass was at 63% shade, and the highest alkaloid content was with 30% shade. Davis (7) recommends 63% shade under natural light conditions for forest field simulated cultivation. These attempts have been in native soils and no reports of soilless mixes and containers are known.

However, even minimal attempts to remove *Hydrastis* from its native environment, i.e. artificial shade structures, have for the most part failed, as evidenced by declining shade cloth acreage and increasing wild simulated production (9). This situation combined with habitat loss resulting from land development led the US Fish and Wildlife Service to recommend, in 1997 the placement of goldenseal on the Convention on International Trade in Endangered Species (CITES) List II in an effort to "buy time for the plant while sustainable production methods are developed". Only with a premium for cultivated goldenseal vs wild collected material would there be an incentive for growers to invest time or effort to cultivate the plant. The cleanliness of medicinal quality plant material might offer that economic incentive.

Plant growth chambers offer the ability to control light, temperature, humidity, and CO₂ concentration. The expense of the chambers almost disqualify their use in production, but they can be used to identify appropriate conditions for cultivation in an indoor electrically-lit "plant factory", sunlit greenhouse, or modified forest field structure. The objective of this study was to observe goldenseal growth during a fifteen-week season with supplemented CO₂ at 380, 880, and 1280 ppm in growth chambers, and compare to growth in forest field. The quality of the harvested rhizomes and roots were also compared between growth chamber and forest field crops.

Materials and Methods

Hydrastis canadensis rhizomes were originally collected from Overton County, Tennessee in 2000, transplanted to Sleepy Hollow Herb Farm in Dalton, GA, and cultivated using certified organic methods. Fruit collected in the Summer of 2008, were fermented and de-pulped, and the seed was immediately replanted in woodland beds. Seedlings germinated in the Spring of 2009 were harvested in Spring, 2012. These were the forest field cultivated control plants.

In March 2012, a couple hundred plants were unearthed, shipped to Clemson University, and dormant roots and rhizomes stored in moist, dark conditions at 4 °C. Three Conviron E-15 growth chambers (Conviron Environments Ltd, Winnipeg CA) provided 3 concentrations of CO₂ (380, 880, 1280 ppm). Two textures of media (peat, pine bark, vermiculite, dolomitic limestone) were used, one coarse and one medium in texture (Metromix 8852 and Fafard 3-B, respectively, Sun Gro Horticulture, Agawam MA). Both mixes were made more

porous by adding 30% perlite (by volume), or remained non-amended. Media were moistened and partially filled Anderson plant bands (AB 36 Stuwe and Sons Inc., Tangent OR). The mass of each seedling was taken at planting and one seedling was placed in each band, and the medial level was topped off carefully to cover root with bud at media surface. Twenty four planted bands (2 media x 2 perlite x 6 replicates) were placed in each of three trays (Tray 5, Stuwe and Sons Inc., Tangent OR), with the 24 planted bands and one empty band in the center of the 25-unit tray.

The experiment was a completely randomized design with 3(chambers) x 2 (media) x 2 (perlite) factorial arrangement with 6 seedlings per treatment factor. Data were analyzed using JMP v 10.0 (Statistical Analysis System, Cary, N.C.). ANOVA and hypotheses were tested at $P = 0.05$.

One tray was placed into each of the 3 chambers. Chambers were set with 16 hr days, 400 μM fluorescent light, 22 °C day; 15 °C night, and 75% relative humidity. Sub-irrigation was performed two or three times a week by removing the trays from the chamber and giving them an overnight soak in 2" of fertilizer solution (100 ppm N 15-15-15 4Ca 2Mg, Jack's professional LX., J.R. Peters, Inc.), so all soils and trays received the same amount of irrigation and fertilizer.

Plant growth was measured by harvesting, washing and blotting dry fresh roots and rhizomes, and separating leaves of each plant after 15 weeks in chambers. The dry mass of the root/rhizome and the leaf/stem was measured after plants were oven dried in paper bags at 40°C for 4 days. Goldenseal root/rhizome and leaf/stem were used to quantify alkaloid concentration using a modification of Brown and Roman (10). Reagent grade berberine sulfate purchased from Sigma (St. Louis, MO) was used as standard.

Results

Plants emerged rapidly after being placed in the growth chambers. After 7 weeks under treatment conditions, all of the plants had attained full stature and had a good green color (Figure 1). Following 14 weeks in the chamber, many plants had begun to senesce. At that time, senescence was more prevalent in the medium weight media than the coarse media, with or without additional perlite, regardless of CO₂ treatment (chamber) (Figure 2). In the field, senescence is coincident with shorter days and lower night temperatures at the end of summer. This seasonal change did not occur in the environmental control chambers. Therefore, senescence is under endogenous control of the plant and was associated with the type of soilless mix, implicating the status of the root/rhizome in determining the onset of senescence.

The fresh mass of the plants in the chamber increased during the 15 weeks in treatment conditions. In the coarse media, with delayed senescence, plants more than doubled their fresh mass, and without additional perlite, the fresh mass more than tripled (Table 1). There was less growth in the medium texture mix. The initial plant mass was not a factor in the rate of increase. Plant mass in the field doubles every two to three years, and so 15-weeks in the chamber can have much more growth than compared to the field, depending on the soil mix.

Table 1. Goldenseal Grown for 15 Weeks in Controlled Environment, Growth Chamber in Different Soils and CO₂-Levels Varied in Size, Propagation Rate, and Alkaloid Content

Factors		Plant growth				Alkaloid concentrations (%)				Chemical yield (mg/plant)		
Media Texture	Perlite	Mass increase (out/in) ^a	Bud increase (out/in)	Dry mass root(g)	Dry mass leaf(g)	Hydrastine root	Berberine root	Hydrastine leaf	Berberine leaf	Root	Leaf	Total
Medium	No	1.6	0.7	1.3	0.5	3.0	3.6	1.0	2.3	8.5	1.7	10.1
Medium	Yes	1.9	0.9	1.8	0.4	2.8	3.1	1.2	2.3	10.2	1.7	13.6
Coarse	No	3.3	1.2	2.6	0.7	2.3	2.9	1.1	1.9	13.2	1.9	15.1
Coarse	Yes	2.2	1.8	1.6	0.5	2.7	3.8	1.2	2.0	10.1	1.6	11.9
ANOVA Terms ^b												
Media		**	*	*	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Perlite		n.s.	n.s.	n.s.	*	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.
M x P		*	n.s.	**	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.
CO ₂		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
M x C		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
P x C		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
M x P x C		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Continued on next page.

Table 1. (Continued). Goldenseal Grown for 15 Weeks in Controlled Environment, Growth Chamber in Different Soils and CO₂-Levels Varied in Size, Propagation Rate, and Alkaloid Content

Factors		Plant growth				Alkaloid concentrations (%)				Chemical yield (mg/plant)		
Media Texture	Perlite	Mass increase (out/in) ^a	Bud increase (out/in)	Dry mass root(g)	Dry mass leaf(g)	Hydrastine root	Berberine root	Hydrastine leaf	Berberine leaf	Root	Leaf	Total
Initial Mass		n.s.	n.s.	*	**	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
Whole Model Fit (R ²)	0.31	0.21	0.33	0.32	0.22	0.36	0.19	0.17	0.20	0.13	0.17	

^a ‘out’ denotes out in the forest field; ‘in’ denotes in the growth chamber ^b * and ** denotes significantly different means with 95% and 99% confidence, respectively, while n.s. is not significantly different.



Figure 1. One or two leaves per plant had emerged by week 7 in the chamber and plants were nearly full size. There was no noticeable difference between plants grown at the three different CO₂ concentrations (380 left, 880 center, and 1280 right).

The environmental chamber might be used to propagate plants, but the rate of division is low. Coarse medium with perlite almost doubled the number of buds on the rhizomes (Table 1). Initial size of seedling and CO₂ treatment (chamber) were not significant factors. Propagation in this environment would still be expensive for small amounts of plants produced.

The initial mass of the seedling influenced leaf and root dry mass of the plants after 15 weeks (Table 1) with larger seedlings producing more dry mass (data not shown). The largest root and rhizome dry mass was in the coarse medium without perlite, although the medium media could have improved root and rhizome mass by adding perlite. Leaf mass was larger in plants without perlite. The CO₂ concentration (chamber) did not affect dry mass implying other factors were limiting growth (e.g light).

Alkaloid concentrations in the root and rhizome were lower in the plants with the fastest growth. The coarse medium with no perlite addition had the lowest concentrations of berberine and hydrastine. Most of the plant mass (about 75%) was in the roots and rhizomes (Table 1). The leaf contained less berberine and hydrastine than the root, for both mass and concentration since translocation had begun (Fig. 3). The total amount harvested is the product of concentration and dry mass. Since those responses had different maximal treatments, there was no significantly best growth chamber treatment to get the highest amount of alkaloid yield. The different treatments were essentially equivalent.

Alkaloid concentrations of plants grown in the forest field were very similar to plants grown in the chamber (Figure 4). There would be no qualitative difference in using the chamber to finish seedlings. The greater growth in mass may justify the cost. Further improvements in chamber culture may come from improved media blends and irrigation protocols. This is the first report of controlled environment



Figure 2. By week 15, senescence was underway. Leaf drop progressed more slowly in the coarse medium (top) than in the medium weight media (bottom). Additional perlite treatment is shown on the left side of all the paired rows and the three different CO₂ concentrations were shown (380 left, 880 center, and 1280 right).



Figure 3. At harvest most of the plant mass is in the root and rhizome. Best specimens from the three different CO₂ concentrations (380 left, 880 center, and 1280 right) are shown.

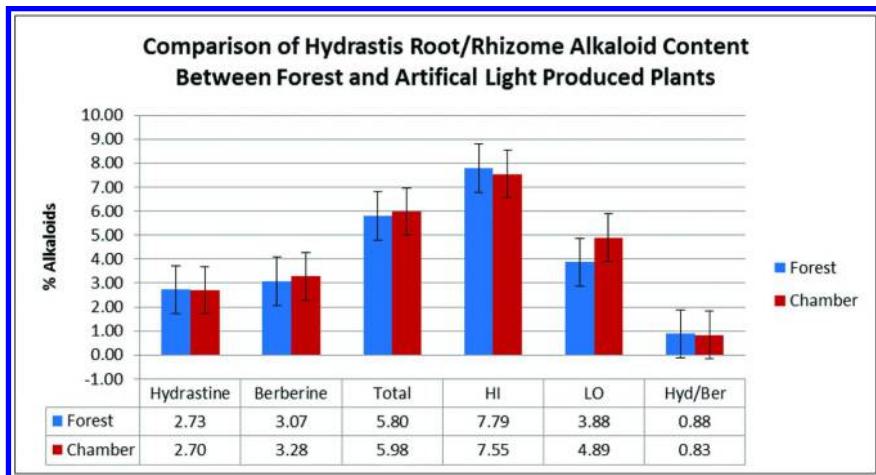


Figure 4. Hydrastine and berberine concentrations in the roots and rhizomes of goldenseal plants cultivated in growth chambers was compared to the concentrations obtained during forest field cultivation.

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Chapter 6

Potential Benefits of Soil Microorganisms on Medicinal and Aromatic Plants

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Inoculating medicinal and aromatic plants with nurturing rhizospheric microorganisms enhances plant growth, development, and secondary metabolite production through increased nutrient and moisture availability, repressed pathogens, improved stress tolerance, and increased phytochemical synthesis. The use of growth promoting bacteria and mycorrhizal fungi reduces the need for chemical fertilizers and pesticides applied to cultivated medicinal and aromatic plant species. Only a limited number of commercial rhizospheric microorganisms are currently marketed for medicinal and aromatic plants. As more growers become aware of the beneficial effects of rhizospheric microorganisms, increased demand for microorganism products can be expected.

Introduction

Plants, living in an environment full of bacteria and fungi, interact with these microorganisms. The rhizosphere, the soil area surrounding the plant roots, is especially abundant in microorganisms due to plant root exudates that serve as a nutrient source for microbial growth (1, 2). Rhizospheric microorganisms can interact with plants, causing neutral, negative, or positive effects on plant growth and development (3).

Plant growth promoting rhizobacteria (PGPR), a term introduced by Kloepper in the late 1970s (4), enhance the growth of plants through various mechanisms, including solubilizing phosphorus, fixing nitrogen, producing iron-chelating siderophores, balancing phytohormones, synthesizing volatile organic compounds

(VOCs), degrading quorum-sensing signals in pathogens, and inducing plant resistance to pathogen and abiotic stresses (5–9). Many PGPRs, such as *Azospirillum* spp., *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp., have been identified and sold commercially as biofertilizers and biopesticides (10).

In addition to PGPR, mycorrhizal fungi can also serve as biofertilizers. Mycorrhiza is a symbiotic association where external fungus mycelium supplies soil derived nutrients to a plant root (11). Arbuscular mycorrhizal fungus (AM fungus) is a type of mycorrhiza characterized by the formation of fungal hyphae penetrating root cortex cells (12). AM fungi consist of 9–55% of the biomass of the total soil microorganisms and are the most ubiquitous fungi in agriculture soils (13), residing in the roots of more than 80% of all terrestrial plants (14). AM fungi improve biotic and abiotic stress resistance of the plant through a number of mechanisms, including the delivery of nutrients and water to the plant root through extraradical hyphae networks (15, 16), and increasing metal catalysts for use by plant antioxidant enzymes (17, 18). The AM fungi *Glomus* spp. and non-AMF fungi, such as *Trichoderma* spp. are sold as biofertilizers and biopesticides (19, 20).

Treatment of plant seeds and/or roots with microorganisms can also increase secondary metabolites, including terpenes, phenolics, flavonoids, and alkaloids, by mechanisms which are not fully clarified (20, 21). The microorganisms act as bioelicitors and trigger production of secondary metabolites involved in plant defense systems, partly by stimulating immune receptors on the plant root surface (22, 23). By eliciting medicinally active secondary metabolite synthesis, PGPR and mycorrhizal fungi can improve quality of medicinal and aromatic plant products.

While PGPRs can contribute to plant growth and soil fertility, some studies (24, 25) report possible growth inhibition by PGPR. Production of cyanide, for example, has been reported to demonstrate inhibition of both pathogen and plant growth (24, 25). Careful examinations are needed to avoid possible deleterious effects from PGPRs.

This review focuses on microbes and fungi that promote plant growth and elicit secondary metabolite synthesis in plants, but does not cover bacteria or fungi that directly synthesize medicinally active components, such as bacteria that synthesize paclitaxel and maytansine (26, 27).

Commercial PGPRs and AM fungi are available for various crops and vegetables in the United States, however, few PGPRs and AM fungi are marketed for aromatic and medicinal plants. Additional studies are needed to determine the PGPRs and fungi that are useful for cultivation of aromatic and medicinal plants.

Enhancement of Nutrient Supply by Biofertilizers

Solubilization of Phosphorus

Plants need soluble forms of phosphorus (P) for growth. Although a relatively high abundance of P exists in soils, most of the P is inaccessible to medicinal and other plants because soluble P easily precipitates with calcium in alkaline soils

and with iron and aluminum in acid soils (28). Plants acquire P as orthophosphate anions (mainly as HPO_4^{2-} and H_2PO_4^-) that usually account for less than 1% of the total P (28). Microbes can solubilize P by secreting phosphatases that degrade organic P and by secreting acids that dissolve inorganic P compounds. The increased availability of P enhances plant growth (29, 30).

AM fungi can enhance the plant uptake of P through fungal hyphae that can transport P from distant sources (31). When *Andrographis paniculata*, an annual herbaceous medicinal plant that originated in India, was inoculated with *Glomus mosseae* and *Trichoderma harzianum* and grown under field conditions at two P levels (the recommended P level and 75% of the recommended P level), the growth and medicinal alkaloid (andrographolide) production was significantly improved at both P levels as compared with uninoculated control plants at the recommended P level (32). In a study on the effects of phosphate-solubilizing bacteria and AM fungi, as compared with P fertilizer in a field of rose-scented geraniums (33), inoculation with microorganisms provided yields equivalent to the P fertilizer. Co-inoculation of phosphate-solubilizing bacteria and AM fungi increased the yield of geranium by 33.0%, while P fertilizer increased the yield 36.7% compared with a control planting without microbe inoculation or P fertilizer. The inoculation of the geraniums with microorganisms enhanced the monoterpene (citronellol, geraniol, and geranal) and sesquiterpene content of the geranium plants. Thus, using PGPRs and AM fungi may reduce the amount of P fertilizer needed for aromatic and medicinal plant cultivation.

Fixation of Nitrogen

Nitrogen (N) is a major nutrient required for plant growth. Microbes play an essential role in N cycling and the utilization of N by plants (34). Rhizospheric microbes that fix N in the soil symbiotically or non-symbiotically can reduce the need for N fertilizers in crop production (35). Legumes are well-known for symbiotic N fixation in root nodules by *Rhizobium* bacteria (36, 37). Non-leguminous plants, such as rice (*Oryza* spp.), sugarcane (*Saccharum* spp. hybrids), wheat (*Triticaceae* spp.), and maize (*Zea mays*), have also been reported to have symbiotic N-fixing bacteria, such as *Azotobacter* spp., *Bacillus* spp., and *Beijerinckia* spp. (38–40).

N fixation also occurs in free-living diazotrophs, including *Azospirillum*, *Burkholderia*, *Azoarcus*, *Gluconacetobacter*, *Pseudomonas*, and cyanobacteria (41, 42). These diazotrophic bacteria occur in rhizospheric soil of many medicinal plants. A study by Karthikeyan and coauthors (43) on the diazotrophic population in the rhizosphere region of four medicinal plants (holy basil (*Ocimum sanctum* L.), coleus (*Coleus forskholii* Briq), Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don.), and Aloe (*Aloe vera*)) identified *Azospirillum*, *Azotobacter*, and *Pseudomonas* colonies.

Research by Hellal and coauthors (44) applied a mix of five PGPRs (*Azotobacter chroococcum*, *Azospirillum lipoferum*, *Bacillus polymyxa*, *Bacillus megaterium*, and *Pseudomonas fluorescens*) to plots of dill (*Anethum graveolens* L.) with three levels of N fertilizer (one-third, two-thirds and a full recommended

dose). Maximum plant growth of dill occurred with the treatment of PGPRs plus two-thirds of the recommended dose of N, suggesting that biofertilizers can reduce the use of N fertilizers.

Production of Siderophores

Iron-chelating siderophores produced by microbes also contribute to plant growth by supplying iron to host plants and by inhibiting the growth of pathogenic microbes in the rhizosphere (45). Siderophores can convert iron from the insoluble mineral phase to soluble ferric complexes that can be absorbed by plants (46). Examples of siderophore-producing species of bacteria are *Pseudomonas*, *Enterobacter*, *Bacillus*, and *Rhodococcus*. These bacteria also suppress phytopathogens by removing iron from the environment (45, 47). Khamna and coauthors (48) isolated a total of 445 actinomycetes from 16 medicinal plant rhizospheres. Of the total isolated microbes, 89% belonged to the genus *Streptomyces* with 75 isolates producing siderophores on chrome azurol S (CAS) agar.

Plant Growth Enhancement

Production of Phytohormones

PGPRs can affect plant growth by producing phytohormones, such as auxins, cytokinins, and gibberellins (49, 50), and by decreasing ethylene concentrations in plant cells (51). An estimated 80% of bacteria in the rhizosphere can produce indole-3-acetic acid (IAA), a major plant growth hormone (52). Bacterial-produced IAA can stimulate the development of the host plant root system, further enhancing nutrient absorption by plant roots (53). IAA production by root symbionts has been suggested as important for the formation of root nodules in the aquatic legume, water mimosa (*Neptunia oleracea*) (54) used medicinally for treating cancer (55).

Enzymes produced by PGPRs that degrade 1-aminocyclopropane 1-carboxylic acid (ACC), an ethylene precursor, enhance plant growth by lowering ethylene concentrations in the plants (56–58). Low ethylene concentrations, those below threshold levels that vary among plant species, promote plant growth. When the ethylene levels are elevated, plant growth is inhibited (59). Stress conditions are known to elevate ethylene concentration, inhibiting plant growth (59). Barnawal and coauthors (60) treated holy basil (*Ocimum sanctum*) grown under water-logged stress with ten different PGPRs that have ACC-deaminase activity and observed maximum growth and yield of the basil in the plots treated with the PGPR *Achromobacter xylosoxidans*, which had decreased the ethylene level the most. In the salt stress studies with basil (*Ocimum basilicum*) (61), corn mint (*Mentha arvensis*) (62), and Madagascar periwinkle (*Catharanthus roseus*) (63), PGPRs with ACC-deaminase activity have enhanced plant growth compared with plants not treated with PGPRs.

Production of Volatile Organic Compounds (VOCs)

Some bacteria produce VOCs that can enhance plant growth. Bacterial VOCs are mixtures of small molecules that easily volatilize, including low molecular alcohols, aldehydes, esters, terpenoids, and thiols (64). For example, the VOCs 2,3-butanediol, acetoin, and tridecane promote the growth of *Arabidopsis thaliana* seedlings (5, 65). VOCs released from *Bacillus subtilis* GB03 increased growth and essential oil content of basil (*Ocimum basilicum*) (66). A comparison of the VOCs from *Pseudomonas fluorescens*, *Bacillus subtilis* and *Azospirillum brasilense* by Santoro and coauthors (67) showed that the VOCs from *Pseudomonas fluorescens* significantly improved growth and increased the yield of two major essential oil constituents, (+) pulegone and (-) menthone, in peppermint (*Mentha piperita*).

Disrupting Quorum Sensing Signals in Pathogens

Bacteria can detect their population density through a cell-cell communication system termed quorum sensing (QS) (68). QS signals regulate various gene expressions, including virulence factors. Common QS signaling molecules are *N*-acyl homoserine lactones (AHLs) (69) that regulate gene expressions depending on population density. Degradation of QS signals, known as ‘quorum quenching’ (QQ), can significantly reduce bacterial virulence (70). *Bacillus* species such as *B. thuringiensis* produce *N*-acyl homoserine lactone lactonases that hydrolyze AHLs, significantly impairing pathogenicity (71). QS degrading enzymes have been identified in several other bacterial species, including *Pseudomonas*, effective for disease control that enables vigorous plant growth.

Stress Resistance

Resistance toward Pathogens

PGPRs can improve plant health by increasing plant immunity to pathogens (72, 73). Using pattern recognition receptors (PRRs), plant immunity systems recognize microbe-associated molecular patterns (MAMPs). The MAMPs are essential structures for the survival of microbes and are conserved among pathogens and non-pathogenic microorganisms (74). Bacterial flagellin, elongation factor Tu, peptidoglycan, lipopolysaccharides, fungal chitin, and β -glucan from oomycetes are MAMPs perceived by PRRs. The surface-localized PRRs, receptor-like kinases (RLKs) and receptor-like proteins (RLPs), initiate immune signaling in plants (75). PGPRs, such as *Pseudomonas*, *Bacillus*, and *Bradyrhizobium* activate plant immunity, enabling plants to respond faster and more strongly to subsequent pathogen attacks (76–78).

PGPRs can also directly inhibit the growth of pathogenic microorganisms by producing antibiotics or competing for nutrients and colonization niches (10). Microorganisms including *Streptomyces* spp., *Bacillus subtilis*, *Trichoderma*

spp., and *Ampelomyces quisqualis* are commercially sold as biopesticides for pathogenic infections, such as powdery mildew, *Fusarium*, *Phytophthora*, and *Rhizoctonia* (10, 79).

Resistance toward Abiotic Stresses

Environmental stresses, such as drought, floods, salinity, extreme temperatures, and heavy metals, can reduce plant growth, and yield (80–82). PGPRs and AM fungi can stimulate medicinal plant growth despite these environmental stresses (9, 83). For example, under a water deficit stress, the yield of Madagascar periwinkle (*Catharanthus roseus*) was improved when the plants were grown from seeds inoculated with *Pseudomonas fluorescens* as compared with the control group of plants grown from uninoculated seeds (84). A study of PGPRs on basil (*Ocimum basilicum* L.) under water stress revealed an increase in proline and soluble carbohydrate accumulations in the leaves as compared with basil not treated with PGPRs (85). Dual inoculation with *Piriformospora indica* (a novel endophytic fungus isolated from desert soil in India) (86) and *Pseudomonas fluorescens* improves the survival rate of transplanted musli (*Chlorophytum* spp.) (87).

Deleterious Effects of PGPR

While PGPR play essential roles in soil fertility, plant growth, and plant health, some chemical compounds produced by PGPRs can have adverse effects on plant growth (46, 88). Bacterial production of hydrogen cyanide (HCN), auxin, and rhizobitoxine, an ethylene synthesis inhibitor, can have a positive or negative effects on plant growth and development, depending upon the environment and the plant species (9). Adverse effects can be specific to the combination of plants, microbes, and environmental conditions. The selection of PGPRs compatible with the plant material, soil conditions, and the environment is necessary to maximize expected advantages in plant growth and yield with PGPRs.

Secondary Metabolite Induction

Increases in phytochemical production in medicinal and aromatic plants has been associated with the presence of PGPRs and AM fungi in the rhizosphere (89–93). Although the mechanism by which PGPRs and AM fungi increase phytochemical production is not well understood, such an increase could be due to more vigorous plant growth or a direct promotion of metabolic pathways. Bacterial produced phytohormones and VOCs can serve as induction signals for phytochemical production (66, 94). Banchio and coauthors (66) have demonstrated that VOCs produced by *Bacillus subtilis* GB03 increased the concentration of α -terpineol and eugenol in basil. When inoculated with polysaccharides from *Bacillus cereus*, the hairy roots of *Salvia miltiorrhiza* accumulate tanshinones (diterpenoid quinones) (95). Treating sivakaranthai (*Sphaeranthus amaranthoides*), a siddha holistic herb, with *Glomus walkeri*

and several other PGPRs enhances production of secondary metabolites (total phenols, ortho-dihydroxy phenols, flavonoids, alkaloids, and tannins) in leaves (96). Experimental results indicate that PGPRs and AM fungi can improve the quality of cultivated medicinal plants while promoting plant growth and enhancing stress resistance (Figure 1).

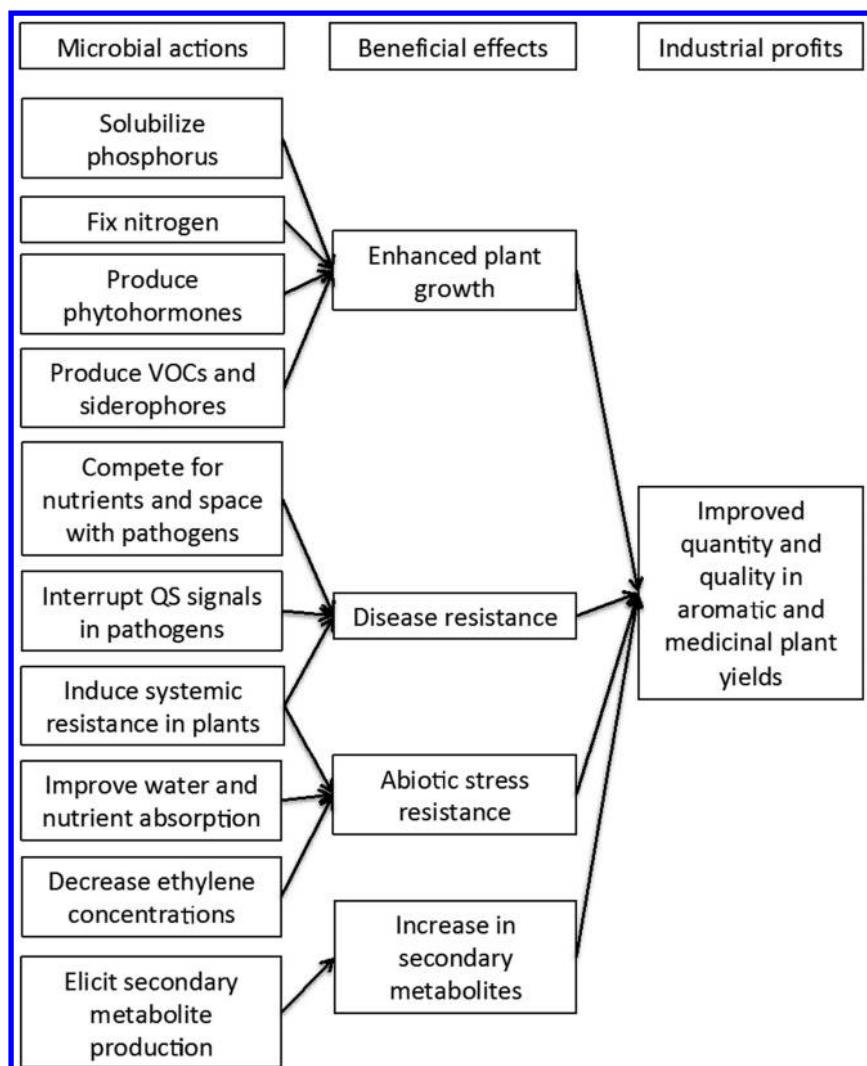


Figure 1. Microbial actions associated with rhizospheric microorganisms.

Table 1. Commercial Microorganism Inoculants Available in the United States

Microorganisms	Trade Name/ Company Name	Plants	Nutritional effect
<i>Bradyrhizobium japonicum</i>	BYSI-N /Brett-Young	Soybean	Nitrogen fixation
<i>Delftia acidovorans</i>	BioBoost/ Brett-Young	Canola	Sulfur oxidizer
<i>Glomus</i> spp., <i>Rhizopogon</i> spp.	Root Growth/ TheSeedSupply	Not specified	General plant health and nutrient absorption
<i>Rhizobium leguminosarum</i>	Nitragin/AgraQuest	Alfalfas, sweet clovers	Nitrogen fixation
Microorganisms	Trade Name/ Company Name	Plants	Pathogen, Pest, Damage control
<i>Agrobacterium radiobacter</i> K84	Galltroll-A/ AgBioChem	Ornamental nursery stock	Crown gall infection
<i>Bacillus pumilus</i> QST 2808	Sonata/ BayerCropScience	Pome grape, tomato	Downy, powdery mildews, rusts
<i>Bacillus subtilis</i> QST 713	Serenade Soil/ BayerCropScience	Tomato, cucurbits, peanuts, potato, strawberry, citrus, onion	<i>Pythium</i> , <i>Rhizoctonia</i> , <i>Fusarium</i> , some strains of <i>Phytophthora</i>
<i>Bacillus subtilis</i> GB03	Companion/ Growth Products	Berries, citrus, cole crops, cucurbits, grapes, herbs and spices, vegetables, fruits, legumes	<i>Phytophthora</i> , <i>Pythium</i> , <i>Fusarium</i> , <i>Rhizoctonia</i> , <i>Sclerotinia</i> , <i>Xanthomonas campestris</i>
<i>Bacillus thuringiensis</i> aizawai	Agree WG/ Certis USA	Vegetables, ornamentals, fruits, corns, beans	Lepidopterous larvae
<i>Chromobacterium subtsugae</i>	Grandevol/ Marrone Bio Innovations	Vegetables, ornamentals, fruits, herbs, spices	Foliar-feeding pests, including caterpillars and coleopteran
<i>Coniothyrium minitans</i>	Contans WG/ BayerCropScience	Most vegetables, ornamentals, transplants, herbs	<i>Sclerotinia</i> spp.
<i>Streptomyces lydicus</i> WYEC 108	Actinovate/ Novozyme	Greenhouse vegetables and herbs	<i>Pythium</i> , <i>Rhizoctonia</i> , <i>Phytophthora</i> , <i>Verticillium</i> , <i>Fusarium</i>
<i>Streptomyces griseoviridis</i> K61	MycoStop/ Verdera	Container grown ornamentals, vegetables, trees, forest seedlings	<i>Fusarium</i> , <i>Alternaria</i> , <i>Phomopsis</i>
<i>Trichoderma harzianum</i> T-22, <i>Trichoderma virens</i> G-41	RootShield plus/ BioWorks	Most pot plants, cuttings, bulbs, woody propagation	<i>Pythium</i> , <i>Fusarium</i> , <i>Rhizoctonia</i> , <i>Thielaviopsis</i> , <i>Cylindrocladium</i>
<i>Trichoderma virens</i>	SoilGard/ Certis USA	Ornamentals, transplants, field crops	<i>Pythium</i> , <i>Rhizoctonia</i> , <i>Fusarium</i> , Root rots

Commercial PGPRs for Aromatic and Medicinal Plants

Most commercial microbial inoculants that are available in the United States are registered for ornamental or vegetable crops, but not for aromatic or medicinal plants (Table 1). While progress has been made in screening and mass production of beneficial living microbes, the cost of registration of these materials for application to medicinal and aromatic plants may remain a major obstacle for the development of new products (10, 97, 98). More studies on beneficial microorganisms and commercial product development for aromatic and medicinal plants can contribute to a sustainable supply of natural products.

Conclusion

PGPRs and AM fungi can improve plant growth by increasing the amount of P and N available to plants, and through the production of siderophores, phytohormones, and VOCs. Soil microorganisms can also increase secondary metabolites, improving the overall quality and value of aromatic and medicinal plants.

Reassessment of traditional holistic health care has created surging demands for natural products. For example, according to the United Nations Comtrade Statistics (99), the size of essential oil fragrance and flavor global market was estimated to be U.S. \$26 billion in 2013, with an average growth rate of 8.1% in the past five years. The United States is the largest importer (U.S. \$3,020 million) and the third largest exporter of essential oils (U.S. \$2,284 million) in the world. Applying chemical fertilizers can enhance the yield of medicinal plants, but an estimated 60 to 90% of the applied fertilizers are lost in the surrounding environment, contaminating water systems (100). The use of PGPRs and mycorrhizal fungi that function as environmental friendly biofertilizers, can enhance the growth and secondary metabolite production in medicinal and aromatic plants.

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Chapter 7

Potential Herbicides for Weed Control in Clary Sage (*Salvia sclarea*)

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Clary sage is a major essential oil crop in Eastern Europe and has been grown in the United States. A limiting factor in Clary sage production is weed control. Two-year field experiments were conducted to evaluate herbicides for weed control in Clary sage. The treatments of linuron and linuron plus quizalofop resulted in 15 and 9% increase in yield of Clary sage as compared to the control. Yields of fresh inflorescences from the metribuzin and from the metribuzin + primisulfuron treatments were 27-28 % lower than yields from the unweeded control. Essential oil content was highest in the control with a single removal of weeds, and in the linuron + quizalofop treatments, but lowest in the metribuzin treatment. Compared to the untreated control, the essential oil yields increased by 35% in the single removal of weeds, by 56% in the linuron treatment, by 37 % in the linuron + quizalofop treatment, and by 12 % in the quizalofop treatment. The application of metribuzin reduced essential oil yields by 40%, while the application of metribuzin + primisulfuron reduced oil yields by 35% relative to the unweeded control. The application of metribuzin + primisulfuron reduced linalool content in the

oil, and the application of metribuzin reduced terpinen-4-ol compared to the control with single removal of weeds. The other treatments did not alter the essential oil composition. This study demonstrated some herbicides could provide efficient weed control in Clary sage plantations without negative effect on essential oil content or composition.

Introduction

Clary sage (*Salvia sclarea* L.) is grown for production of essential oil which is used as aromatic agent in perfumery, aromatherapy, cosmetics and pharmaceutical products (1, 2). Clary sage essential oil has been reported to have antimicrobial (3–6), as well as anti inflammatory and analgesic properties (5). Clary sage is a major essential oil crop in Bulgaria and in some other Central and Eastern European countries (1, 7), and is also being tested in India in addition to other countries (8). Clary sage has also been grown for essential oil production in the United States. Individual specialty crops growers indicated interest towards this cash crop in the US, although the plant is listed as a class A noxious weed in Washington state (9). Currently, Clary sage in Central and Eastern Europe and in Russia is grown as 2-4 year perennial crop for production of essential oil (obtained via steam distillation of fresh inflorescences) or concrete (obtained via solvent extraction of fresh inflorescences). Both Clary sage essential oil and concrete have a stable international market. A major limiting factor in Clary sage production is weed competition (1). While several herbicides have been tested and registered for weed control in Clary sage fields in other countries (10–17), there were no registered herbicides for weed control in Clary sage crop in Bulgaria, other European countries, or in North America. While there has been some essential oil production of Clary sage in North Carolina, the plant is listed as a class A noxious weed in Washington state (9).

The objective of this study was to evaluate several herbicides and their combinations for weed control in Clary sage, and to assess treatment effects on crop productivity and on the essential oil yields and quality.

Materials and Methods

Two-year field experiments were conducted at the Experimental Fields of the Agricultural University in Plovdiv, Bulgaria. The experimental design was a completely randomized block design, with a size of the experimental plots 30 m², in four replications. Buffer zones of 2 m were maintained around each of the plots. The experiments were conducted on a drained carbonate meadow soil with a pH of 7.1–7.2. The depth of humus horizon was 25–28 cm, the organic matter content was 4.2–4.4%, the clay content in the three horizons reached up to 50%. The maximum specific water retention capacity was 32% for the top 20 cm of soil layer, and 28% for the 0–60 cm soil layer.

Clary sage ‘Trakijka’, the most productive and widely grown cultivar of Clary sage in Bulgaria (18) was used for the field experiments. Certified seeds of cv “Trakijka” were provided by the Research Institute for Roses, Aromatic and Medicinal Plants in Kazanlak, Bulgaria.

The following treatments were tested: (1) control without weeding; (2) control with single mechanical removal of weeds; (3) linuron at 1 kg ai/ha; (4) quizalofop-methyl quizalofop at 150 g ai/ha; (5) metribuzin at 350 g ai/ha; (6) primisulfuron at 22.5 g ai/ha; (7) linuron 1 kg ai/ha plus quizalofop 150 g ai/ha; and (8) metribuzin 350 g ai/ha plus primisulfuron 22.5 g ai/ha. Linuron and metribuzin were applied as preemergence, while quizalofop and primisulfuron were applied as postemergence herbicides, when weeds reached 15–20 cm height. In the control with single weeding, the weeds were mechanically removed once, at the same time when postemergence herbicides were applied (20–25th of April each year).

Fertilizers (N-80 kg/ha; P₂O₅ - 150 kg/ha; K₂O - 150 kg/ha) were applied with the deep plowing in the fall of the previous year. Early in the spring (beginning of April) another split application of N (80 kg/ha) was applied to plants from all treatments. The fertilizer rates were in accordance with the recommendation for Clary sage fertilization in the region (1) and the amount of available P and K in soil prior to seeding. The soil was plowed in late summer (end of August) at 25–28 cm depth, with subsequent disking and harrowing in the preceding year before initiation of the experiment. Clary sage was seeded on March 5th at interrow space of 60 cm, at 3 cm depth, and seeding rate of 7.5 kg/ha (1). Clary sage is usually grown as non-irrigated crop. Due to its deep tap root system, and anatomical features such as trichomes on the leaves, Clary sage withstands summer droughts very well on most soils.

Plants were harvested at the end of flowering and beginning of seed formation, when the essential oil content and quality are the highest (1, 19). Plants were harvested with small plot research combine by cutting the inflorescences, at the height of the top pair of leaves. The essential oil was extracted from fresh inflorescences via steam distillation in semi-industrial apparatus with capacity of 10 kg of fresh material, following the standard distillation protocol for this species. Essential oil content in fresh inflorescences was determined by hydrodistillation of 50 g samples in four replicates from each plot in Clevenger type apparatuses, as described previously (20).

The essential oil samples from all plots were analyzed on a PYE Unicam, 204 series gas chromatograph (GC) fitted with a 20 m X 0.25 mm capillary column with hydrogen as carrier gas as described previously (21). The injection sample was 1 µL. The column oven temperature was programmed as follows: 10 min at 50 °C, 2 °C/min increase to 60 °C, 10 min at 60 °C, at 10 °C/min increase to 190 °C, and 2 min at 190 °C. All data analysis was performed using Proc GLM or GLM procedure in SAS 8.0 (22). Mean separations were conducted at P < 0.05, using the least squares means (LSMEANS).

Results

The first count of weeds in April showed that the major weeds were dicotyledonous, both annual and perennials, and contributed to over 95% of the weed infestation in Clary sage (Table 1). During the first weed count, there were significant differences in weed infestation among various treatments (Table 2). The lowest infestation of dicotyledonous weeds was found in the linuron + quizalofop, and in the metribuzin + primisulfuron treatments. The highest infestation of dicotyledonous weeds was found in the control without weeding, and in the quizalofop treatment. These results are logical because quizalofop controls only monocotyledons plants and have no effect on dicotyledonous weeds.

The single removal of weeds treatment (control with weeding) increased the yields of fresh inflorescences by 15% relative to the control without weeding (Table 3). The application of metribuzin or metribuzin + primisulfuron reduced fresh inflorescence yields by 27-28% relative to the control without weeding. The application of the other herbicides and herbicide combinations increased fresh yields of inflorescences by 13-33% relative to the control without weeding. In comparison with the weeded control, higher yields were achieved in the linuron, and in the linuron + quizalofop treatments. The application of linuron alone or in combination with quizalofop increased fresh inflorescence yields by 15 and 9%, respectively, relative to the weeded control. The application of quizalofop or quizalofop + primisulfuron did not alter fresh yields relative to the weeded control, while the application of metribuzin either alone or in combination with primisulfuron reduced fresh yields by 37% relative to the weeded control.

The treatments of the single removal of weeds and the application of linuron alone increased essential oil content in fresh inflorescences by 17-18% relative to the control without weeding (Table 4). Essential oil content in fresh inflorescences from the linuron + quizalofop treatment was also increased by 10% relative to the control without weeding. Essential oil content was reduced by the application of metribuzin, and by the combination of metribuzin and primisulfuron.

Single mechanical removal of weeds increased essential oil yields by 35% relative to the control without weeding. Also, application of linuron alone or in combination with quizalofop lead to 37-56% increase of essential oil yields relative to the control without weeding. Application of quizalofop also increased oil yields by 12 % relative to the control without weeding. Application of primisulfuron did not increase oil yields, while metribuzin application either alone or in combination with primisulfuron reduced oil yields by 35-40% relative to the control without weeding.

Metribuzin + primisulfuron reduced linalool content in the oil, and metribuzin reduced terpinen-4-ol relative to the control with single removal of weeds (Table 5). Although chemical constituents varied somewhat, the oil profile remained within typical oil composition for Clary sage (18, 23).

Table 1. Effect of Treatments on Weed Density in Clary Sage Crop (Pooled Data for the Two Years), First Weed Counting

Weed type	Treatments							
	C-1	C-2	Lin	Quiz	Met	Prim	Lin + Quiz	Met + Prim
-----Number of weeds/m ² -----								
Annuals								
- monocots	5a ¹	1b	6a	5a	6a	3ab	3ab	4ab
- dicots	57ab	68a	58ab	72a	65a	60ab	64a	50b
Perennials								
- monocots	0	1	0	0	2	0	0	0
- dicots	44bc	60a	60a	35c	36c	52ab	38c	48b

¹ Means with the same letter within row (a weed group in annuals or perennials) are not significantly different at $P \leq 0.05$ using Duncan's grouping. C-1 = control without weeding C-2 = control with single removal of the weeds Lin = Linuron at 1 kg/ha Quiz = Quizalofop at 150 g/ha Met = Metribuzin at 350 g/ha Prim = Primesulfuron at 22.5 g/ha Lin + Quiz = Linuron 1 kg/ha + Quizalofop 150 kg/ha Met + Prim = Metribuzin 350 g/ha + Primesulfuron 22.5 g/ha

Table 2. Effect of Treatments on Weed Density in Clary Sage Crop (Pooled Data for the Two Years), Second Weed Counting

Weed type	Treatments							
	C-1	C-2	Lin	Quiz	Met	Prim	Lin + Quiz	Met + Prim
-----Number of weeds/m ² -----								
Annuals								
- monocots	6a ¹	0b	0b	0b	1b	0b	0b	0b
- dicots	141a	21c	18c	77b	26c	20c	8d	9d
Perennials								
- monocots	0	0	0	0	2	0	0	0
- dicots	35a	3b	9b	38a	2b	7b	2b	4b

¹ Means with the same letter within row (a weed group in annuals or perennials) are not significantly different at $P \leq 0.05$ using Duncan's grouping. C-1 = control without weeding C-2 = control with single removal of the weeds Lin = Linuron at 1 kg/ha Quiz = Quizalofop at 150 g/ha Met = Metribuzin at 350 g/ha Prim = Primsulfuron at 22.5 g/ha Lin + Quiz = Linuron 1 kg/ha + Quizalofop 150 kg/ha Met + Prim = Metribuzin 350 g/ha + Primsulfuron 22.5 g/ha

Table 3. Yields of Fresh Inflorescence of Clary Sage As Affected by Various Treatments

Treatments	1995	1996	Mean	% relative to	
	t/ha	t/ha		C-1	C-2
Control without weeding (C-1)	5.21b	7.5b	6.35	100	87
Control with single removal of the weeds (C-2)	6.12ab	8.56a	7.34	115	100
Linuron at 1 kg/ha,	7.94a	8.94a	8.44	133	115
Quizalofop at 150 g/ha,	6.03b	8.32a	7.18	113	98
Metribuzin at 350 g/ha,	3.26c	5.85c	4.55	72	62
Primingulfuron at 22.5 g/ha,	5.81b	7.95ab	6.91	109	94
Linuron 1 kg/ha + Quizalofop 150 kg/ha,	6.93ab	9.07a	7.99	126	109
Metribuzin 350 g/ha + Primisulfuron 22.5 g/ha	3.18c	6.08c	4.63	73	63

Means with the same letter within a year (column) are not significantly different at $P \leq 0.05$ using Duncan grouping.

Table 4. Essential Oil Content in Fresh Inflorescences (%) and Essential Oil Yield (kg/ha) of Clary Sage As Affected by Various Treatments

Treatments	Year 1	Year 2	Mean	% to C-1	Year 1	Year 2	Mean	% to C-1		
	Ess. Oil content				Essential oil yield					
	%	%	kg/ha		kg/ha	kg/ha	kg/ha			
C-1)	0.191ab	0.195ab	0.193	100	9.94	14.63	12.29	100		
(C-2)	0.233a	0.220a	0.226	117	14.25	18.84	16.54	135		
Linuron at 1 kg/ha,	0.230a	0.225a	0.227	118	18.26	20.12	19.20	156		
Quizalofop at 150 g/ha,	0.186ab	0.195ab	0.191	99	11.22	16.21	13.72	112		
Metribuzin at 350 g/ha,	0.148b	0.170b	0.159	82	4.82	9.95	7.39	60		
Primisulfuron at 22.5 g/ha,	0.165ab	0.185	0.175	91	9.69	14.71	12.20	99		
Linuron 1 kg/ha + Quizalofop 150 kg/ha,	0.218a	0.205a	0.212	110	15.1	18.59	16.85	137		
Metribuzin 350 g/ha + Primisulfuron 22.5 g/ha	0.165b	0.175ab	0.170	88	5.24	10.65	7.95	65		

Means with the same letter within a year (column) are not significantly different at $P \leq 0.05$ using Duncan grouping.

= Control with single removal of the weeds

C-1 = Control without weeding; C-2

Table 5. Variation of Major Constituents of the Essential Oil of Clary Sage As Affected by Various Treatments

<i>Treatments</i>	<i>Variation in major constituents, % of the total oil</i>		
	<i>Linalool</i>	<i>Linalyl acetate</i>	<i>Terpinen-4-ol</i>
Control without weeding (C-1)	19.4a	72.2a	1.62ab
Control with single removal of the weeds (C-2)	19.05a	73.1a	1.89a
Linuron at 1 kg/ha,	19.36a	73.7a	1.39ab
Quizalofop at 150 g/ha,	19.03a	71.8a	1.43ab
Metribuzin at 350 g/ha,	19.51a	73.1a	1.28b
Primisulfuron at 22.5 g/ha,	19.10a	71.5a	1.60ab
Linuron 1 kg/ha + Quizalofop 150 kg/ha,	18.75ab	73.2a	1.41ab
Metribuzin 350 g/ha + Primisulfuron 22.5 g/ha	17.21b	72.6a	1.73ab

Means with the same letter within a compound (column) are not significantly different at $P \leq 0.05$ using Duncan grouping.

Discussion

Our results suggest that linuron is one of the most efficient herbicides for weed control in Clary sage, supporting previous reports from other countries (10, 17, 24). Mitchell and Abernethy (14) also found that linuron applied as PRE at 0.5-1.5 kg ai/ha provided good weed control in Clary sage. If applied postemergence, linuron resulted in leaf damage on Clary sage (14). Other authors, however, have reported satisfactory weed control and no damage on Clary sage when linuron was applied post emergence (13).

Results from this study suggest that metribuzin is not suitable for weed control in Clary sage production, primarily because of reduction in fresh inflorescence yields, essential oil content, and essential oil yields. The results from this study agree with the report of Mitchell and Abernethy (14). The latter authors tested metribuzin as postemergence, but the herbicide provided unsatisfactory weed control, since metribuzin failed to control germinating broadleaf dock (*Rumex obtusifolius*). Our results also suggest that linuron, quizalofop, metribuzin, primisulfuron and the combination of linuron + quizalofop do not alter significantly essential oil composition of Clary sage. These results are consistent with the report of Nagy et al. (15), who found that some preemergence herbicides (chlorbromuron, thiobencarb, prometrin, phenobenzuron plus aminotriazole) had no negative effects on Clary sage essential oil composition. The combination of metribuzin + primisulfuron in our study reduced linalool content in the oil, and metribuzin reduced terpinen-4-ol relative to the control with single removal of weeds. Still, the overall composition remained within the typical range of essential oil quality of Clary sage (18, 23). Hence, several tested herbicides and herbicide combinations would not affect marketability of Clary sage essential oil, which is important from industry and market perspective. Although in this study we used Bulgarian cultivar of Clary sage, the results are consistent with findings from other countries on weed control in the same species. Therefore, the results from this study will be relevant to weed control in Clary sage grown in the United States or other countries around the world with similar environmental conditions.

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Chapter 8

Screening of Preemergence and Postemergence Herbicides for Weed Control in Dill (*Anethum graveolens*), Fennel (*Foeniculum vulgare*), Coriander (*Coriandrum sativum*), and Basil (*Ocimum basilicum*)

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One of the limiting factors in aromatic crop production in Canada and in the United States is the lack of registered herbicides for weed control. Greenhouse experiments were conducted to screen 30 postemergent and five preemergent herbicides for weed control in the aromatic crops dill, coriander, basil, and fennel. Our results suggested that the following herbicides could be used for weed control in the four crops: pyridate, linuron, trifluralin and pendimethalin in dill, sethoxadim, isoxaben, linuron, trifluralin and pendimethalin in coriander, sethoxadim, fluzifop-p-butyl and propyzamide in basil, and ethofumesate, fluzifop-p-butyl, sethoxdim, linuron, trifluralin and pendimethalin in fennel.

Introduction

Weed competition is a limiting factor in the production of aromatic crops; weeds can affect plant yields and essential oil content (1–4). Furthermore, weed competition can reduce the quality of the essential oil (5). The slow emergence rates of many aromatic crops such as dill (*Anethum graveolens* L.), fennel (*Foeniculum vulgare* Mill.), coriander (*Coriandrum sativum* L.), and basil (*Ocimum basilicum* L.), allow for weed species to become established before the crop canopy is fully developed, resulting in crop yield suppression.

Dill, fennel, coriander, and basil have been grown throughout the world as aromatic crops for many years (6, 7), and there is a recent increase in production of these crops in Canada and in the United States (8–10). However, registered herbicides for weed control in these crops are limited. Currently, in Canada there are no herbicides registered for use in dill, fennel, or coriander and there is only one herbicide approved for use in basil, that being napropamide (11). The increasing interest of North American growers towards these essential oil crops prompted some recent plant nutrition and essential oil studies in Atlantic Canada and in Southeastern US (8–10, 12–15). Therefore, there is a need for screening of herbicides and either minor or major registration of herbicides for weed control in dill, fennel, coriander, and basil in Canada and in the United States.

Several herbicides have been used in other countries for weed control in these specific aromatic plant species and similar crops. Recent study (16) evaluated the effect of pendimethalin and oxadiargyl, alone or in combination and at different rates on yield, quality and economics in dill seed production system in India. In Bulgaria for example, linuron is commonly used to control weeds in fennel, dill and coriander fields (4) and in Germany ethofumesate is used in fennel and caraway production (17). Other herbicides used for weed control in fennel production include pendimethalin in Italy and Switzerland and trifluralin in Italy and Spain. A recent study by Yousefi and Rahimi (18) evaluated the application of trifluralin and pendimethalin at reduced rates in fennel production in Iran and in India (19). the application of pendimethalin in a field experiment was studied. Furthermore, trifluralin, oxyfluorfen, pendimethalin and fluazifop-p-butyl have been applied to Japanese mint, a member of the Lamiaceae family, similar to basil, with good weed control and no reduction in yield, oil productivity or oil quality (20) and terbacil is used in Bulgaria, Hungary, Italy, Germany, Spain, and former Yugoslavia for mint (17). A study (21) assessed the preemergent application of pendimethalin on the growth and seed yield of coriander, and another one (22) studied the use of pendimethalin and oxadiardyl on weed management in coriander production in India. The effect of the herbicides ronstar and gallant and planting density of dill was studied in Iran (23).

The North American regulations require registration of a herbicide for use in specific crops. Chemicals for minor crops must go through a registration process, including chemical screening for crop toxicity (24). The objectives of this study were: to screen 30 postemergent and five preemergent herbicides for weed control in dill, coriander, basil, and fennel.

Materials and Methods

Two sets of completely randomized design experiments with four replicates were carried out in the greenhouse facilities at the Nova Scotia Agricultural College, in Truro, Nova Scotia, Canada. There was one set for postemergent herbicides and one for preemergent herbicides.

Experiment One: Postemergent Herbicides

There were four postemergent experiments, one for each of the plant species (dill, fennel, coriander, and basil). Each experiment had 31 treatments (type of herbicide, with one level being a control of no herbicide application). Each treatment was replicated four times.

Transplant trays filled with potting mix Promix®BX (Premier Tech Horticulture, Quebec, Canada), were planted with seeds from basil (cv. 'Broadleaf Italian'), coriander (cv. 'Jantar'), fennel (cv. 'Sweet Fennel'), and dill (cv. 'Dukat'). After emergence it was ensured that there were four seedlings per treatment. The growing conditions in the greenhouse were as follows: a day-time temperature of 20°C and a night-time temperature of 15°C with 14 hours of ambient light.

Herbicides tested for weed control on basil, coriander, dill, and fennel and the corresponding rate of application are described in Table 1. The herbicides investigated in this study were applied once when seedlings reached 10-12 cm in height and had their first true leaves. The herbicides were applied with a hand held sprayer with XR TeeJet® XR 8002 nozzles from a height of 46 cm and under a pressure of 32 psi using carbon dioxide. The herbicides were mixed with distilled water and the sprayer was cleaned with ammonia and double water rinses between each treatment applications. For each of the herbicides tested in this study one rate was used (Table 1), based on herbicide recommendations for basil, fennel, dill, and coriander or similar crops in other countries. The rates of the herbicides were based on the recommended rate (in kg or L/ha) for open field as specified in the label by the manufacturers. Surfactants were added to some of the herbicides as recommended by the manufacturer. Water was the control treatment and was applied with a spray tank in the same manner used for the other treatments. Visual plant phytotoxicity was rated as a percentage of total foliar damage, including both chlorosis and necrosis at 7, 14, 21, and 28 days after treatment (DAT). Ratings were made on a scale of 0 to 100% where 0 indicates no injury and 100 indicates complete kill of the treated seedlings.

Table 1. Formulation and Rate of Postemergence Herbicides Tested on Basil, Coriander, Dill, and Fennel

<i>Chemical Name</i>	<i>Amount of Active Ingredient, g/L</i>	<i>Application Rate (kg/ha)</i>
Terbacil	800	1.250 kg/ha
Cyanazine	480	2.500 kg/ha
Metribuzin	750	1.500 kg/ha
Linuron	500	4.500 kg/ha
Ethametsulfuron-methyl	750	0.020 kg/ha 0.200 % vol/vol
Propyzamide	500	3.250 kg/ha
Pyridate		2.000 kg/ha
Primsulfuron	750	0.030 kg/ha 0.200 % vol/vol
Chlorimuron-ethyl	250	0.036 kg/ha 0.200% vol/vol
Nicosulfuron	750	0.033 kg/ha 0.200 % vol/vol
Nicosulfuron/	187	0.033 kg/ha
Rimsulfuron	187	0.200 % vol/vol
Thifensulfuron methyl	750	0.008 kg/ha 0.200 vol/vol
Triflusulfuron Methyl	500	0.175 kg/ha 0.200 vol/vol
Triasulfuron	750	0.040 kg/ha 0.200 vol/vol
Rimsulfuron	250	0.060 kg/ha 0.200 % vol/vol
Thifensulfuron-methyl/ tribenuron methyl	500 or 250	0.020 kg/ha 0.200 % vol/vol
Isoxaflute	750	0.140 kg/ha
Quinclorac	750	0.165 kg/ha
Isoxaben	750	1.000 kg/ha
Sulfosulfuron	750	0.0275 kg/ha 0.500 L/ha
Isoxaflute	240	0.520 L/ha
Dithiopyr	480	2.250 L/ha
Quinclorac	125	2.000 L/ha

Continued on next page.

Table 1. (Continued). Formulation and Rate of Postemergence Herbicides Tested on Basil, Coriander, Dill, and Fennel

<i>Chemical Name</i>	<i>Amount of Active Ingredient, g/L</i>	<i>Application Rate (kg/ha)</i>
Isoxaben	450	1.100 L/ha 0.500 L/ha
Sulfosulfuron	240	2.500 L/ha
Pendimethalin	374	4.200 L/ha
Ethofumesate	190	4.660 L/ha
Mesotrione	400	0.210 L/ha
Dithiopyr	127	4.500 L/ha
Fomesafen	240	1.000 L/ha 0.250 % vol/vol

Experiment Two: Preemergence Herbicides

Three completely randomized design experiments (one each for dill, fennel, and coriander) were conducted, with one factor, the preemergent herbicides with six treatments (type of herbicide) in four replications. For control no herbicide was added to the water in the spray tank.

Eight-inch pots were filled with field soil and perlite, to provide aeration, in a 1:3 ratio (v/v). Each pot contained 2 kg of the soil/perlite mixture. The soil was classified as spodosols from the great group of haplorthods. Also, the soil was classified as Canning soil at Province level Soil Series designation (for Nova Scotia, Canada). Soil pH was 6.8, with no lime addition, with an organic matter of 2.8%, and contained P₂O₅ at 838 kg/ha and K₂O at 233 kg/ha. The soil originated from the Plumdale field at the Nova Scotia Agricultural College, classified as Truro soil series and loamy sand texture (25). Herbicides were applied using recommended rates for the same crops and herbicides currently used in other countries (Table 2). Metribuzin and trifluralin were applied to the container and incorporated into the soil prior to planting of the crops. The other three herbicides (linuron, terbacil, and pendimethalin) and the control were applied after seeding using the same sprayer and conditions as described for experiment one. Between treatments the sprayer was cleaned with ammonia and a double water rinse. Ten seeds were planted in each pot and water was applied to the soil. Greenhouse temperatures were 25°C during the day and 18°C during the nights. For the duration of the experiment there were 14 hours ambient light conditions each day.

Seedling emergence data was collected on two dates. The number of emerged seedlings in each pot was recorded at 14 DAT and 32 DAT. Plant phytotoxicity was rated based on visual observations at 39 DAT. As with the other experiment, ratings in this experiment were made on a scale of 0 to 100% where 0 indicates no injury and 100 indicates complete kill of the treated seedlings.

Statistics

An analysis of variance (general linear models procedure) was performed to analyze the data from all sets of experiments. Tukey's test, with a 95 % level of confidence was used to separate treatment means (26).

Table 2. Preemergent Herbicides Tested on Coriander, Dill, and Fennel

Chemical	Application Rate	
	Active Ingredient (kg/ha)	Product
Linuron	2.26	4.52 kg/ha
Metribuzin	1.12	1.50 kg/ha
Trifluralin	1.55	2.40 L/ha 480g/L
Terbacil	1.40	1.75 kg/ha
Pendimethalin	1.68	4.20 L/ha

Results and Discussion

Postemergent Herbicides: Fennel

Several of the herbicides tested showed potential for use in fennel production. Ethofumisate, fluzifop-p-butyl, propyzamide, and sethoxadim caused no phytotoxic symptoms (Table 3). In comparison, a number of the herbicides tested (metribuzin, fomesafen, isoflurole, acifluorfen, thifensulfuron-methyl, triasulfuron, nicosulfuron-rimsulfuron, rimsulfuron, cyanazine, primisulfuron, chlorimuron-ethyl, terbacil, nicosulfuron, mesotrione, dithiopyr, thifen-triben-methyl, sulfosulfuron and pyridate) had a negative effect on the growth of fennel, with nearly complete tissue damage.

Linuron, which is used in fennel production in various countries (17, 27) showed potential as a postemergent herbicide. There was slight plant damage, 10 % at 28 DAT. Of the herbicides tested for chemical weed control in fennel production and based on the ratings for phytotoxic effects, ethofumisate, fluzifop-p-butyl, propyzamide and sethoxadim show potential for use in fennel production.

Postemergent Herbicides: Dill

Sethoxadim, fluazifop-p-butyl and ethofumisate did not affect the growth of dill plants. Sethoxydim has previously been reported as a potential chemical control of weeds in dill, with no phytotoxic damage (28). Bentazon, propyzamide, linuron, and dithiopyr inflicted only minor damage four weeks after their application (Table 3). Similar to the finding of Precheur and Garrabrant (28), there was initial damage to dill leaves from the bentazon application, however, plant growth was not inhibited and the total damage at 28 DAT was minimal.

In contrast to other umbelliferous plants, such as carrots, dill may be negatively affected by the application of linuron. A possible cause for this difference is that in carrots the chemical is translocated in the roots, while in dill the linuron remains in the foliage of dill (29). The other herbicides were rejected for use in dill production based on the significant damage to the plant tissue by 28 DAT.

Unlike the results reported in (27), results from this study suggest that metribuzin at 1.5 kg/ha would not be acceptable for use in dill. The difference in results may be attributed to higher rate of the herbicide used in this study, compared to 600 g/ha as indicated in (27). Therefore, there may be a potential to use metribuzin in dill production, however, the rate would have to be lower. Bentazon was toxic to dill in this study, in contrast to (27), however, the rate tested here was higher than the rate previously found to be non-toxic (27). Linuron as a post emergent application, initially (7-21 DAT) caused limited damage (10-15 %), however, by 28 DAT 75 % damage to the plants was observed. The contrast to the findings of (27) can be attributed to the higher application rate of linuron in this study.

For postemergence application isoxaben and sethoxadim show potential for use in dill production, as these herbicides did not cause phytotoxic effects. Other herbicides with potential use in dill production include dithiopyr, bentazon, and propyzamide, which caused slight damage of 10%, and ethofumisate, pendimethalin and fluazifop-p-butyl with damage of 5%, as determined by the visual ratings.

Postemergence Herbicides: Basil

Basil was not affected by the application of sethoxadim, propysamide or fluazifop-p-butyl at 28 DAT (Table 4). Bentazon, triflusulfuron-methyl, thifensulfuron-methyl and ethametsulfuron affected the growth of basil plants only slightly (10%). In contrast, metribuzin, linuron and cyanazine caused complete necrosis on the basil tissue. The level of tissue damage caused by the remaining herbicides was significant enough to conclude they would be unsuitable for postemergent application on basil. The chemical herbicides that show promise for use in basil production are sethoxadim, propysamide or fluazifop-p-butyl as there was no damage to the plant tissue, nor a reduction to their growth as a result of their application.

Postemergent Herbicides: Coriander

Phytotoxicity shown by the coriander plants treated with sethoxadim or isoxaben were no different from the control plants at 28 DAT as the low level of initial damage was outgrown (Table 4). Ethofumisate, pendimethalin and fluazifop-p-butyl caused slight damage (5%) by 28 DAT as did propyzamide, bentazon and dithiopyr (10%) (Table 4). The remaining herbicides tested were highly toxic to the coriander plants (Table 4) and would not be suitable for coriander production systems.

Table 3. Phytotoxic Effects of Postemergence Herbicides on Fennel and Dill Seedlings

Herbicide	Phytotoxicity (%)							
	Fennel				Dill			
	7 DAT	14 DAT	21 DAT	28 DAT	7 DAT	14 DAT	21 DAT	28 DAT
Metribuzin	15 d ^l	100 a	100 a	100 a	70 a	100 a	100 a	100 a
Bentazon	10 e	15 m	5 l	10 k	70 a	90 b	100 a	10 l
Fomesafen	70 b	95 c	95 b	95 b	70 a	40 f	45 g	85 c
Ethofumisate	15 d	5 o	5 l	0 m	30 b	20 j	60 f	40 h
Pendimethalin	5 f	15 m	10 k	20 j	30 b	35 g	15 j	15 k
Isoxaflute	20 c	40 k	40 I	80 d	30 b	80 c	100 a	100 a
Oxyfluorfen	80 a	55 I	40 I	30 h	30 b	25 I	20 I	15 k
Fluzifop-p-butyl	5 f	5 o	5 l	0 m	20 c	10 l	75 d	50 g
Acifluorfen	20 c	70 f	75 f	60 e	20 c	45 e	40 h	50 g
Thifensulfuron-methyl	15 d	90 d	95 b	100 a	15 d	25 I	60 f	75 e
Triasulfuron	15 d	98 b	90 c	100 a	15 d	15 k	20 h	85 c
Nicosulfuron-rimsulfuron	10 e	80 e	80 e	100 a	15 d	40 f	90 b	85 c
Rimsulfuron	10 e	80 e	85 d	100 a	15 d	30 h	40 h	90 b
Cyanizine	10 e	90 d	100 a	100 a	10 e	80 c	100 a	100 a
Propyzamide	5 f	10 n	0 m	5 l	10 e	10 l	10 k	10 l
Primsulfuron	10 e	70 f	70 g	100 a	10 e	40 f	65 e	80 d

Herbicide	Phytotoxicity (%)							
	<i>Fennel</i>				<i>Dill</i>			
	7 DAT	14 DAT	21 DAT	28 DAT	7 DAT	14 DAT	21 DAT	28 DAT
Chlorimuron-ethyl	20 c	70 f	80 e	100 a	10 e	15 k	20 I	20 j
Terbacil	10 e	80 e	100 a	100 a	10 e	20 j	100 a	100 a
Ethametsulfuron	10 e	45 j	20 j	50 f	10 e	10 l	10 k	40 h
Linuron	5 f	0 p	10 k	10 k	10 e	15 k	10 k	75 e
Nicosulfuron	5 f	65 g	75 f	100 a	10 e	10 l	10 k	25 I
Quinclorac	5 f	5 o	20 j	60 e	10 e	15 k	20 I	80 d
Mesotrione	10 e	30 l	60 h	85 c	5 f	30 h	80 c	100 a
Triflusulfuron-methyl	10 e	10 n	10 k	40 g	5 f	35 g	40 h	60 f
Ioxabenz	5 f	0 p	10 k	25 I	5 f	10 l	0 l	20 j
Sethoxydim	5 f	5 o	0 m	0 m	5 f	0 n	0 l	40 h
Dithiopyr	5 f	5 o	0 m	10 k	5 f	10 l	0 l	80 d
Thifensulfonyl-methyl	10 e	30 l	70 g	100 a	5 f	10 l	0 l	60 f
Sulfosulfuron	15 d	60 h	80 e	100 a	5 f	50 d	90 b	100 a
Pyridate	5 f	15 m	10 k	80 d	0 g	5 m	10 k	0 m
Control	0 g	0 p	0 m	0 m	0 g	0 n	0 l	0 m

^a Means with the same letter in the same column are not significantly different at p>0.05.

Table 4. Phytotoxic Effects of Postemergence Herbicides on Basil and Coriander

Herbicide	Phytotoxicity(%)							
	Basil				Coriander			
	7 DAT	14 DAT	21 DAT	28 DAT	7 DAT	14 DAT	21 DAT	28 DAT
Metribuzin	70 b ^l	100 a	100 a	100 a	50 c	100 a	100 a	100 a
Linuron	70 b	95 b	100 a	100 a	5 g	5 k	5 m	70 e
Cyanizine	20 f	95 b	100 a	100 a	0 h	90 c	100 a	100 a
Oxyfluoren	90 a	90 c	90 b	95 b	80 a	60 e	55 f	30 I
Dithiopyr	0 I	0 m	40 h	95 b	0 h	0 l	0 n	10 k
Isoxaflute	20 f	30 g	85 c	95 b	15 e	20 h	30 I	35 h
Fomesafen	70 b	40 e	80 d	90 c	70 b	95 b	95 b	100 a
Nicosulfuron-rimsulfuron	5 h	75 d	80 d	90 c	10 f	60 e	70 d	85 c
Pyridate	20 e	75 d	80 d	85 d	20 d	90 c	100 a	100 a
Triasulfuron	10 g	20 I	45 g	80 e	5 g	20 h	60 e	90 b
Quinclorac	10 g	15 j	30 I	80 e	15 e	60 e	40 h	80 d
Chlorimuron-ethyl	10 g	30 g	30 I	80 e	5 g	20 h	30 i	85 c
Mesotrione	10 g	20 I	70 e	80 e	10 f	30 f	60 e	65 f
Rimsulfuron	10 g	25 h	50 f	80 e	5 g	15 I	40 h	35 h
Ethofumisate	40 c	40 e	40 h	70 f	15 e	5 k	5 m	5 l
Terbacil	40 c	35 f	85 c	60 g	10 f	90 c	100 a	100 a

Herbicide	Phytotoxicity(%)							
	<i>Basil</i>				<i>Coriander</i>			
	7 DAT	14 DAT	21 DAT	28 DAT	7 DAT	14 DAT	21 DAT	28 DAT
Pendimethalin	5 h	5 l	15 k	40 h	5 g	10 j	10 l	5 l
Primisulfuron	5 h	10 k	20 j	40 h	15 e	20 h	40 h	90 b
Acifuorfen	30 d	35 f	20 j	30 I	50 c	70 d	80 c	60 g
Nicosulfuron	5 h	5 l	15 k	30 I	5 g	20 h	10 l	30 I
Thifen-triben-methyl	5 h	15 j	15 k	30 I	5 g	10 j	50 g	85 c
Isoxaben	5 h	5 l	10 l	25 j	5 g	0 l	15 k	0 m
Sulfosulfuron	5 h	10 k	10 l	10 k	5 g	10 j	30 I	60 g
Bentazon	30 d	20 I	10 l	10 k	15 e	20 h	10 l	10 k
Triflusulfuron-methyl	5 h	15 j	10 l	10 k	5 g	5 k	20 j	25 j
Thifensulfuron-methyl	5 h	5 l	0 n	10 k	5 g	5 k	20 j	60 g
Ethametsulfuron	5 h	0 m	5 m	10 k	5 g	25 g	20 j	35 h
Sethoxadim	5 h	0 m	0 n	0 l	5 g	0 l	0 n	0 m
Propyzamide	0 I	0 m	10 l	0 l	0 h	0 l	0 n	10 k
Fluazifop-p-butyl	5 h	0 m	0 n	0 l	0 h	5 k	5 n	5 l
Control	0 I	0 m	0 n	0 l	0 h	0 l	0 n	0 m

[†] Means with the same letter in the same column are not significantly different at p>0.05.

The degree of damage caused by linuron in this experiment was in contrast to other studies. Smith and Cromack (30) recommended the application of linuron at 1.25 kg/ha. The application rate of linuron used in this study was 2.25 kg/ha, and negatively affected the growth of coriander. However, the findings from this study for isoxaben and propyzamide, were similar to the findings of Smith and Cromack (30) who reported no damage to coriander from the use of isoxaben and propyzamide. Similar to the results reported in (27) sethoxadim and fluazifop-p-butyl have potential for use as postemergent herbicides in coriander.

Ioxaben and sethoxadim show potential for postemergence application, as these herbicides did not cause phytotoxic effects. Other potential herbicides include dithiopyr, bentazon, and propyzamide, which caused phytotoxic damage of 10% as determined by the visual ratings, and also ethofumisate, pendimethalin and fluzifop-p-butyl with a phytotoxic damage of 5%.

Preemergence Herbicides: Dill

Compared to the control treatment, trifluralin and pendimethalin showed no significant differences in either the emergence of dill or phytotoxic symptoms (39 DAT) (Table 5). Previously, trifluralin has been reported as a safe herbicide for application to dill (27, 28). Precheur and Garrabrant (28) reported no difference from the control for trifluralin plus dithiopyr and trifluralin plus sethoxydim. In this study, the preemergent application of terbacil and metribuzin was highly toxic to dill plants, with no emergence of seedlings (Table 5). Linuron reduced the emergence of dill seedlings (14 DAT and 32 DAT). However, by 39 DAT the damage was equal to that in the control. Linuron had an initial reduction of emergence of dill seedlings, however, the crop damage at 21 days was no greater than the control treatment. Linuron has been reported to cause damage to dill crops in Canada, as a postemergent application (31). However, Zheljazkov (27) stated that linuron can be used in dill. Perhaps linuron could be applied at reduced rates, however, more research is needed to estimate optimal rates for dill. Pendimethalin and trifluralin were found to cause no negative effect on emergence or growth of dill plants. Therefore, these two herbicides show potential for use as herbicides in dill. Trifluralin had previously been found to control weeds without affecting the dill crop (31). . In another study (16) the use of pendimethalin in dill seed production was evaluated and was found that the highest net returns were when pendimethalin was applied preemergence at 0.5 kg/ha followed by postemergence application of oxadiardyl at 75 g/ha 20 days after sowing.

Preemergence Herbicides: Fennel

The phytotoxic injury to fennel from linuron, trifluralin, terbacil and pendimethalin applications was not different from the control treatment (Table 6). The results obtained from this study from the applications of trifluralin and linuron are in agreement with Zheljazkov (27). The application of metribuzin was toxic to fennel, with no emerged seedlings; and total plant damage was recorded

at 100 %. The damage caused by terbacil at 39 DAT was 75 %. The preemergence application of linuron, trifluralin, and pendimethalin did not inhibit germination and did not cause phytotoxic damage to fennel. Pendimethalin was reported to provide better weed control than trifluralin in a field study with fennel (18) and the application at 75% of the label recommended rate produced consistently high yields and reduced the weed biomass.

Table 5. Effects of Preemergence Herbicides on Dill Seedling Emergence and Phytotoxicity

<i>Herbicide</i>	<i>Seedlings Emerged (no.) 14 DAT</i>	<i>Seedlings Emerged (no.) 32 DAT</i>	<i>Phytotoxicity (%) 39 DAT</i>
Metribuzin	0 b ¹	0 b	100 a
Terbacil	0 b	0 b	100 a
Linuron	1 b	1 b	35 b
Trifluralin	6 a	8 a	29 b
Pendimethalin	7 a	10 a	3 b
Control	6 a	10 a	0 b

¹ Means with the same letter are not significantly different at P>0.05.

Table 6. Effects of Preemergence Herbicides on Fennel Seedling Emergence and Phytotoxicity

<i>Herbicide</i>	<i>Seedlings Emerged (no.) 14 DAT</i>	<i>Seedlings Emerged (no.) 32 DAT</i>	<i>Phytotoxic injury to plants % 39 DAT</i>
Metribuzin	0 c ¹	0 b	100 a
Terbacil	0 bc	2 b	75 ab
Linuron	4 ab	4 ab	4 b
Trifluralin	2 abc	3 ab	60 b
Pendimethalin	5 a	8 a	5 b
Control	2 abc	6 a	0 b

¹ Means with the same letter are not significantly different at P>0.05.

Preemergence Herbicides: Coriander

Several of the tested preemergent herbicides linuron, trifluralin, and pendimethalin did not affect the coriander emergence or caused any visual tissue damage (Table 7). The preemergent application of metribuzin and terbacil completely inhibited emergence of coriander seedlings. Our results are in agreement with (2) who reported that pendimethalin did not reduce seed or essential oil yield or lowered the quality of coriander essential oil. Other conducted studies (21, 22) also support the above statement. The authors reported that maximum net return in coriander production was obtained with pendimethalin applied at preemergence at 1 kg/ha. The application of metribuzin to coriander cannot be recommended, as there was 100% plant damage, which is in agreement with Zheljazkov and Zhalnov (4) who reported reductions in coriander yields from a mixture of metribuzin and pendimethalin. In contrast, pendimethalin applied alone did not affect the yield of coriander (4, 30). Results from the present study indicated that both trifluralin and linuron could be used as preemergence herbicides for weed control in coriander, confirming the report of (4, 27, 30, 32).

Table 7. Effects of Preemergence Herbicides on Coriander Seedling Emergence and Phytotoxicity

Herbicide	Seedlings Emerged (no) 14 DAT	Seedlings Emerged (no) 32 DAT	Phytotoxic injury to plants, % 39 DAT
Metribuzin	0 b ¹	0 b	100 a
Terbacil	0 b	0 b	100 a
Linuron	8 a	10 a	0 b
Trifluralin	10 a	10 a	5 b
Pendimethalin	10 a	10 a	2.5 b
Control	8 a	10 a	0 b

¹ Means with the same letter are not significantly different at P>0.05.

Conclusions

Of the numerous herbicides tested on basil, coriander, dill, and fennel in this study, several for each crop show potential for use in dill, coriander fennel or basil production in North America. In summary, for fennel, sethoxydim, fluzifop-p-butyl, ethofumesate, bentazon, and dithiopyr show potential for postemergence application while linuron, trifluralin and pendimethalin could be used as a preemergence weed control application with no phytotoxic damage. For weed control in dill crops, pyridate and bentazon applied postemergence and linuron, trifluralin and pendimethalin applied preemergence, could be used.

Promising weed control herbicides for basil are sethoxadim, propyzamide and fluazifop-p-butyl. For weed control in coriander postemergence application of isoxaben and sethoxadim and preemergence application of pendimethalin, linuron and trifluralin could be used.

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Chapter 9

Identification and Characterization of Biopesticides from *Acorus tatarinowii* and *A. calamus*

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Acorus species are rich in secondary compounds and possess high contents of essential oils in their rhizomes. We report on the isolation, characterization and antifungal and pesticidal activity of essential oil and eleven compounds obtained from *A. tatarinowii* Schott. and *A. calamus* L. Five of the compounds had weak antifungal activity against the plant pathogens *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides* where 50% growth inhibition occurred at < 300 µM. The microdilution broth assay showed that these compounds are

not sufficiently active to be potential agricultural fungicides. The essential oils from *A. tatarinowii* and *A. calamus* were more active as mosquito larvacides than any of the isolated compounds with 100% mortality at 31 ppm. Isocalamusenone was more active at a dose of 0.78 µg/mosquito as an adult insecticide than the essential oils or other purified compounds. *A. tatarinowii* essential oil was found to be a better mosquito repellent than oil from *A. calamus*. Several of the compounds were moderately phytotoxic to bentgrass (*Agrostis stolonifer*) and duckweed (*Lemna paucicostata*), but not to lettuce (*Lactuca sativa*). Results indicated that compounds or mixtures of compounds from *Acorus* species may yield new biopesticide leads.

Introduction

Increasing resistance to pesticides and the loss of existing pesticides because of economic and/or toxicology issues are factors that drive the need to search for new natural product-based pest management chemicals. The necessity for a larger number of insecticides to prevent transmission of vector-borne pathogens is also needed. Although there are a number of effective synthetic mosquito repellents, the public desires safe, natural product-based products. Since registration in the USA is generally less expensive and less complex for biochemical biopesticides than conventional pesticides, interest in natural compounds for pest management has increased. Natural product leads offer an approach to discover new chemical classes of pesticides with reduced toxicological and environmental impact. Bioprospecting for natural products allows for the discovery of new products for pest management.

Acorus tatarinowii Schott. and *A. calamus* L. are widely distributed in southern China, India, and Thailand as perennial wetland monocots of the Acoraceae. *Acorus* spp. are rich in chemical resources and possess high contents of essential oils in their rhizomes. Rhizomes of *A. tatarinowii* are produced mainly in Zhejiang and Jiangsu Provinces. *A. calamus* is distributed naturally in Liaoning, Hubei, Hunan, and Sichuan Provinces. After collection in the early spring, leaves are removed, rhizomes cleaned, dried in the sun, and used unprepared according to Chinese Pharmacopoeia (1, 2).

Acorus essential oils contain a variety of phenylpropanoids, terpenoids, fatty acids and phenolic compounds. Excellent pharmacological and chemical reviews of *A. calamus* are provided by Mukherjee *et al.* (3), Balakumbahan *et al.* (4), and Paithankar *et al.* (5). Because of the long history of *Acorus* spp. in traditional medicine most papers describe chemistry and biological activity with a focus on the pharmacological applications. Authors describing antifungal activity primarily contain information related to human fungal diseases, and only a few reports contain biological activity against plant pathogens that cause important crop diseases worldwide. Nawamaki and Kuroyanagi (6) reported on the anti-germination effects against lettuce seeds, and Deng *et al.* (7) studied in

vitro antifungal activity of *A. tatarinowii* extracts and some compounds against seven plant pathogenic fungi. Methanolic extracts of *A. gramineus* were tested by Lee against six plant diseases and were active against only two diseases (8). These methanolic extracts possessed strong fungicidal activity against *Phytophthora infestans* (Oomycetes) and *Rhizoctonia solani* (Basidiomycota) at 2000 mg/L, and no activity was seen against *Botrytis cinerea* at 1000 mg/L. Further study showed that asaronaldehyde produced 68% activity of the chlorothalonil control against *R. solani* disease on rice, 100% activity of the chlorothalonil control against *P. infestans* on tomato, and no activity against *B. cinerea* on cucumber. α -Asarone (Figure 1, 6) produced 85% inhibition of *P. infestans* at 1000 mg/L and 53% inhibition of *P. infestans* at 500 mg/L and was more than 20 times less active than the fungicide control but he stated that α -asarone may still be a useful lead compound for new types of fungicides.

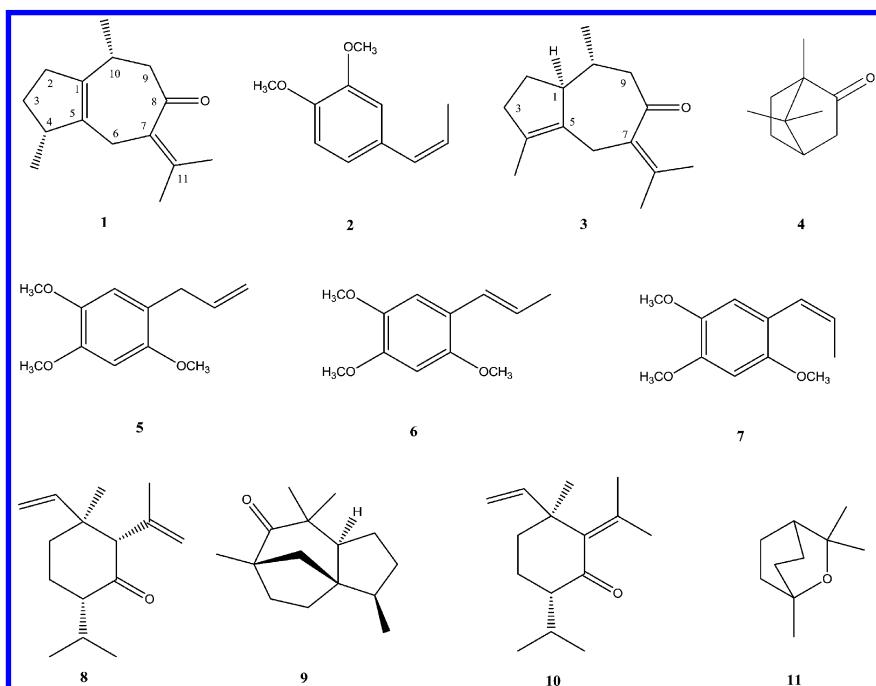


Figure 1. Structures of compound 1–11. Calamusenone (1), (Z)-Methylisoeugenol (2), (γ -Epi-Isocalamusenone (3), Camphor (4), γ -Asarone (5), α -Asarone (6), β -Asarone (7), Shyobunone (8), Eremophila ketone (9), Isoshyobunone (10), Eucalyptol (11).

A preliminary study by He *et al.* (9) demonstrated inhibitory effects of *A. tatarinowii* ethanol extracts against *Colletotrichum gloeosporioides* and *Fusarium oxysporum* f. sp. *Cubense*. Because of our ongoing work with *C. gloeosporioides* and *F. oxysporum*, these reports intrigued us. Therefore, the aim

of this research was to pursue a more in-depth study of the potential fungicidal and biopesticidal activity of *Acorus* spp. This is the first report of antifungal bioassay-guided fractionation of *Acorus* species using direct-bioautography and the subsequent evaluation of pure compounds in a 96-well microdilution broth assay with agriculturally important fungal plant pathogens. We also present data to evaluate the potential for new insecticides, insect repellents, and herbicides.

Materials and Methods

Plant Material and Distillation

Rhizomes of *Acorus tatarinowii* (Voucher #20110920) and *A. calamus* (Voucher #2011018) were obtained from Lianqiao, Traditional Chinese Medicine Materials Market in Hunan Province which is the regional center for cultivated plant material used in Chinese medicine, Hunan, China in 2011. Samples were identified by Dr. Tasi Liu at Hunan University of Chinese Medicine (HUCM), and voucher specimens were placed in the HUCM herbarium, Changsha, China. Rhizomes of *A. tatarinowii* (20 kg) and *A. calamus* (20 kg) were crushed separately, soaked in water for 2 h, and then the entire (20 kg) hydrodistilled with a Clevenger-type apparatus for 6 h, providing 100 mL of each essential oil. *A. tatarinowii* essential oil was used for antifungal bioassay-guided fractionation due to its relative abundance of the biologically active constituents in the Thin Layer Chromatography (TLC).

General Experimental

¹H and ¹³C NMR spectra were recorded in CDCl₃ on a Varian ANOVA 400 MHz spectrometer (Palo Alto, CA). All ¹³C multiplicities were deduced from 90° and 135° DEPT experiments. High-resolution mass spectra were obtained using an Agilent 1100 HPLC coupled to a JEOL AccuTOF (JMS-T100LC, Peabody, MA) as previously described (10). Column chromatography was performed using a Biotage, Inc. Horizon™ Pump (Charlottesville, VA) equipped with a Horizon™ Flash Collector and fixed wavelength (254 nm) detector. LogP values were calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2015 ACD/Labs) (10).

Chromatographic Analysis

HPLC method development was performed using an Agilent 1100 system equipped with a quaternary pump, autosampler, diode-array detector, and vacuum degasser. Semi-preparative HPLC purifications were performed using a Waters Delta-Prep system (Milford, MA) equipped with a diode-array detector and a binary pump. The essential oils, fractions and purified compounds were analyzed by GC-MS on a Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS. GC was equipped with a DB-5 column (30 m × 0.25 mm fused silica capillary column, film thickness of 0.25 μm) operated using the following conditions: injector temperature, 240 °C; column temperature, 60–240 °C at 3 °C /min and then held

at 240 °C for 5 min; carrier gas, He; and injection volume, 1 μ L (splitless). The MS ionization energy was set to 70 eV. TLC analysis for extracts of *Acorus* essential oil was performed with hexane and diethyl ether (8:2) as solvent system, visualized by spraying vanillin sulfuric acid reagent (500 mg vanillin, 200 mL 100% ethanol, 10 mL sulfuric acid) followed by heating. Uniplate™ silica gel GHLF plates (scored 10 × 20 cm, 250 microns) were used.

Antifungal Bioassay-Guided Fractionation

The oils, fractions, and pure compounds were evaluated for antifungal activity against strawberry anthracnose-causing plant pathogens, *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides* using the direct overlay bioautography.

A. tatarinowii Oil Fractionation

Initially, a portion (1.012 g) of the essential oil from the rhizomes of *A. tatarinowii* were separated on a Biotage XP-sil, 100 g SNAP cartridge (40-63 μ m, 60 Å, 40 × 150 mm) running at 40 mL/min using a hexane/diethyl ether step gradient system beginning with 98:2 to 50:50 over 2000 mL and finishing with 50:50 to 0:100 over 400 ml and a 200 mL methanol wash. The fractionation was repeated twice then combined into similar fractions. Twenty-two fractions were collected and recombined based on TLC similarities into nine distinct fractions labeled from A to H. Fraction B provided 211.8 mg of pure compound **1** and fraction F provided 57.3 mg of pure compound **2**. There were two compounds in fraction D as determined by GC-MS. Fraction D (60.3 mg) was further purified using preparative TLC (Silica gel 60 RP-18 F₂₅₄S, 10 × 20 cm) using methanol:H₂O (90:10), a dark zone was detected under UV ₂₅₄ which provided 33.6 mg of pure compound **3** after filtering, however, compound **4** had no UV absorption. Compound **4** was identified using GC-MS and Kovats Index methods (*II*). Fraction H (573.8 mg) was subjected to the HPLC system running a linear gradient with hexane:diethyl ether from 95:5 to 80:20 over 20 min while monitoring at 254 nm. This resulted in the isolation of compound **5** (61.4 mg). Fraction H also contained the other two main compounds, **6** and **7**, which were identified using GC-MS and Kovats Index methods (*II*). Fraction A (55.8 mg) was subjected to HPLC (Zorbax Rx-Sil, 9.4 × 250 mm, 5 μ m) running a linear gradient with hexane: ethyl acetate from 100:0 to 85:15 over 20 min while monitoring at 254 nm. This resulted in the isolation of compound **8** (8.9 mg) and 10.4 mg of pure compound **9** (10.4 mg).

A. calamus Oil Fractionation

A portion (1.0056 g) of essential oils from the rhizomes of *A. calamus* were separated on a Biotage XP-sil, 100 g SNAP cartridge (40-63 μ m, 60 Å, 40 × 150 mm) using the same conditions as described previously. Twenty-two fractions

were collected and recombined based on TLC similarities into nine distinct fractions labeled A to H. Fraction A (64.7 mg) was subjected to HPLC (Zorbax Rx-Sil, 9.4 × 250 mm, 5 µm) running a linear gradient with hexane:ethyl acetate from 100:0 to 85:15 over 20 min while monitoring at 254 nm. This resulted in the isolation of 4.8 mg of pure compound **8**, 5.2 mg of pure compound **9**, 12.4 mg of pure compound **10**, and 2.8 mg of pure compound **11**. Fraction B provided 289.6 mg of pure compound **1** and fraction F provided 40.7 mg of pure compound **2**. Fraction D (60.3 mg) was further purified using preparative TLC as described above. Fraction H was subjected to HPLC (Zorbax Rx-Sil, 9.4 × 250 mm, 5 µm) running a linear gradient from 95:5 (hexane: diethyl ether) to 80:20 (hexane:diethyl ether) over 20 min while monitoring at 254 nm. This resulted in the isolation of 70.6 mg of pure compound **5** and fraction H-1 yielded two main compounds, **6** and **7**. Fractions and pure compounds from both *Acorus* oils were compared by TLC and GC-MS, and we found that both essential oils have the compounds **1–9** with slightly difference in yields.

Calamusenone (1)

^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 138.8 (C-1), 34.2 (C-2), 30.9 (C-3), 44.9 (C-4), 137.7 (C-5), 27.2 (C-6), 134.5 (C-7), 205.2 (C-8), 48.8 (C-9), 33.2 (C-10), 140.7 (C-11), 22.6 (C-12), 23.0 (C-13), 19.8 (C-14), 19.7 (C-15). The spectroscopic data agree with the published values (*12*).

cis-Methylisoeugenol (2)

^1H NMR (400 MHz, CDCl_3) and ^{13}C NMR (100 MHz, CDCl_3) data agree with the published values (*13*).

Isocalamusenone (3)

^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 53.6 (C-1), 23.2 (C-2), 37.0 (C-3), 132.7 (C-4), 135.6 (C-5), 27.5 (C-6), 135.8 (C-7), 210.1 (C-8), 47.8 (C-9), 30.9 (C-10), 133.9 (C-11), 22.0 (C-12), 20.6 (C-13), 16.6 (C-14), 14.0 (C-15). The spectroscopic data agree with the published values (*12, 14*).

Camphor (4)

Compared TLC, GCMS, and NMR data with purchased standards. (purity ≥96%, SIGMA-ALORICH, CAS NO.:76-22-2). ^1H NMR (400 MHz, CDCl_3) and ^{13}C NMR (100 MHz, CDCl_3) data agree with the published values (*15*).

γ-Asarone (5)

¹H NMR (400 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃) data agree with the published values (16).

α-Asarone (6)

Compared TLC, GCMS, and NMR data with purchased standards (purity ≥98%, SIGMA-ALORICH, CAS NO.:2883-98-9). ¹H NMR (400 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃) data agree with the published values (17).

β-Asarone (7)

Compared TLC, GCMS, and NMR data with purchased standards (purity ≥95%, EXTRASYNTHÈSE, CAS NO.:5273-86-9). ¹H NMR (400 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃) data agree with the published values (18).

Shyobunone (8)

¹³C NMR (100 MHz, CDCl₃): δ (ppm) 209.9 (C-1), 66.7 (C-2), 46.1 (C-3), 39.5 (C-4), 24.8 (C-5), 56.3 (C-6), 140.0 (C-7), 116.7 (C-8), 21.2 (C-9), 146.5 (C-10), 111.0 (C-11), 24.4 (C-12), 26.2 (C-13), 18.8 (C-14), 18.6 (C-15). The spectroscopic data agree with the published values (19, 20).

ent-Prelacinan-7-one (9)

¹³C NMR (100 MHz, CDCl₃): δ (ppm) 53.3 (C-1), 38.8 (C-2), 31.1 (C-3), 21.3 (C-4), 59.1 (C-5), 45.9 (C-6), 220.1 (C-7), 52.9 (C-8), 35.3 (C-9), 22.0 (C-10), 46.8 (C-11), 14.3 (C-12), 21.7 (C-13), 29.2 (C-14), 24.6 (C-15). The spectroscopic data agree with the published values (21).

Isoshyobunone (10)

¹³C NMR (100 MHz, CDCl₃): δ (ppm) 210.1 (C-1), 140.2 (C-2), 44.7 (C-3), 39.7 (C-4), 21.6 (C-5), 55.2 (C-6), 142.0 (C-7), 23.6 (C-8), 24.4 (C-9), 146.5 (C-10), 110.9 (C-11), 24.5 (C-12), 29.6 (C-13), 20.9 (C-14), 18.4 (C-15). The spectroscopic data agree with the published values (22).

Eucalyptol (II)

Compared TLC and GCMS data with purchased standards (purity ≥99%, Sigma-Aldrich, CAS NO.:470-82-6).

Biological Assays

Bioautography Assay

Bioautography procedures were described in our previous studies (23–25).

Microdilution Antifungal Assay

A standardized 96-well microdilution broth assay developed by Wedge and Kahajek was used to evaluate antifungal activity of pure compounds from *Acorus* spp. that were identified as active by bioautography (26). This assay was used to evaluate antifungal activity of pure compounds towards *Botrytis cinerea* Pers.:Fr, *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz., *F. oxysporum*, *Phomopsis viticola* and *P. obscurans* in comparison with known fungicide standards (27–29). Commercial, technical grade azoxystrobin and captan (without formulation) were used as validation controls in all microdilution broth assays. Each fungus was treated with 75, 150, and 300 µM of each compound. Captan and azoxystrobin standards were run at 0.3, 3.0, and 30.0 µM. Microtiter plates (Nunc MicroWell, untreated; Roskilde, Denmark) were covered with a plastic lid and incubated in a growth chamber as described previously for fungal growth. Fungal growth was then evaluated by measuring absorbance of each well at 620 nm using a microplate photometer (Packard Spectra Count, Packard Instrument Co., Downers Grove, IL). Mean absorbance values with standard errors were used to evaluate fungal growth at 48 h and 72 h. Due to the slow germination and growth of *P.* and *P. viticola* measurements were made at 120 and 144 hrs. Means for percent inhibition/stimulation of each fungus at each dose of test compound relative to the untreated positive growth controls were used to evaluate fungal growth. The SAS, Proc ANOVA was used to identify significant factors (30), and Fisher's protected LSD was used to separate means (31). All experiments were repeated at least once in time.

Bioassays against Aedes aegypti

The Orlando strain of *Ae. aegypti* (established 1952) was reared in the insectary of the Mosquito and Fly Research Unit at Center for Medical, Agricultural, and Veterinary Entomology, USDA-ARS according to the procedures described in Pridgeon *et al.* (32). Briefly, eggs were hatched in a flask with de-ionized water, left overnight, and transferred to a plastic tray containing distilled water. A powdered diet (2:1 pot belly pig chow: brewer's yeast) was

added to each tray. Mosquitoes were reared in an environmental chamber at 28°C, 80% RH, and a photoperiod of 14 h:10 h (Light:Dark). Adults were held in a screened cage and provided 10% sucrose *ad libitum*. Female adults were used for all adult bioassays. For larval assays, eggs were hatched under vacuum and larvae were reared in containers as described above.

Bioassays for Herbicide Activity

The *Lactuca sativa* (lettuce) and *Agrostus stolonifera* (bentgrass) bioassays of Dayan *et al.* were used with minor alterations to evaluate phytotoxicity against dicots and monocots, respectively (33). The more quantitative method of Michel *et al.* with *Lemna pausicostata* (duckweed) was used with minor alterations to analyze the active compounds for herbicidal activity (34).

Results and Discussion

Essential Oil Components

Essential oils from the rhizomes of *A. tatarinowii* and *A. calamus* were obtained by hydrodistillation, subjected to biotage separation, and then characterized by gas chromatography, gas chromatography/mass spectrometry, and NMR. The rhizomes of *A. tatarinowii* and *A. calamus* visually appear very different and are thus easy to identify. Our TLC profiles indicated that the *A. tatarinowii* contained more of constituents **1** and **3** and similar to *A. calamus* content for the other visible constituents (**5**, **6** and **7**) (Figure 2). However, the TLC and GC-MS chromatograms were quite similar and the presence of multiple asarone stereoisomers make the isolation and purification in these species complicated (Figures 1 and 3).

Fungicide Assays

Direct bioautography was used to initially study fungicide activity of the essential oils from the rhizomes of *A. tatarinowii* and *A. calamus* against the three *Colletotrichum* species. *A. calamus* essential oil produced the largest zone of growth inhibition. One dimensional TLC bioassay-guided fraction of *A. tatarinowii* demonstrated four antifungal fractions (B, D, F, and H) with different Rf values (data not shown). Further chemical separation resulted in the purification of the five weakly active antifungal compounds. The main compounds in *A. tatarinowii* were evaluated individually at 2 µl and 8 µl for antifungal activity against *C. acutatum*, *C. fragariae*, and *C. gloeosporioides* (Table 1). Subsequent testing of pure compounds indicated that all compounds were less active than four commercial fungicide standards. Methylisoeugenol appeared to be more active against *C. acutatum*. β-Asarone (**7**) appeared slightly more antifungal than α-asarone (**6**), and both compounds seemed to be more

active against *C. fragariae* than the other two *Colletotrichum* species. These results were sufficiently positive for us to study these compounds in greater detail using the microdilution broth assay.

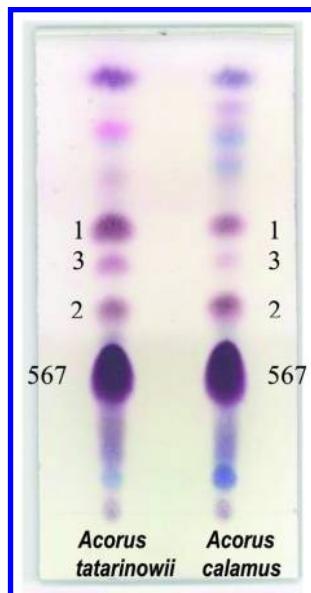


Figure 2. Thin layer chromatography for extracts of Acorus essential oils used hexane with diethyl ether 8:2 solvent system with 5% vanillin in sulfuric acid and ethanol as the developing agent. Calamusenone (1), (Z)-Methylisoeugenol (2), (-)-Epi-Isocalamusenone (3), γ -Asarone (5), α -Asarone (6), β -Asarone (7).

The microdilution broth assay indicated that the antifungal activity of *A. tatarinowii* compounds was strongest against *B. cinerea*, *F. oxysporum*, and *P. obscurans*. *Botrytis cinerea* appeared to be the most sensitive fungal pathogen to the chemistry from *Acorus* species, followed by *F. oxysporum* and then *P. obscurans*. There was no significant activity against the three *Colletotrichum* species and *P. viticola*. All compounds showed classical dose-dependent activity at 75, 150, and 300 μ M with slight differences of activity across the three species (Figures 4-6). α -Asarone (6) at 300 μ M appeared to be the most antifungal compound, causing 57.7% growth inhibition of *B. cinerea*, 43.6 % inhibition of *F. oxysporum*, and 41.5% inhibition of *P. obscurans*. Isocalamusenone (3) appeared to be the second most active compound and caused 54.3% growth inhibition of *B. cinerea*, 35.5 % inhibition of *F. oxysporum*, and 30.8% inhibition of *P. obscurans*. Calamusenone (1) appeared to be the least active antifungal compound.

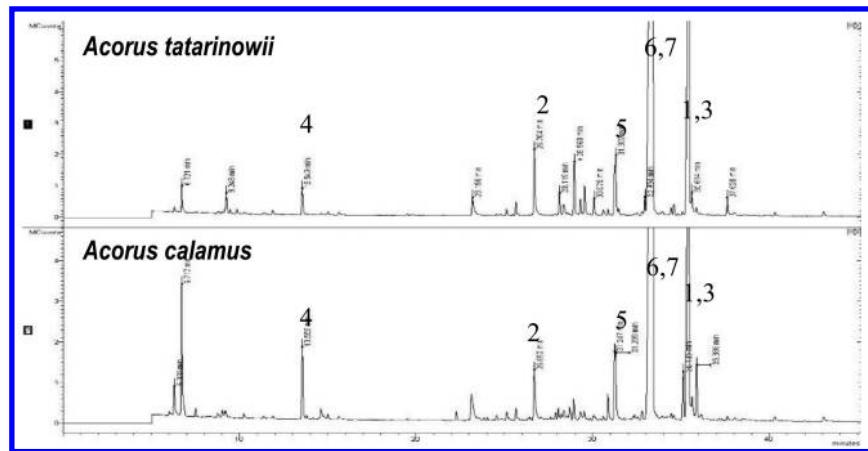


Figure 3. TIC of gas chromatograph for the essential oils from *A. tatarinowii* and *A. calamus*. Calamusenone (**1**), (*Z*)-Methylisoeugenol (**2**), (-)-Epi-Isocalamusenone (**3**), Camphor (**4**), γ -Asarone(**5**), α -Asarone (**6**), β -Asarone (**7**).

By species and decreasing order of activity at 300 μM , α -asarone (**6**) caused 57.7% growth inhibition of *B. cinerea*, isocalamusenone (**3**) caused 54.3% inhibition, *cis*-methylisoeugenol (**2**) caused 45.2% inhibition, β -asarone (**7**) caused 33.7% inhibition, and calamusenone (**1**) caused 31.9% inhibition (Figure 4). In *F. oxysporum* and decreasing order of activity, α -asarone (**6**) caused 43.6% growth inhibition, isocalamusenone (**3**) caused 35.5% inhibition, β -asarone (**7**) caused 33.1% inhibition, *cis*-methylisoeugenol (**2**) caused 24.9 % inhibition, and calamusenone (**1**) caused 23.3% inhibition (Figure 5). In *P. obscurans*, α -asarone (**6**) caused 41.5% growth inhibition, β -asarone (**7**) caused 38.0% inhibition, isocalamusenone (**3**) caused 30.8% inhibition, calamusenone (**1**) caused 29.0% inhibition, and *cis*-methylisoeugenol (**2**) caused 22.3% inhibition. The growth inflection point where *Phomopsis* growth inhibition appears to decrease from 29.6% to 22.3% between 150 μM and 300 μM is probably due to slight precipitation in the well at the higher concentration (Figure 6). The structure activity results indicated that the α -asarone (**6**) was more antifungal than the β -asarone (**7**). Unfortunately, we obtained insufficient amounts of γ -asarone (**5**) to conduct the antifungal studies.

Table 1. Average Fungal Growth Inhibitory Zones (mm) for Pure and Semi-Pure Compounds from *Acorus* Species Tested against *Colletotrichum gloeosporioides* (Cg162), *C. fragariae* (Cf63), and *C. acutatum* (Ca Goff) Using Direct Bioautography Repeated Once

Sample Name	Zone size (mm)					
	Cg162		Cf63		CaGoff	
	2 μ L	8 μ L	2 μ L	8 μ L	2 μ L	8 μ L
<i>A. tatarinowii</i> ^a	6	5	5	8	5	8
<i>A. calamus</i> ^a	8	9	4	7	4	4
Calamusenone (1)	3	5	4	5	5	8
Isocalamusenone (3)	-	3	2	4	2	4
Methylisoeugenol (2)	3	6	3	6	7	14
β -Asarone (7)	6	7	4	16	3	5
α -Asarone (6)	2	5	6	9	2	4
Benomyl ^b	21	-	18	-	14*	-
Captan ^b	25	-	21	-	15	-
Cyprodinil ^b	39	-	24	-	19	-
Azoxystrobin ^b	33	-	32	-	17*	-

* Indicates diffuse growth inhibition zone ^a Essential oils were tested at 40 mg/mL in a 4 μ L solution ^b Technical grade agrochemical fungicides without formulation were used as internal standards at 2 mM in 2 μ L.

Saxena *et al.* demonstrated that the fungitoxicity of major constituents from essential oil from *A. calamus* to *Helminthosporium oryzae* were due to β -asarone, asaraldehyde, and acoradin (three asarone derivatives) (35). *A. calamus* oil at 500 ppm and containing 40% β -asarone (**7**) caused 96 % inhibition of *H. oryzae*. Details of the bioassay, more detailed information on constituents, and concentrations used in these studies were not available but it was proposed that β -asarone (**7**) was primarily responsible for the activity. Our results indicated that β -asarone (**7**) was least active of the compounds tested against *B. cinerea* (test concentrations converted to ppm were *ca.* 22.5, 45, and 90 ppm).

Using the amended agar technique, He *et al.* demonstrated that the ethanol extract of *A. tatarinowii* at 20 mg/mL was effective at inhibiting growth of *F. oxysporum* f. sp. *cubense* (from banana) by approximately 92% and two strains of *C. gloeosporioides* by approximately 93%. No further chemical isolation or testing was conducted on the extracts. More recently, Deng *et al.* used bioassay-guided fractionation to isolate 1,2-dimethoxy-4(2-propenyl) benzene and studied the effects of *A. tatarinowii* extract (unknown solvent extract) on hyphal growth and spore germination of several fungi and showed the highest activity against *F. oxysporum* f. sp. *niveum* (from watermelon) (**7**).

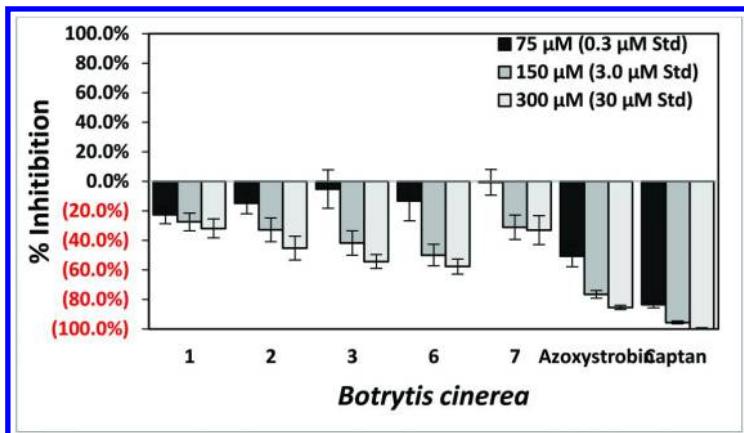


Figure 4. Fungal growth inhibition of *Botrytis cinerea* using a 96 well microdilution broth assay at 48 hrs. Commercial fungicides azoxystrobin and captan were used as standards and the test compounds were calamusenone (**1**), methylisoeugenol (**2**), isocalamusenone (**3**), α -asarone (**6**), and β -asarone (**7**).

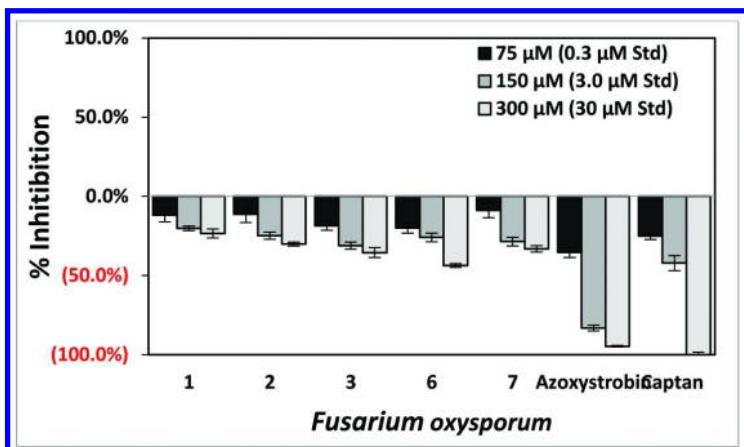


Figure 5. Fungal growth inhibition of *Fusarium oxysporum* using a 96 well microdilution broth assay at 48 hrs. Commercial fungicides azoxystrobin and captan were used as standards and the test compounds were calamusenone (**1**), methylisoeugenol (**2**), isocalamusenone (**3**), α -asarone (**6**), and β -asarone (**7**).

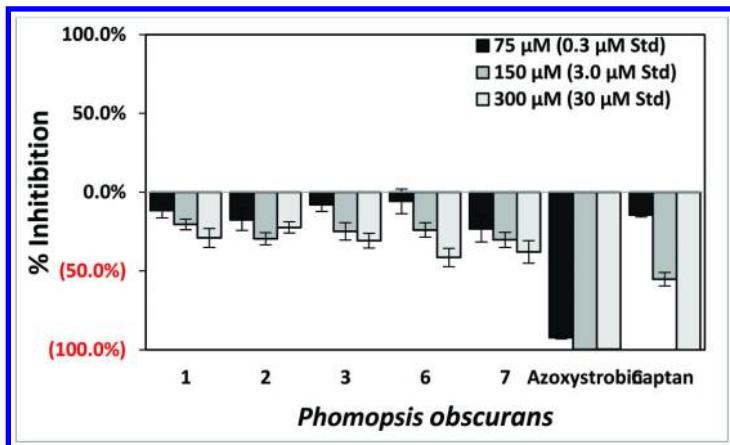


Figure 6. Fungal growth inhibition of *Phomopsis obscurans* using a 96 well microdilution broth assay at 120 hrs. Commercial fungicides azoxystrobin and captan were used as standards and the test compounds were calamusenone (**1**), methylisoeugenol (**2**), isocalamusenone (**3**), α -asarone (**6**), and β -asarone (**7**).

The active agent was attributed to 1,2-dimethoxy-4(2-propenyl) benzene and induced 98.8% inhibition of *Thielaviopsis paradoxa* spores and 100% inhibition of *F. oxysporum* f. sp. *niveum* spores at 0.4 mg/mL. Other *A. tatarinowii* constituents were not found to possess antifungal activity using their bioassay-guided isolation method, but they felt further biopesticide studies were warranted. However, we did not obtain this compound during our extraction from the *A. tatarinowii* essential oil. The microdilution broth assay used in our studies utilizes spores from each species and we therefore were able to detect both spore germination and mycelial growth inhibitors. We did not observe any significant spore germination inhibition in our studies.

Mosquito Assays

There was a distinct difference in activity of the nine compounds assayed, depending on the mosquito life stage tested (Tables 2 & 3). Essential oils from *A. tatarinowii* and *A. calamus* were the most active at the lowest concentrations tested against larval *Ae. aegypti* (Table 2) but these oils were the least active against adult *Ae. aegypti* (Table 3). The most active purified compounds for larval *Ae. aegypti* at the lowest concentration tested were β -asarone (**7**) and γ -asarone (**5**) (Table 2) but they were not highly active against the lower concentrations tested against adult *Ae. aegypti* (Table 3). Isocalamusenone (**3**) was the most active compound tested in female *Ae. aegypti* adult topical mortality assay.

Table 2. Mean % Mortality for Compounds Tested in First Instar *Ae. aegypti* Larval Assay

Compounds	250 ppm	125 ppm	62.5 ppm	31.2 ppm	15.6 ppm	logPa
<i>Acorus tatarinowii</i> (EO)	100±0.0	100±0.0	93.3±6.7	100±0.0	80.0±11.5	
<i>Acorus calamus</i> (EO)	100±0.0	100±0.0	100±0.0	100±0.0	66.7±17.6	
β-asarone (7)	100±0.0	90.0±10.0	80.0±16.3	20.0±12.6	0	3.5±0.33
γ-asarone (5)	50.0±22.6	50.0±22.6	50.0±22.6	20.0±12.6	0	2.60±0.34
methylisoeugenol (2)	100±0.0	100±0.0	73.3±13.3	0	0	3.05±0.24
α-asarone (6)	100±0.0	100±0.0	6.7±4.2	3.3±3.3	0	3.41±0.33
isocalamusenone (3)	56.7±20.3	53.3±21.1	3.3±3.3	0	0	4.38±0.33
camphor (4)	16.7±16.7	10.0±10.0	0	0	0	2.09±0.30
calamusenone (1)	3.3±3.3	0	3.3±3.3	0	0	4.60±0.41
Permethrin ^b	100	100	100	100	100	
acetone solvent control	0					

^a logP (calculated by Scifinder via ACD/Labs). ^b LC₅₀ of permethrin is 0.28 ppb.

Table 3. Mean % Mortality for Compounds Tested in Female *Ae. aegypti* Adult Topical Mortality Assay

Compounds	3.125 µg	1.563 µg	0.781 µg
isocalamusenone (3)	76.7±3.3	96.67±3.3	60.0±15.3
methylisoeugenol (2)	43.3±6.7	16.7±8.8	40.0±5.8
α-asarone (6)	56.7±18.6	46.7±3.3	26.7±8.8
β-asarone (7)	56.7±6.7	30.0±5.8	26.7±6.7
camphor (4)	33.3±8.8	26.7±3.3	26.7±3.3
calamusenone (1)	20.0±5.8	16.7±8.8	20.0±10
γ-asarone (5)	46.7±12.1	30.0±0.0	13.3±6.7
<i>Acorus calamus</i> (EO)	80.0±10.0	26.7±12.1	10.0±5.8
<i>Acorus tatarinowii</i> (EO)	53.3±6.7	6.7±6.7	6.7±3.3
Permethrin ^a	100	100	100
acetone	0		

^a LD₅₀ for permethrin is 0.15 ng/org.

The susceptibility of this *Ae. aegypti* strain to multiple insecticides has been characterized previously (32). There is a general trend for this set of compounds demonstrating an inverse range of activities depending on whether they were tested against larval or adult mosquitoes. The adult topical assay requires the compounds to cross the cuticular barrier to enter the mosquito while in the larval assay they can enter the mosquito either through the cuticle or be ingested and enter through the midgut. The difference in biological activity between adults and larvae may be due to differences in cuticle uptake and/or movement to the target site in the insect. The molecular masses and logP values of all of the compounds are within the ranges found for the overwhelming majority of insecticides (36). Likewise, the number of H-bond donors was less than 2 for all compounds and H-bond receptors were 1 to 3, all values within the ranges of Tice for these parameter for most all insecticides. LogP values of β-asarone (**7**) (3.5) and α-asarone (**6**) (3.41) are similar, but their larvicidal activity is quite different (Table 2). There was less difference in their activity on adults. There was no correlation between the activity and the logP of the three Asarone analogues. Thus, the differences in activity are more likely to involve steric differences between the molecules. It is interesting to note that often only mosquito larvae are used in assessing the biological activity of compounds. Based on these findings, it is important to assay both larvae and adults in order to identify overall biological activity and not eliminate potential candidate compounds. For example, an effective mosquito adulticide that has low toxicity for larvae (and possible other non-target aquatic organisms) would be highly desirable as negative environmental impacts would be greatly reduced.

Among the four compounds assayed for repellency, *A. tatarinowii* essential oil had the lowest minimum effective dosage (MED) aside from DEET (Table 4). Since both the α -asarone (**6**) and β -asarone (**7**) had a MED that was considerably higher than that for *A. tatarinowii*, this indicated that each of these constituents alone is less repellent than the essential oil. Thus, they may not be principally involved in producing the repellent effect observed for the essential oil or there exists synergism between these compounds or possibly with additional compounds within the oil. β -Asarone (**7**) was twice as active as a repellent as α -asarone (**6**), and it was more repellent than the *A. calamus* essential oil. The favorable repellent effect produced by the *A. tatarinowii* essential oil warrants testing of other minor component chemicals for the source of the potent repellency.

Table 4. Mean Minimum Effective Dosage for Repellent Compounds Estimated on Cloth with Three Human Volunteers against Female *Ae. aegypti* Mosquitoes

<i>Ae. aegypti</i> Minimum Effective Dosage (mg/cm ²)						
Compounds	M4	M5	M8	Average	Std. Dev.	High Dose (mg/cm ²)
DEET	0.006	0.011	0.011	0.009	0.003	1.5
<i>Acorus tatarinowii</i> (EO)	0.094	0.094	0.047	0.078	0.027	1.5
β -asarone (7)	0.187	0.375	0.750	0.437	0.287	1.5
<i>Acorus calamus</i> (EO)	0.047	1.500	0.187	0.578	0.802	1.5
α -asarone (6)	0.750	0.187	1.500	0.812	0.659	1.5

Herbicide Assays

Essential oils of both *Acorus* species were phytotoxic to bentgrass at 1 mg/ml, but much less so to lettuce (Table 5). Fractions B, D, F, and H from *A. tatarinowii* completely inhibited germination of bentgrass, but had little or no effect on lettuce, suggesting that they are selective for monocots. However, the only pure compounds from *Acorus* species that had moderate phytotoxicity were *cis*-methylisoeugenol (**2**) and β -asarone (**7**) which severely stunted growth of bentgrass at 1 mM (Table 6).

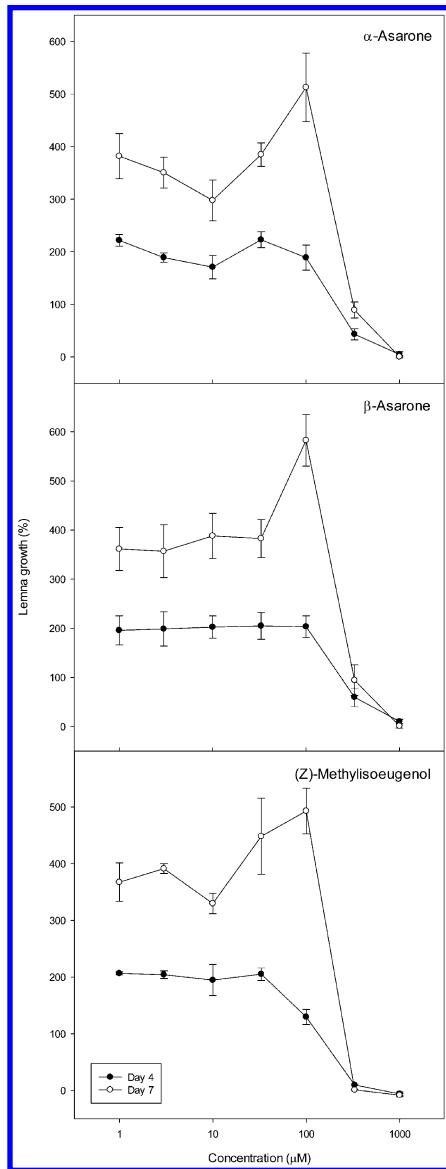
Table 5. Phytotoxicity of *A. tatarinowii* and *A. calamus* Essential Oils (EO) and Fractions

Sample	Conc.	Day	Activity*	
			Lettuce	Bentgrass
<i>Acorus tatarinowii</i> (EO)	1 mg/ml	7	2	4-5
<i>Acorus calamus</i> (EO)	1 mg/ml	7	2	4-5
Fraction A shyobunone (8), eremophila ketone (9)	1 mg/ml	6	0	3
Fraction B calamusenone (1)	1 mg/ml	6	0	5
Fraction D epi-Isocalamusenone (3), camphor (4)	1 mg/ml	6	0	5
Fraction F methylisoeugenol (2)	1 mg/ml	6	1	5
Fraction H γ -asarone (5) α -asarone (6), β -asarone (7)	1 mg/ml	6	1	5

* Ranking based on scale of 0 to 5. 0 = no effect. 5 = no germination. Acetone was used as a solvent control.

At this concentration, there was little effect on lettuce. For comparison, two positive control herbicides (glyphosate at 1 mM and atrazine at 0.333 mM) had phytotoxicity ratings of 2 and 1 on lettuce and bentgrass, respectively. *cis*-Methylisoeugenol (**2**), α -asarone (**6**), and β -asarone (**7**) were also moderately phytotoxic to duckweed, inhibiting growth almost entirely at 0.333 mM (Figure 7). All three compounds stimulated duckweed growth at 0.1 mM. Stimulation of growth by a subtoxic concentration of a toxicant (hormesis) is common with natural phytotoxins (37). The concentrations of the three compounds that cause 50% growth inhibition were between 0.1 and 0.333 mM. This is comparable to the activities of the commercial herbicide clomazone ($IC_{50} = 0.126$ mM) and is better than that of the commercial herbicides glyphosate ($IC_{50} = 0.388$ mM) and asulam ($IC_{50} = 0.407$ mM) in the same bioassay (34). The essential oil of *A. calamus* has been tested for phytotoxicity before and found to inhibit germination of both lettuce and *Lolium perrene* by 50% at concentrations of about 0.5 mg per mL (38), a result that is more encouraging than ours. Asarone was reported by Nawamaki and Kuroyanagi to inhibit germination of lettuce by 89% at 125 μ g/mL after 7 days (6). However, the authors found that another constituent of a methanol extract of *A. calamus*, 1-hydroxyepiacorone, to completely inhibit germination of lettuce seeds at this concentration. This compound was not isolated in our

study, because the isolation of the compounds was based on fungicide activity. *cis*-Methylisoeugenol (**2**) has been patented as a plant growth regulator effective during dormancy inhibitor (39), but we find no other mention of it as a plant growth inhibitor.



*Figure 7. Effects of three purified compounds from *Acorus* species on frond growth of *Lemna pausicostata*. The points at 1 μM are the solvent control values.*

Table 6. Phytotoxicity of Pure Compounds from *Acorus* spp. on Lettuce and Bentgrass Germination and Seedling Growth after 7 Days of Treatment

Compound	Conc.	Activity*	
		Lettuce	Bentgrass
Isocalamusenone (3)	0.1 mM	0	0
Isocalamusenone (3)	0.333 mM	1	1
Isocalamusenone (3)	1 mM	1	2
Methylisoeugenol (2)	0.1 mM	0	1
Methylisoeugenol (2)	0.333 mM	1	3
Methylisoeugenol (2)	1 mM	1	4
α -Asarone (6)	0.1 mM	0	0
α -Asarone (6)	0.333 mM	0	1
α -Asarone (6)	1 mM	1	3
β -Asarone (7)	0.1 mM	0	0
β -Asarone (7)	0.333 mM	0	1
β -Asarone (7)	1 mM	1	4

* Ranking based on scale of 0 to 5. 0 = no effect. 5 = no germination. Acetone was used as a solvent control.

Summary

Use of natural product-based agrochemicals provides an opportunity for better management of our natural resources by reducing dependence on synthetic and often more toxic chemicals. Natural products provide potential replacements to pesticides scheduled for market removal. However, for the fungicide market, commercial agrochemical companies will only pursue novel chemistry that is active in the μ molar range and have broad spectrum activity, and can be applied as g quantities per hectare such as the strobilurin class of chemistry (e.g., azoxystrobin 0.171 kg a.i./ha) (40). Chemistry that would need to be applied in kg quantities per hectare such as captan at 2.2 kg a.i./ha would probably not be developed or approved by governmental agencies. While the asarones possess low toxicity to potential host plants, we believe that they do not possess sufficient antifungal activity needed for commercial development by the agrochemical industry. The essential oil of *A. tatarinowii* was approximately nine times less potent repellent than the standard DEET. This result indicates that this natural oil exhibits a fair amount of repellency towards *Ae. aegypti* mosquitoes. Neither α -asarone (**6**) nor β -asarone (**7**), the principal constituents of the oil, exhibited a repellent effect that was as potent as the oil itself. The oil of *A. calamus* was 7-8 times less repellent than the oil of *A. tatarinowii*. Natural products as new insect repellents or biopesticides that have potential market in minor crops and fruits can be facilitated in their development by the IR4 program in the USA.

Acknowledgments

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Chapter 10

Chemical Profile and Bioactivity of Essential Oil Fractions as a Function of Distillation Time

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A number of medicinal and aromatic plants contain essential oils; some are grown on large areas as high-value crops for commercial production of essential oils. Plant-derived essential oils are natural products with diverse applications in various industries such as in food and beverages, perfumery and cosmetics, in pharmaceutical products, in aromatherapy, and also as eco-friendly pesticides. The aroma, composition, and the bioactivity of the essential oils within single plant species may vary significantly, due to genetic and environmental factors, but may also depends on postharvest management and extraction procedure. The end users demand consistency in supply and quality. The specific chemical profile and aroma of the essential oil forms the basis for its utilization and price on the international markets. For most of the commercially grown essential oil crops, the conditions for essential oil extractions that would guarantee consistency of quality but also economics have been researched and identified. Steam and hydrodistillation have been traditionally used as economical and simple methods for extraction of the essential oil from a

number of aromatic plants. Still there is no agreement in the literature regarding the optimal distillation time. Our research in the last five years have demonstrated that by varying the steam or hydro-distillation time, one could obtain oils with defined specific composition, opening the possibilities to produce unique essential oils from the same batch of biomass or seed. Furthermore, by manipulating the extraction time and conditions, one can obtain oils or oil fractions with differential bioactivity. The resulting products (essential oil fractions) could have diverse industrial, medical, or environmental applications. These findings could be used by industry to develop new products, to reduce energy inputs and oil losses, and speed up oil extraction. Our results clearly demonstrated that the conditions for oil extractions must be reported when the essential oil content and composition of a specific plant has been reported. This will facilitate comparisons of oil yield and composition. Herewith, we summarize the recent research on how steam or hydrodistillation time and conditions alters essential oil yield and composition from some of the most widely grown and utilized essential oil crops.

Introduction

There are a number of aromatic plants that contain essential oils, which have commercial applications in industries such as the food and beverages, perfumery and cosmetics, are also used in pharmaceutical products, in aromatherapy, and as eco-friendly pesticides. The essential oil profile and bioactivity may depend on various factors such as species, but also cultivar, and environmental conditions. The essential oils are complex products that may contain several major constituents and up to 200 minor constituents. The concentration of the major and minor oil constituents, as well as the specific ratio between the oil constituents is important and may be the basis for the commercial production of specific oil.

There is great variation of essential oil content and profile within a single aromatic species. For example, in a study with 38 genotypes of sweet basil (*Ocimum basilicum* L.), Zheljazkov et al. (1) found significant variation not only in essential oil and composition but also in phenotypic traits; the 38 accessions had various phenotypes, ranging in color from purple to green, with various shapes and sizes of inflorescences, leaves, and plant height. The oil content of the 38 sweet basil accessions varied from 0.07 to 1.92% in dry herbage. Based on the oil composition, the 38 sweet basil accessions were divided into seven groups: (1) high-linalool chemotype (19-73% (-)-linalool), (2) linalool-eugenol chemotype (six chemotypes with 28-66% (-)-linalool and 5-29% eugenol), (3) methyl chavicol chemotype (six accessions with 20-72% methyl chavicol and no (-)-linalool), (4) methyl chavicol-linalool chemotype (six accessions with 8-29% methyl chavicol and 8-53% (-)-linalool), (5) methyl eugenol-linalool chemotype (two accessions with 37 and 91% methyl eugenol and 60 and 15%

(-)-linalool), (6) methyl cinnamate-linalool chemotype (one accession with 9.7% methyl cinnamate and 31% (-)-linalool), and (7) bergamotene chemotype (one accession with bergamotene as major constituent, 5% eucalyptol, and less than 1% (-)-linalool) (1). The authors concluded that availability of various chemotypes offers the opportunity for production of basil to meet the market requirements of specific basil oils or individual compounds such as (-)-linalool, eugenol, methyl chavicol, methyl cinnamate, or methyl eugenol. Several of the sweet basil oil constituents quantified by Zheljazkov et al. (1) have been shown to have applications as medicinal ingredients, flavors, fragrance. For example, (-)-linalool, is used as a fragrance in domestic products such as soaps, detergents, shampoos, and lotions and serves as a valuable reactant in the synthesis of vitamin E and other important compounds (2). (-)-Camphor is used as a plasticizer for cellulose nitrate, as a moth repellent, as an antimicrobial substance, in embalming, and in fireworks. (-)-Camphor (together with menthol) is the active ingredient in vapor-steam products and is effective as a cough suppressant (3).

In another study with two basil species, sweet basil (*Ocimum basilicum* L. (cvs. German and Mesten) and *Ocimum sanctum* L. (syn. *O. tenuiflorum* L.) (cv. Local), Zheljazkov et al. (4) demonstrated that biomass and oil yields, oil content, and composition as well as bioactivity may depend on cultivar (genetic factor), but also on harvest stage. Zheljazkov et al. (4) reported that essential oils of both species grown in Mississippi showed *in vitro* activity against *Leishmania donovani* (IC_{50} 37.3 – 49.6 $\mu\text{g}/\text{ml}$). Furthermore, the authors found that minor basil oil constituents (+)-d-cadinene, 3-carene, -humulene, citral, and (-)-*trans*-caryophyllene had antileishmanial activity, while other constituents were ineffective (4).

The essential oil composition and bioactivity can be altered by steam or hydrodistillation conditions.

Antioxidant Capacity of the Essential Oils

Unless otherwise specified, the antioxidant capacity of the essential oils reviewed in these studies was measured in triplicate using the ORAC_{oil} (oxygen radical absorbance capacity in bulk oil) method (5, 6) with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as the standard and is expressed as micromol Trolox/g.

Testing for Antimicrobial Activity of the Essential Oils

Antimicrobial testing of the essential oils from the studies reviewed here (unless otherwise specified) was performed at the National Center for Natural Product Research (NCNPR), University, MS. Primary screening for antimicrobial activity of various essential oils was tested against *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Escherichia coli*, *Pseudomonas aerogenosa*, and *Mycobacterium intracellulare*, at a concentration of 50 $\mu\text{g}/\text{ml}$, and % inhibition was calculated as described previously (7).

Antileishmanial and Antimalarial Activity Testing

Unless it is otherwise specified, the antileishmanial activity of the oils in the reviewed studies was tested *in vitro* on a culture of *Leishmania donovani* promastigotes. In a 96-well microplate assay, the oil at concentration of 80 ug/mL was added to the *Leishmania* promastigotes culture (2×10^6 cells/mL). The plates were incubated at 26 °C for 72 h, and growth of *Leishmania* promastigotes was determined by Alamar Blue assay (8). Pentamidine and Amphotericin B were used as the standard antileishmanial agents. IC₅₀ (the concentrations causing 50% inhibition in growth) and IC₉₀ (the concentrations causing 90% inhibition in growth) were computed from the growth inhibition curve. Antimalarial activity was tested using a previously described procedure (7).

Lamiaceae

Plants from the Lamiaceae (Labiatae), also known as mint family, are found in the temperate regions worldwide (9). According to Almeida and Albuquerque (10), Lamiaceae family includes approximately 220 genera and around 3500-4000 species. Many of the species are aromatic plants which contain essential oil (EO) with diverse composition, fragrance, bioactivity, and commercial uses. Terpenoids and flavonoids of Lamiaceae family have been extensively studied although other compounds such as alkaloids, iridoids and ursolic acid are also produced by plants of this family. Lamiaceae family includes plants such as basil, mints, rosemary, garden sage, lavender, oregano, thyme, and others, that are commercially grown for production of essential oil and will be discussed later in the chapter in relation to changes in essential oil composition caused by varying the duration of the distillation time (DT).

*Japanese Cornmint (*Mentha canadensis* L. syn *M. arvensis* L.)*

Japanese cornmint is an industrial crop cultivated for production of essential oil that contains high concentrations of menthol and is the only commercially viable source for production of natural menthol (11, 12). The essential oil composition of Japanese cornmint is influenced by genetic and environmental conditions, but also by the distillation time (DT) (13). Zheljazkov and Astatkie (13) studied the effect of eight different steam DT (1.25, 2.5, 5, 10, 20, 40, 80, and 160 min) on essential oil profile extracted from dried herbage of Japanese cornmint. The authors found that distillation of the dried herbage beyond 20 min did not result in additional increase of oil yield (13). Zheljazkov and Astatkie (13) also found that dissimilar composition of essential oil can be obtained from same biomass of Japanese cornmint by adjusting DT. The major constituents of Japanese cornmint essential oil is menthol. In the study conducted by Zheljazkov and Astatkie (13), highest menthol concentration (79%) was obtained at 160 min DT and the lowest (74.3%) at 1.25 min DT. Similarly, other oil constituents of Japanese cornmint EO are also affected by the duration of distillation time during essential oil extraction. In the same study by Zheljazkov and Astatkie

(13), the concentrations of α -pinene, β -pinene, 3-octanal, limonene, eucalyptol, isopulegone, and isomenthone were high at the initial shorter DT (1.25–5 min) and decreased with increasing DT (160 min). Higher concentrations of sabinene were at 1.25 to 40 min DT, lower at 80 min, and the lowest at 160 min DT. The concentrations of menthone, myrcene and piperitone were highest at 1.25 min DT, 5 to 80 min DT, and 10–20 min DT.

Pandey et al. (14) hydro distilled Japanese cormmint aerial part for 6 hr and were able to get only 71 % of menthol in oil profile. Similarly, Shiwakoti et al. (12) had menthol concentration in the range of 67–73 % in a study on diurnal effects on Japanese cornmint. The longest DT tested by Zheljazkov and Astatkie (13) was 160 min and 74% was minimum percentage of menthol obtained in all DT.

*Peppermint (*Mentha piperita L.*)*

Peppermint is a very popular herb which is used extensively and in numerous forms (oil, leaf, leaf extract, and leaf water). All above ground parts are considered economic as they contain essential oil. The major constituents of peppermint essential oil are menthol, menthone, and menthofuran (15).

Cannon et al. (16) studied the effect of steam DT (1.25, 2.5, 5, 10, 20, 40, 80, and 160 min) on peppermint oil yield and composition. Most of the essential oil in peppermint was extracted for 20 min; further increase in DT had no effect in the yield. Cannon et al. (16) reported that menthofuran concentration in the oil was affected by DT, but the concentrations of menthol and menthone were not. Menthofuran concentration was lowest in the early distillates and higher in the 40 min DT or 160 min DT. Eucalyptol concentration decreased with every increase in DT whereas *t*-caryophyllene gradually increased with DT reaching highest at 160 min. Menthyl acetate concentration was not affected by DT (16).

*Rosemary (*Rosemarinus officinalis L.*)*

Rosemary is one of the important essential oil crops of Lamiaceae family that has been utilized by humankind for thousands of years (17). Aboveground parts of rosemary contain essential oils. The major constituents of rosemary essential oil are eucalyptol, camphor and α -pinene (15). Distillation time (DT) was also found to affect essential oil yield and composition of rosemary. In a study with DT (1.25, 2.5, 5, 10, 20, 40, 80, and 160 min and steam distillation) on rosemary, Zheljazkov et al. (18) reported that the majority of the oil was eluted in the first 20 min DT. The higher concentration of α -pinene, β -pinene, camphene, and eucalyptol were at between 1.25–2.5 min DT compared to oils obtained later. The concentrations of camphor and borneol were higher in the oils obtained at longer DT (80 or 160 min) than the oils obtained at shorter DT (1.25 or 5 min). The concentration of myrcene, verbenone and linalool were not affected by DT. DT did not affect antimicrobial, antileishmanial and antioxidant activity of the EO (18). The antioxidant activity using ORAC_{oil} method of the rosemary oil was reported to be 4,108 micromolVE/L (18).

*Wild Thyme (*Thymus serpyllum* L.)*

Wild thyme (mother of thyme) is a dominant *Thymus* species; and is predominantly found in Northern and Central Europe. The wild thyme essential oils consists of highly variable amounts of phenols (thymol and carvacrol), alcohols, and monoterpene hydrocarbons (19, 20). Wesołowska et al. (21) reported that there is no distillation effect on the oil content and composition of essential oil of wild thyme. Their group performed hydrodistillation of dry herbage for 2 hours, 3 hours, and 4 hours of DT. Most probably, the lack of response of EO yield and composition to hydro DT was due to the fact that all the chemicals could have been recovered before 2 hours DT.

*Lavender (*Lavandula angustifolia* Mill.)*

Lavandula angustifolia is known as English lavender. The main constituents of lavender oil are cineole, fenchol, camphor, and linalool acetate (15). Lavender is one of the most widely grown essential oil crops, mainly in Europe (France, Bulgaria, Serbia, Ukraine, Russia) and Mediterranean countries. Zheljazkov et al. (22) conducted a research to evaluate the effect of various steam DT (1.5 min, 3 min, 3.75 min, 7.5 min, 15 min, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min, and 240 min) on essential yield and composition of dried lavender flowers (inflorescences). The latter authors reported that 60 min DT is the best DT for optimal oil yield from dried lavender inflorescences. DT had significant effect on the composition of major constituents (fenchol, linalool acetate, and cineole) except for camphor. High concentration of camphor can be achieved at 7.5-15 min DT while very short (1.5 min) DT yielded highest cineole and fenchol concentration. High concentration of linalool acetate could be obtained at 30 min DT (22).

*Garden Sage (*Salvia officinalis* L.)*

Garden sage is one of the valuable herbs used widely in traditional medicine (17). Monoterpene (58.2% to 84.1%) represent the major percentage of essential oil followed by sesquiterpenes (4.0% to 16.1%), and diterpenes (0.3% to 7.6%) (23).

Zheljazkov et al. (23) reported that distillation time (DT) was significant on yield and on concentration of oil constituents. The different distillation times they tested were 1.25, 2.5, 5, 10, 20, 40, 80, and 160 min via steam distillation. According to this research, 10 to 20 min steam DT is optimum to achieve maximum essential oil yield; further increase in DT will not increase the essential oil yield. Zheljazkov et al. (23) documented that high concentration of oxygenated monoterpenes (eucalyptol, cis-thujone, transthujone, camphor, borneol, and bornyl acetate) and higher concentration of monoterpene hydrocarbons (α -pinene, camphene, β -pinene, myrcene, and limonene) could be obtained when garden sage is distilled for 20 min. High concentration of camphor and cis-thujone can

be obtained in 2.5 to 5 min steam DT. Oil with a high concentration of diterpene manool could be obtained when garden sage is distilled for 80 min whereas oil with a high concentration of sesquiterpenes (β -caryophyllene, α -humulene, and verdifloral) could be obtained when garden sage is distilled for 160 min (23).

Oregano (Origanum vulgare L.)

Oregano is one of the medicinal plants used since ancient times. Carvacrol is the main constituents of oregano essential oil. Zheljazkov et al. (24) evaluated the effect of DT (1.25, 2.5, 5, 10, 20, 40, 80, 160, 240, 360 min) on essential oil yield, and composition and found that distillation times (DT) had significant effect on oil content and composition of oregano essential oil. The dried leaves were used in steam distillation in this study. Maximum essential oil yields can be achieved at 240 min DT (24). According to Zheljazkov et al. (24), the major constituents of oregano essential oil (carvacrol) continued to increase with increasing DT up to 240 min. However, the concentrations of most essential oil constituents were higher at the shorter DT (1.25 or 2.5 min) than longer DTs. The essential oil of oregano from this study did not have significant antimicrobial activity using the method developed at the NCNPR; at 50 mg mL⁻¹ the oil had lower than 50% growth inhibition of *Leishmania donovani*, *Plasmodium falciparum* clones D6 and W2, *Candida krusei* (6% inhibition), *Candida glabrata* (3% inhibition), *Escherichia coli* (6% inhibition), *Pseudomonas aeruginosa*, *Cryptococcus neoformans* (8% inhibition), *Mycobacterium intracellulare* (4% inhibition), or *Aspergillus fumigatus* (5% inhibition) (24).

Apiaceae

Apiaceae is also known as Umbilleferae. Fennel, dill, anise, coriander, cumin, and caraway belong to this family (15). Seeds (fruits) are the most important part of majority of Apiaceae plants. Seed contains different amount of essential oil with diverse composition, aroma, and end use. Generally, the essential oil is extracted from seed by hydrodistillation.

Fennel (Foeniculum vulgare Mill)

Fennel is indigenous to Mediterranean region, but is widely naturalized in many parts of the world. The major constituents of fennel essential oil differ depending on the plant part they are obtained from(e.g. steam distillation of fennel herbage, (25), steam distillation of fennel whole seed or fruits (26) or hydrodistillation of fennel ground seed (27).

Moser et al. (26) studied the effect of 15 steam DT (15, 30, 60, 120, 240, 360, 480, 600, 720, 840, 960, and 1080 min) on whole fennel seed (fruit) essential oil. Moser et al. (26) in a study with steam distillation of whole (unground) fennel seed (fruits) reported that EO yield increased with increasing DT to a maximum of 1.375% at 1080 min. Steam DT between 15 and 960 min did not affect the

antioxidant activity of the fennel fruit essential oil, which ranged between 13.9 and 15.6 micromol Trolox/g. However, the longest DT of 1080 min increased the fennel fruit oil antioxidant capacity to 20.6 micromol Trolox/g (26).

Zheljazkov et al. (25) conducted a study on the effect of 8 steam DT (1.25, 2.5, 5, 10, 20, 40, 80, and 160 min) on fennel herb essential oil. In this study, the authors used fennel herb (all aboveground plant parts). The authors reported that fennel essential oil yield reached a maximum of 0.68% in dried herbage at 160 min DT. Zheljazkov et al. (25) reported that the concentration of trans-anethole (32.6–59.4% concentration range in the oil) was low at 1.25 min DT, and increased with an increase of the DT. The concentration of alpha-phelandrene (0.9–10.5% range) was the lowest at 1.25 min DT and higher at 10, 80, and 160 min DT. Alpha-pinene (7.1–12.4% range) and beta-pinene (0.95–1.64% range) were higher in the shortest DT and the lowest at 80 min DT. Myrcene (0.93–1.95% range), delta-3-carene (2.1–3.7% range), cis-ocimene (0–0.23% range), and gamma-terpinene (0.22–2.67% range) were the lowest at 1.25 min DT and the highest at 160 min DT. In contrast, the concentrations of paracycmenene (0.68–5.97% range), fenchone (9.8–22.7% range), camphor (0.21–0.51% range), and cis-anethole (0.14–4.66% range) were highest at shorter DT (1.25–5 min DT) and the lowest at the longest DT (80–160 min DT). Fennel oils from the 20 and 160 min DT had higher antioxidant capacity (164 micromol Trolox/g) than the fennel oil obtained at 1.25 min DT (131 micromol Trolox/g).

Burkhardt et al. (27) collected essential oil fractions from fennel seed at ten different hydrodistillation times, HDT (at 2, 7, 15, 30, 45, 75, 105, 135, 165, and 195 min). The fennel seed used in this study was ground in water to prevent essential oil evaporation. The authors reported that the main constituent of ground fennel seed oil was estragole and ranged from 42.8 to 84.4% of the total oil. (−)-alpha-pinene (0.84%–0.12%) and (R)-(+) -limonene (33.5%–6.3%) were relatively high in the oil fractions captured at initial HDT and then decreased with increase of HDT. (−)-fenchone (4.1%–0.5%) showed a near linear decrease in the oil fractions until approximately the 165 min HDT. Burkhardt et al. (27) reported that if high (−)- α -pinene and (R)-(+) -limonene oil is desirable, then the oil fraction needs to be taken early in the HDT, at 2 min. Oil fraction with highest estragole concentration could be obtained by capturing the oil eluted between 30 and 45 min HDT. The authors reported that HDT affected the antioxidant capacity of the oil fractions; the oil from the control (195 minutes uninterrupted HDT) had the highest antioxidant capacity (481 μ mole Trolox equivalents/g), and the oil fraction collected at 45 min HDT had the lowest antioxidant capacity (148 μ mole Trolox equivalents/g) (27).

Anise (Pimpinella anisum L.)

Anise is native to eastern Mediterranean region and was used in Egypt as early as 1500 B.C. Anise whole or ground seed is used as spice and contains essential oil (15, 28). Traditionally, the anise essential oil is extracted from anise seed (fruits) via steam or hydrodistillation and is widely used as an aromatic agent in the food and liquor industry (15, 17, 28, 29).

Zheljazkov et al. (30) evaluated the effect of nine steam DT (5, 15, 30, 60, 120, 180, 240, 360, and 480 min) on essential oil yield and essential oil composition of anise seed. The authors used whole anise seed (fruits) in this study. The authors statistically analyzed variations in the concentration and yield of linalool, methyl chavicol, para-anis-aldehyde, trans-anethole, gamma-himachalene, trans-pseudoisoeugenyl-2-methyl, and epoxy-pseudoisoeugenyl-2-methyl as a function of the steam distillation of whole anise seed, using a one-way analysis of variance. In addition, the authors developed regression models to predict essential oil yield, and the concentration of individual constituents as a function of DT. The authors reported the highest essential oil yield of 2.0 g/100 g seed (2%) was achieved at 360 min DT. The major oil constituent was trans-anethole with concentration in the total oil ranging from 93.5% to 96.2%. Trans-anethole concentration was high in the oil collected at 15-60 min DT and low in the oil collected at 240-480 min DT. Overall, steam DT of whole anise seed also significantly affected the concentration of the other oil constituents. The concentrations of linalool and methyl chavicol were higher in the shortest DT than longer DT; the concentration of para-anis-aldehyde was highest at short DT (30 min) and lowest at 306 min DT and the concentrations of trans-pseudoisoeugenyl-2-methyl and epoxy-pseudoisoeugenyl-2-methyl showed positive linear relation to increasing DT (30). The authors concluded that by varying the duration of steam DT of whole anise seed, one could obtain anise essential oil with various composition that would benefit the essential oil industry and could be utilized for specific purposes.

Dill (Anethum graveolens L.)

Dill is an annual herb of Apiaceae family and a native of Asia Minor and the Mediterranean region. Herbage and seed oil produce two different types of essential oil (31, 32). Dill is cultivated as culinary herb, medicinal plant, and essential oil crop. The essential oil can be extracted either from whole aboveground herbage harvested at flowering (dill herb oil) or from the seeds (fruits) (dill seed oil). Dill has been grown as essential oil crop in a number of countries in the Mediterranean region and Eastern Europe (Bulgaria, Serbia, Romania, Ukraine), and Russia and India are major producers of dill essential oil (33, 34). The major oil constituents of dill seed oil are carvone and D-limonene, whereas alpha-phellandrene is the main oil constituent of dill herb oil (35). In a hydrodistillation study crushed dill seed, Sintim et al. (36) collected oil fractions at 10 (2, 7, 15, 30, 45, 75, 105, 135, 165, and 195 min) hydrodistillation times (HDT) and evaluated the effect on dill seed oil yield, composition, and bioactivity. The dill seed oil constituents (D-limonene, p-cymenene, cis-dihydrocarvone, trans-dihydrocarvone, carvone and apiole) were identified in oil samples by Kovat analysis and comparison of mass spectra with those reported in the NIST mass spectra database. According to Sintim et al. (36), there is no need to extend the HDT for high essential oil yield of crushed dill seeds beyond 135 min. D-limonene concentration and p-cymenene were higher at the short HDT than in the oil fractions from the longer HDT whereas carvone, cis-dihydrocarvone

and trans-dihydrocarvone increased in their concentrations as DT increased. The authors reported that different oil fractions showed differential antioxidant capacity: the oil collected at 2 min had the highest antioxidant activity (21.8 μ mole Trolox equivalents/g). The same dill oil showed significant antileishmanial activity in the initial screening, however, dill oils from the repeated study did not show any antileishmanial activity. Neither of the dill oil fractions showed significant antimicrobial activity against the 10 microorganisms that were tested at the NCNPR.

*Coriander (*Coriandrum sativum L.*)*

Coriander is a native to regions ranging from southern Europe and northern Africa to southwestern Asia. Coriander is grown as a culinary, medicinal, and as an essential oil crop (37, 38). In North America, coriander generally means seed and leaf is known as cilantro. The desired parts for the essential oil extraction of coriander are the coriander seeds (fruits), although the whole plant also contains aromatic oil (39). Linalool is the major constituent of essential oil composition of coriander seeds. Coriander essential oil may depend on genetic and environmental factors (37, 40), but also on the extraction process (41) al., 2008). Zheljazkov et al. (42) conducted a study on the effect of hydrodistillation time (HDT, 1.25, 2.5, 5, 10, 20, 40, 80, 160, and 240 min) on essential oil yield and composition from crushed coriander fruits. The authors identified the following constituents in coriander oil: tricyclene, alpha-thujene, alpha-pinene, camphene, sabinene, beta-pinene, myrcene, alpha-terpinene, para-cymene, limonene, eucalyptol, gamma-terpinene, cis-sabinene hydrate, terpinolene, linalool, camphor, citronellal, 4-terpinenol, alpha-terpineol, geraniol, geranial, and geranyl acetate. The minor constituents (those that stay consistently below 1% of the oil) were excluded from the statistical analyses. Previous studies used less than 60 min (39), to 180 min (40), or 240 min (43) to extract essential oil of coriander seed but Zheljazkov et al. (42) demonstrated that longer DT than 40 min is not beneficial for high oil yield. Alpha- pinene, camphene, beta-pinene, and myrcene concentrations were higher at shorter DT than longer DT (42). According to Zheljazkov et al. (42), the concentrations of para-cymene and linalool were highest at 240 min DT; the concentrations of gamma-terpinene and limonene were higher at 1.25- 2.5 min DT; and the concentration of camphor was maximum at 20 min HDT. Distillation time altered the antioxidant activity of coriander oils. The antioxidant activity of coriander oils from the 20 and 240 min DT, 34.8 and 35.9 μ M Trolox/g, respectively, was higher than the one at 80 min DT (31.1 μ M Trolox/g).

*Cumin (*Cuminum cyminum L.*)*

Cumin is native to Levant and Upper Egypt, and is one of the most widely used spices in the world since ancient times. Cumin aldehyde is the major component of cumin oil. Cumin essential oil is extracted from cumin seed (fruits). In Iranian

ancient medical system, cumin seed and cumin oil have been used for the treatment of various conditions such as toothache, diarrhea, and epilepsy (44), whereas in the traditional ayurvedic medicine it is used as a stimulant, and medicine against indigestion, flatulence, and diarrhea (45).

Zheljazkov et al. (46) collected 9 different essential oil fractions from 9 hydrodistillation time frames (0-2, 2-7, 7-15, 15-30, 30-45, 45-75, 75-105, 105-135, and 135-165 min) to study the effect of hydrodistillation times (HDT) of crushed cumin seed on oil content and composition. The authors reported that oil collected at different time points resulted in significantly different profile, with the concentration of oil constituents fluctuating as follows: (as percentage of total oil): α -pinene (0.2-2.1%), β -pinene (5-35.8%), myrcene (0.3-1.7%), para-cymene (12.0-26.4%), γ -terpinene (4.8-25.9%), cumin aldehyde (3.8-51.1%), α -terpinen-7-al (0.2-11.2%), and γ -terpinen-7-al (1.3-13.1%). The antioxidant capacity (ORAC) of the collected oil fractions varied from 25 (the oil fraction collected at 0-2 min HDT) to 394 micromol Trolox Equiv. g oil (oil fraction collected at 105-135 HDT timeframe). The authors found that ORAC values of the oil fractions were positively correlated to the concentration of cumin aldehyde (0.962), α -terpinene (0.889) and γ -terpinene (0.717). The latter finding suggest that these oil constituents in cumin oil may be the source for the measured differential antioxidant capacity (46).

*Caraway (*Carum carvi L.*)*

Caraway is one of the widely utilized crops for flavoring in cookery, confectionery and liqueurs, it is a spice and essential oil crop (15). Caraway seeds (fruits) contain 2-5% essential oil. The major constituents of caraway seed oil are carvone (40-70% of the oil) and limonene (35-40% of the oil) (15). Carvone and limonene ratio is the main quality determinant of essential oil of caraway seed.

Shiwakoti et al. (47) conducted distillation study to elucidate the effect of various hydrodistillation time frames (HDT, 0-2, 2-7, 7-15, 15-30, 30-45, 45-75, 75-105, 105-135 min and a control of 135 min) on essential oil fractions yield and composition of ground caraway seed. The process involved was hydrodistillation. The authors identified the following 26 oil constituents in the caraway seed oil: alpha-pinene, sabinene, myrcene, limonene, trans-ocimene, linalool, trans-para-mentha-2,8-dien-1-ol, cis-limonene oxide /cis-para-mentha-2,8-dien-1-ol, trans-limonene oxide, alpha-terpineol, cis-dihydro carvone, trans-dihydro carvone, iso-dihydro carveol, trans-carveol, neoiso-dihydro carvone, carvone, geranal, perilla aldehyde, trans-carvone oxide, methyl geranate, iso-caryophyllene, β -caryophyllene, germacrene D, and caryophyllene oxide. Of these, the authors conducted statistical analyses of the 5 constituents with the highest concentrations in the oil fractions: myrcene, limonene, trans-carveol, carvone, and β -caryophyllene. Carvone and limonene were major oil constituents, comprising 96.4 to 98.0 % of total oil composition in various oil fractions collected at different time frames. The authors reported that most of the oil was eluted early and only negligible amount was eluted after

105 min HDT. Myrcene and limonene concentrations were high in the early DT whereas trans-carveol, β -caryophyllene, and carvone concentrations were high in the late HDT. Carvone to limonene ratio increased with the increasing HDT.

Asteraceae

Asteraceae is also known as compositae and contains large number of flowering plants. Lettuce, sunflower, chamomile, artichoke are some examples of plants belonging to this family.

Chamomile (Matricaria chamomilla L. syn. M. recutita)

Chamomile flowers (inflorescences) are commonly used for making chamomile tea known for its soothing effect. Chamomile essential oil is extracted from chamomile flowers or whole abovevegraound plant parts and is utilized in the pharmaceutical and cosmetic industries (48, 49). β -farnesene and α -bisabolol oxide A are the major constituents of total oil composition. The compositon of chamomile essential oil varies widely depending on the origin, genetics, cultivar, fertilization and growing conditions. Chamomile oil has been traditionally extracted via steam distillation, however, various researchers and commercial units have been utilizing different duration of the steam distillation process. The knowledge gap on how steam distillation duration would affect chamomile oil yield and composition was addressed by Gawde et al. (50). Chamomile essential oil yield reached maximum at 720 distillation time (DT); the essential oil yield obtained at 30, 60, 90, 120, 180, 240, 360, 480, 600, and 720 min showed gradual and significant increase to reach a maximum of 3.1 g oil per 1000 g of flowers at 720 min. Gawde et al. (50) identified and quantified the major oil constituents anethole, β -farnesene, spathulenol, α -bisabolol oxide B, α -bisabolone oxide A, chamazulene, α -bisabolol oxide A, and spiroether. DT had significant effect on β -farnesene which decreaseded with the increasing duration of DT, and also on α -bisabolol oxide A, spiroether, and chamazulene which rapidly increased up to 240 min. DT affected the concentration of anethole in the oil; anethole decreased with increasing the duration of the DT. The DT did not affect the antioxidant activity of chamomile oil measured using the ORAC oil method. The authors reported an average activity of 905 μ mol Trolox equivalents/g of extract in chamomile essential oil (50). Chamomile oil did not showsignificant antimicrobial activity using the method developed by the NCNPR at the University of Mississippi. The chamomile essential oil exhibited low inhibition against *Candida krusei*, *Cryptococcus neoformans*, and *Mycobac-terium intracellularare* with percent inhibitions of 38, 39, and 35, respectively. Also, the chamomile oil in this study had some activity against *Candida globrata* and *Pseudomonas aeruginosa*, with percent inhibition of 14 and 10, respectively, but negligible activity against *E. coli* and *Candida albicans*, (3% and 5%, respectively) and zero activity against *Aspergillus fumigatus*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (50).

*Sweet Sagewort (*Artemisia annua*)*

Sweet sagewort, also known as sweet wormwood, is widely used medicinal plant, it contains several natural products, and is currently the only commercial source of natural artemisinin (51). The essential oil of sweet wormwood is extracted via steam distillation of the aboveground herbage; the crop is commercially grown on large areas in Eastern European countries such as Bulgaria, Hungary, and Romania, and also in Italy and Switzerland. In a study with sweet wormwood biomass, Zheljazkov et al. (52) evaluated the effect of eight (1.25 min, 2.5 min, 5 min, 10 min, 20 min, 40 min, 80min, and 160 min) different distillation times (DT) on the essential oil yield, composition, and its antioxidant capacity. The tested DT altered the essential oil yield, which varied from 0.005 to 0.23% in fresh biomass; maximum yield was obtained at 160 min DT. DT affected the concentration of alpha-pinene (0.5-2.1% of the oil), camphene (0.9-3.5%), para-cymene (1.2-4.7%), eucalyptol (1.1-5.9%), and camphor (8.7-50%), borneol (0.3-2%), beta-caryophyllene (4.1-14.3% range), trans-beta-farnesene (2.9-5.6% range), and germacrene-D (5.5-23.1% range), beta-chamigrene (1.1-2.1%) gamma-himachalene (1.2-1.9%), trans-muurola-4(15), 5-diene (0.3-5.7% range), spathulenol (0-2.6% range), caryophylene oxide, and cis-cadin-4-en-ol. The concentration of the oil main constituent, camphor (8.7-50%), was highest at the shorter DT and reached a minimum at 160 min DT. The sweet wormwood essential oil had the lowest antioxidant capacity at 5 min and 80 min DT, and the highest at 20 min DT (52).

Ferreira et al. (53) studied the effect of steam distillation time (DT, 1.25, 2.5, 5, 10, 20, 40, 80, 160, and 240 min.) of sweet wormwood biomass on essential oil yield, artemisinin concentration, and antioxidant capacity of the plant residue from distillation (PRD). This was a study on dual utilization of sweet wormwood; for essential oil and for artemisinin production. The artemisinin concentration was measured in the biomass after the extraction of the essential oil via steam distillation.

The authors reported that most of the artemisinin in the PRD was apparently degraded during distillation; it was observed that artemisinin decreased by 84% in PRD that had been distilled for 1.25 min (only 0.18% artemisinin left), compared to the undistilled control (1.15 g 100 g⁻¹ artemisinin). Furthermore, the results showed that the concentration of artemisinin in PRD decreased with increasing DT up to 20 min. The PRD distilled for 40 min did not show detectable amount of artemisinin (53). However, the DT did not drastically reduced the antioxidant capacity of PRD; the antioxidant capacity of the leaves decreased by 6% after 40 min of DT and 25% at 240 min DT. Therefore, the authors concluded that the byproduct from the oil extraction via steam distillation (PRD) could be used as a source of antioxidants. Such PRD could potentially be used for animal feeding and even as ingredient in functional foods and may have other human health applications (53).

Cupressaceae

Creeping Juniper (Juniperus horizontalis)

Creeping juniper is a low growing evergreen shrub and is commonly grown as an ornamental. Creeping juniper contains highly aromatic essential oil that has been traditionally used in fragrance and pharmaceutical industries. The major constituents of essential oil of creeping juniper needles are α - pinene, sabinene, and limonene (54). Recent research has shown that creeping juniper contains podophyllotoxin, an important anticancer drug precursor (54, 55). Podophyllotoxin is a precursor to the semi-synthetic anti-cancer drugs etoposide and teniposide, which are used for the treatment of lung cancer, testicular cancer, neuroblastoma, hepatoma, other tumors (56, 57). Also, other derivatives of podophyllotoxin are utilized in the treatment of psoriasis and malaria, and also for rheumatoid arthritis (58, 59).

The essential oil of creeping and other junipers is traditionally extracted via steam distillation. However, the optimal steam distillation time was not known. Cantrell et al. (54) evaluated 11 (20, 40, 80, 160, 180, 240, 480, 600, 720, 840, and 960 min) different steam distillation times steam (DT) on essential oil yield and composition of creeping juniper. The authors reported that creeping juniper essential oil yield increased with increasing the duration of the steam DT from a minimum of 0.023% at 20 min to a maximum of 1.098% at 960 min. DT has significant effect on the concentration of sabinene (46.6% at 80 min to a low of 30.2% at 960 min) and alpha-pinene (9.6% at 20 min DT and 4.2% at 960 min DT), however, the concentration of limonene was largely unaffected by the DT (54). The authors reported that steam distillation did not degrade podophyllotoxin; podophyllotoxin remaining in the plant tissue after distillation varied from a 0.281% to 0.364%, whereas the podophyllotoxin concentration of the undistilled control sample was 0.217% (54). The authors concluded that DT can be utilized to obtain creeping juniper essential oil with specific profile and also plant residue from distillation can be utilized as a source of podophyllotoxin. Therefore, creeping juniper can be utilized to obtain two natural products: essential oil and podophyllotoxin (54).

Rocky Mountain Juniper (Juniperus scopulorum Sarg.)

According to Adams (60) the genus *Juniperus* includes many species found throughout the world, with a wide ecological adaptation. Rocky Mountain juniper (*Juniperus scopulorum* Sarg.) is found throughout the Western US, Canada, and Mexico (60–62). This species is evergreen, long-lived, up to 1,000-3,000 years and forms male and female cones on separate trees (62). Rocky Mountain juniper has been used in the past as medicinal plant by native Indians (62, 63). Because it is widespread on very low fertility soils and in semi-arid zones, where other species may not survive, this juniper plays important ecological role; it provides cover for wildlife species and is used for food by birds, pronghorn antelope, whitetail

deer, elk, bighorn sheep, and occasionally by domestic cattle and sheep when other vegetation is not available (62). As with creeping juniper, the Rocky Mountain juniper contains both essential oil and podophyllotoxin (55, 64). The essential oil profile of Rocky Mountain juniper depends on the ecological conditions, the sex of the tree, time of sampling, and genetics (55, 60, 65, 66).

To find out the effects of distillation time (DT) on essential oil yield and composition and antioxidant capacity of male Rocky Mountain juniper needles, Zheljazkov et al. (67) conducted a study with 15 different distillation times (DT 1.25, 2.5, 5, 10, 20, 40, 80, 160, 240, 360, 480, 600, 720, 840, and 960 min). The authors found that DT significantly affected essential oil yield, which ranged from 0.07% at 1.25 min DT to 1.48% at 840 min DT. DT also significantly altered the concentration of essential oil constituents in the oil. For example, the concentrations of low-boiling constituents such as alpha-thujene (1.76-2.75%), alpha-pinene (2.9-8.7%), sabinene (45-74.7%), myrcene (2.4-3.4%), and para-cymene (0.8-3.1%) peaked in the oil obtained at 1.5 to 5 min DT, but were low in the oil obtained later in the distillation process. Some oil constituents peaked in the oil collected at 40 min DT; cis-sabinene hydrate (0.5-0.97%) and linalool plus trans-sabinene (0.56-1.6%). Other constituents also varied depending on the DT: limonene (2.3-2.8%), pregeijerene-B (0.06-1.4%), 4-terpinenol (0.7-5.7%), alpha-terpinene (0.16-2.9%), gamma-terpinene (0.3-4.9%), terpinolene (0.3-1.4%), delta-cadinene (0.06-1.65%), elemol (0-6.0%), and 8-alpha-acetoxylemol (0-4.4%). The concentrations of α -thujene, α -pinene, sabinene, myrcene, and para-cymene had negatively linear relation with DT- concentrations decreased with increasing DT. Highest concentrations of limonene and pregeijerene-B were obtained at 360-480 min DT whereas maximum concentratons of α - terpinene, γ - terpinene, and terpinolene were at 720 min DT (67). DT also affected the antioxidant capacity of Rocky Mountain Juniper oil. The oils collected after 480 min DT had higher antioxidant activity (41.8 μ mole Trolox equivalents/g), whereas oils collected after 40 min DT showed 21.6 μ M Trolox equivalents/g), the oils collected after 160 min DT showed 26.3 μ mole Trolox equivalents/g, and the oils collected at 960 min DT showed 25.4 μ mole Trolox equivalents/g antioxidant capacity; the latter two were not significantly different from each other (67).

Distillation times (DT) have significant effect onthe essential oil yield content and composition of female rocky mountain juniper needles (68). In the latter study, the authors evaluated 11 steam DT (1.25, 2.5, 5, 10, 20, 40, 80, 160, 240, 360, 480 minutes) on essential oil fractions collected from needles of female Rocky Mountain juniper. Results showed that essential oil yield was maximum at 240 min DT; longer DT decreased the concentrations of α -thujene, α -pinene, camphene, myrcene and para-cymene; 40 min DT yielded maximum concentrations of cis-sabinene hydrate and linalool/trans-sabinene hydrate whereas the concentrations of α -terpinene, limonene, γ -terpinene and 4-terpineol reached their maximum at 360 minutes DT; and terpinolene, α -eudesmol/ β -eudesmol and 8- α acetoxylemol reached maximum at 480 minutes DT (68).The antioxidant capacity of the essential oil from this study increased from 20.4 μ mole Trolox/g in the oil at 40 min DT to 54.7 μ mole Trolox/g at 480 min DT (68).

Pinaceae

Ponderosa Pine (Pinus ponderosa Dougl. ex Laws)

Ponderosa pines are also known as long-leaved pine, bull pine, blackjack, yellowbelly, Western yellow pine, red pine, silver pine, Montana black pine, pondo, pino real etc. Ponderosa pine belongs to the genus *Pinus* (Pinaceae family) that includes more than 100 species, and is found throughout the United States, and provides habitat for many wildlife species (69, 70). The major oil constituents of ponderosa essential oil are α - pinene and β - pinene. The ponderosa pine essential oil is characterized by fine aroma, and has various commercial applications. As with other essential oil species, the composition of ponderosa pine essential oil depends on many factors such as environmental and genetics, but is also dependent on the extraction process (71). The essential oil is extracted from pine needles via steam distillation, however, the optimal distillation time (DT) has not been researched.

Zheljazkov et al. (72) evaluated the effect of distillation time (DT) on essential oil composition of fresh needles of ponderosa pine. They tested 1.25, 2.5, 5, 10, 20, 40, 80, 160, 240, and 360 min DT via steam distillation. Essential oil content was highest at late DT and reached maximum at 160 min DT. α - pinene and β - pinene concentrations decreased with increasing DT and were high at the initial DT whereas the concentrations of delta-3-carene, limonene, cis-ocimene, α -terpinyl acetate, germacrene-D, α -muurolene, γ -cadinene, delta-cadinene, and germacrene-D-4-ol increased with increasing DT. The concentration of myrcene did not change significantly with changing DT (72). The authors found that the antioxidant capacity of the essential oils obtained at 20, 80, and 360 min DT were 9.7, 7.0, and 14.5 μ mole Trolox/g of oil, respectively, but they were not statistically different. Therefore, the authors concluded that DT time does not affect the antioxidant activity of ponderosa pine essential oil (72).

Poaceae

Lemongrass (Cymbopogon flexuosus Steud.) and Palmarosa (Cymbopogon martini Roxb.)

Lemongrass and palmarosa belong to the same genus (*Cymbopogon*) and family (Poaceae alias gramineae alias true grass). The essential oils of lemongrass and palmarosa have wide use in the culinary, pharmaceutical, and cosmetics industries; and are also considered an eco-friendly insect-repellant (73–76). The essential oil is extracted with steam distillation, the main components of lemongrass and palmarosa essential oils are neral and geranial. Cannon et al. (16) studied the effect of different distillation times (1.25, 2.5, 5, 10, 20, 40, 80, 160, and 240 min) on the oil content and composition of lemongrass and palmarosa via steam distillation using 500 gm of fresh herbages. According to their report, increasing in DT further more than 20 min is not efficient to achieve high essential oil yield of lemongrass and palmarosa. In lemongrass, short DT yielded higher concentrations of neral and geranial than longer DT and caryophyllene

oxide concentrations increased with increasing DT (16). In palmarosa, the main constituent, geraniol, did not show any significant changes in composition with various DT whereas geranyl acetate concentration increased with increasing DT until it reached 240 min DT (16). As with the other studies discussed, the authors of this study concluded that DT can be used as a tool for obtaining essential oils with specific targeted composition. Also, this and the previous reviewed studies demonstrated that the duration of DT or HDT must be reported when essential oil or composition is reported; such information will make the comparison of data on oil yield composition much more feasible.

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Chapter 11

Effects of Plant-Derived Bio-Active Compounds on Rumen Fermentation, Nutrient Utilization, Immune Response, and Productivity of Ruminant Animals

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Plants produce an extensive array of organic compounds derived from secondary metabolism that may be useful in animal nutrition because of their chemical makeup. These plant-derived bio-active compounds (PBAC), also referred to as phytonutrients or phytobiotics, have been shown to express antimicrobial activities against a wide range of bacteria, yeast, and fungi and have been investigated as alternatives to rumen modifiers, such as ionophoric antibiotics, in animal nutrition. PBAC have also been studied as inhibitors of pathogens that impact animal health and productivity, modulate the immune system, and reduce stress. A large number of *in vitro*, *in situ*, and *in vivo* studies on the effects of PBAC on ruminal fermentation have been published in recent years. Some reports have concluded that PBAC may inhibit deamination of amino acids and methanogenesis and shift fermentation towards propionate and butyrate. Responses, however, have been highly variable. Overall, hydrolysable and condensed tannins may offer an opportunity to reduce rumen methane production, although intake and animal productivity may be compromised. Most of the experiments with PBAC have been conducted *in vitro*. Although *in vitro* data are useful for screening purposes, the true value of PBAC for altering rumen microbial fermentation and ultimately enhancing animal production must be assessed.

in vivo and in long-term trials. Another, relatively new area of research is the effects of PBAC on immunity and animal health. PBAC such as garlic, curcumin, and capsicum have modulatory effects on the adaptive immune system in monogastric species and similar properties may be expected in ruminants. Studies with dairy cows have indicated that some PBAC delivered postruminally increase neutrophil activity and the numbers of immune cells related to acute phase immune response. Overall, some PBAC may be beneficial as rumen modifiers and may positively affect animal immunity, health, and productivity, but more and long-term studies are needed to fully elucidate these effects in ruminant animals.

Introduction

The secondary metabolism of plants produces a variety of organic compounds. These plant-derived bio-active compounds (PBAC) are responsible for color, taste, and odor of plants, and also used for self-defense against insects and microorganisms (1). Particularly, PBAC have been known to exhibit anti-microbial properties on bacteria, yeasts, and fungi and researchers have studied the possibility of using PBAC as modifiers of rumen fermentation in ruminants (2). The inhibitory effects of PBAC on specific groups of microbes in the rumen have altered the pattern of rumen fermentation resulting in decreased methane production in *in vitro* and *in vivo* studies (3, 4). Recent reports have suggested immune-modulatory effects of PBAC in ruminants (5, 6), which could be beneficial for animal health and productivity. This chapter presents a review of the effects of major groups of PBAC, such as essential oils, tannins, and saponins on rumen fermentation, methane production, productivity, and immunity in ruminant animals.

Essential Oils

Essential oils (EO) are secondary metabolites from the volatile fraction in plants. Generally, EO are extracted by hydrodistillation from plant materials (7). The chemical composition, activities, and biological properties of EO are diverse. The active compounds of EO are commonly categorized into terpenoids and phenylpropanoids (2). Terpenoids have a basic structure of C₅ (isoprene, C₅H₈) and are classified according to the number of isoprene. Monoterpenoid (C₁₀) and sesquiterpenoids (C₁₅) are known to be major compounds in terpenoids (8). Phenylpropanoids are characterized by an aromatic ring of 6 carbons with a 3 carbons side chain (Figure 1).

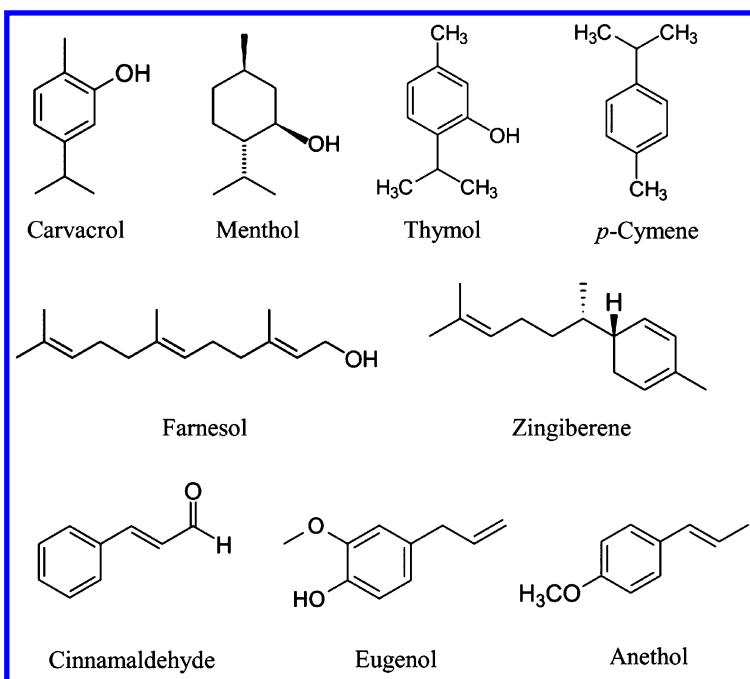


Figure 1. The main terpenoids and phenylpropanoids in essential oils. Carvacrol, menthol, thymol, and *p*-cymene are monoterpenoids; farnesol and zingiberene are sesquiterpenoids; cinnamaldehyde, eugenol, and anethol are phenylpropanoids.

One of the well-known modes of action of terpenoids is the interaction with bacterial cell membranes (9). The hydrophobicity of cyclic hydrocarbons in terpenoids changes the membrane conformation of bacterial cells. This reduces the stability of bacterial cell membrane, allowing leakage of ions across the membrane. In turn, this causes cell death or suppression of growth due to a large amount of energy loss. However, Gram-negative bacteria are not susceptible to the hydrophobicity of terpenoids because of their cell wall structures (10). The layer of lipopolysaccharides in Gram-negative bacteria does not allow terpenoids to interact with bacterial cell membranes. Only small molecules such as thymol and carvacrol having hydroxyl groups may be able to diffuse into the layer of lipopolysaccharides. In addition to the interaction with the cell membrane, phenolic compounds of terpenoids may coagulate cell constituents by protein denaturation (2).

Studies have shown that EO could inhibit hyper-ammonia producing bacteria (HAPB) in the rumen, which decreases deamination of amino acids (11). An *in vitro* study demonstrated that an EO mixture (thymol, eugenol, vanillin, and limonene) reduced the activity of HAPB species such as *Clostridium sticklandii* and *Peptostreptococcus anaerobius* while other bacteria were adapted to EO (12). Supplementation with EO at 100 mg/d in an *in vivo* experiment decreased

the number of HAPB by 77% in sheep fed a low protein diet (13). However, EO were not effective in a high protein diet in the same experiment, which indicates the inhibitory effect of EO on HAPB may depend on diet composition. Dietary supplementation of oregano leaves (750 g/d; 1.05 g/kg live body weight) containing carvacrol and thymol in dairy cows decreased the proportion of *Ruminococcus flavefaciens*, which is one of the major rumen fibrolytic species (14). However, oregano leaves in the same study did not affect the major rumen bacteria. It was reported that dietary supplementation of an EO mixture (750 mg/d; 1.36 mg/kg live body weight) containing thymol, eugenol, vanillin and limonene did not affect total viable cellulolytic bacteria in the rumen of dairy cows (15). Similarly, the same mixture was used in an experiment with sheep and no change in total viable rumen bacteria counts was observed (16). It has been reported that rumen bacteria were differently affected by EO depending on dose amount. The growth of *Selenomonas ruminantium* was selectively inhibited by thymol at a low dose (90 mg/L) *in vitro* (17), whereas thymol inhibited all other ruminal bacteria at a high dose (400 mg/L) in this study.

The effect of EO on protozoa has not been consistent. Extracts of clove containing eugenol decreased the numbers of total protozoa, small entodiniomorphs and holotrichs *in vitro* (18). Decreased counts of holotrichs and entodiniomorphs were also observed in beef cattle fed 2 g/d anise extract (100 g/kg anethol) (19). However, cinnamaldehyde (0.4 to 1.6 g/d) did not affect ruminal protozoa including *Isotricha*, *Dasytricha* and *Entodinium spp.* in steers (20). In dairy cows, 750 mg/d (1.36 mg/kg live body weight) of an EO mixture containing thymol, eugenol, vanillin, guaiacol, and limonene had no effect on ruminal protozoa counts (15).

The inhibitory properties of certain EO on methanogens and methanogenesis have been demonstrated *in vitro* and *in vivo*. In an *in vitro* study using rumen fluid, 1000 ppm of an EO mixture (thymol, eugenol, vanillin, and limonene) inhibited *Methanobrevibacter smithii* while 160 ppm was not effective (12). However, Beauchemin and McGinn (21) found no effect of EO containing thymol, eugenol, vanillin, guaiacol, and limonene on methanogenesis in beef cattle. The inhibitory effects of EO on rumen methanogens may be dependent on dose. High concentration of peppermint oil (1 and 2 mL/L rumen liquid) decreased methanogen population in buffalo rumen liquid, whereas a lower dose (0.33 mL/L) increased the methanogens measured using real-time PCR (22). In a more recent experiment, peppermint oil inclusion in rumen fluid (0.37 to 1.5 µL/mL) linearly decreased methane production in sheep (23). Supplementation on diets with juniper berry oil (0.02 g/kg dry matter intake) containing myrcene (25%), citronellol (18%) and alpha-pinene (14%) exhibited an inhibitory effect on *Methanobrevibacter ruminantium*, a predominant methanogenic species, in ewes (24). Because *Methanobrevibacter ruminantium* has been known to be associated with protozoa such as *Polyplastron multivesiculatum*, it was suggested that the inhibitory effect of juniper berry on rumen archaea in this study was due to decreased protozoal populations (25). Dietary supplementation of EO from oregano decreased methane production in sheep (26). In dairy cows, oregano supplementations decreased methane production in the rumen although methanogens were not affected (14, 27).

Individual EO or EO mixes have been reported to modify rumen fermentation parameters such as pH and volatile fatty acids (VFA) concentration in *in vitro* and *in vivo* studies (2, 11). The results, however, are not consistent among the studies, and the modulatory effects on rumen fermentation differ by type of EO. It was reported that supplementation with thymol (500 mg/L) decreased total VFA and ammonia N concentration and increased acetate:propionate ratio in *in vitro* batch and continuous culture studies using rumen fluid from dairy cows (28). In these studies, a lower dose (50 mg/L) had no effect on the ruminal fermentation. Similar effects were also reported in an *in vitro* study, in which acetate:propionate ratio in rumen fluid from steers was increased by moderate doses (100 to 400 mg/L) of thymol (17). However, thymol decreased acetate:propionate ratio in rumen fluid collected from beef cattle in other study (29). Due to a hydroxyl group in its phenolic structure, thymol is known to be more effective on antimicrobial activity compared with non-phenolic EO (2). In addition, thymol is able to associate with the external cell wall of bacteria because of its small molecular weight. The strong antimicrobial properties of thymol against a wide range of Gram-negative and Gram-positive bacteria make targeted modification of ruminal fermentation difficult. Eugenol is one of the main active components of clove EO and is also effective on both Gram-negative and Gram-positive bacteria. Ethanol and methanol extracts of clove buds, however, did not affect VFA concentration in an *in vitro* gas production test (30). Addition of peppermint oil (*Mentha pulegium*) to rumen fluid (0.37 to 1.5 μ L/mL) linearly decreased total VFA concentration, apparently due to an overall inhibition of the ruminal fermentation (23). Major active components in peppermint oil used in that study were menthol, menthone, p-cymene, and limonene. The effects of EO may depend on rumen pH (29). *Capsicum* oleoresin was tested *in vitro* at two different pH on rumen fermentation using inoculum from beef cattle fed a high concentrate diet (29). *Capsicum* oleoresin decreased total VFA and propionate concentration, but increased acetate and butyrate at pH 7.0. In contrast, at pH 5.5, *Capsicum* oleoresin increased VFA and propionate concentration, but decreased acetate concentration. The authors hypothesized that low pH may increase hydrophobicity of capsaicinoids, the active compounds in *Capsicum*, which may enhance their adverse effect on certain types of bacterial cell membranes (2). Similarly, a blend of EO decreased acetate concentration and the acetate:propionate ratio in the rumen only at lower pH (31).

In *in vivo* studies, inclusion of oregano leaves containing mainly carvacrol in the diet of lactating dairy cows did not affect ruminal pH and VFA concentrations (14, 27). Although milk production was not affected in these studies, milk fat content and yield, and 3.5% fat-corrected milk yield and feed efficiency were increased by the oregano supplementation. A mixture of EO containing thymol, eugenol, vanillin, guaiacol, and limonene was tested in a series of experiments in dairy cows (15, 32, 33). Supplementation of the EO mixture ranged from 750 to 1200 mg/d (1.1 to 1.8 mg/kg live body weight) in these experiments and it did not affect milk yield, except an increase in 3.5% fat-corrected milk (32).

Collectively, EO have been shown to exhibit antimicrobial effects on bacteria, protozoa, and methanogens in *in vitro* studies, which triggered responses in rumen fermentation. Generally, EO decreased ruminal VFA and ammonia concentration,

acetate:propionate ratio, and methane production. These responses, however, have not been consistent among *in vivo*. The effects of EO likely depend on composition of the basal diet, application doses, and chemical properties of EO. Toxicity of EO should be also considered because tolerance to toxicity differs among ruminant species (34). In addition, due to the adaptation of microbes in the rumen, EO should be tested in long-term *in vivo* studies.

This last point deserves some discussion. *In vitro* systems are convenient for screening a large number of treatments, with sufficient replication, and in a short time, at a fraction of the cost of an animal study. Due to various factors inherent to all *in vitro* systems, however, fermentation end-products accumulate (batch culture systems) and the original microbial community may degenerate and protozoa usually disappear. A meta-analysis of 180 continuous culture (an *in vitro* system often used to test feed supplements) studies with more than 1000 individual treatments concluded that continuous culture systems are generally characterized by lower acetate concentrations, extremely low counts or lack of ruminal protozoa, and lower organic matter and neutral-detergent fiber digestibilities compared with *in vivo* digestion (35). This analysis showed that variability was much greater for continuous culture compared with *in vivo* data. *In vitro* systems also cannot address the very important question of long-term adaptability of the ruminal ecosystem to a treatment. As discussed earlier, the rumen ecosystem is very robust and in the long-term may adapt to various interventions, which may be effective in modifying fermentation in the short-term.

Tannins

Tannins are polyphenolic compounds having high molecular mass and widely distributed in forage, grains, fruits, and cereals. Tannins have been considered anti-nutritional factors because of their adverse effects on intake although this depends on their concentration (36). Tannins are also known to possess antimicrobial properties due to the large number of phenolic hydroxyl groups in their molecules and to decrease proteolysis in the rumen by forming complexes with proteins (36). There are mainly two groups of tannins: condensed tannins (CT) and hydrolysable tannins (HT) (Figure 2). The CT are polymers formed by the condensation of flavans and complexes of flavonoid units linked by carbon-carbon bonds (37). The HT have a central core such as glucose, glucitol, quinic acids, and shikimic acids, which is esterified with phenolic groups. Gallic acids and ellagic acids are involved in the esterification. By further esterification and oxidative crosslinking, the galloyl groups become more complex HT (38).

Tannins as polyphenols have antimicrobial properties that inhibit growth of bacteria, protozoa, and methanogens. The antimicrobial activities include morphological changes of bacterial cell wall, inhibition of oxidative phosphorylation, and deprivation of substrates essential for microbial growth (36). It was observed that that CT formed tannin-protein complexes on the cell surface of *Fibrobacter succinogenes* (39). Also, the extracellular endoglucanase activities of the bacteria were inhibited by CT in this *in vitro* study. CT (catechins) was known to damage the lipid bilayer of the cell membrane causing leakage (40).

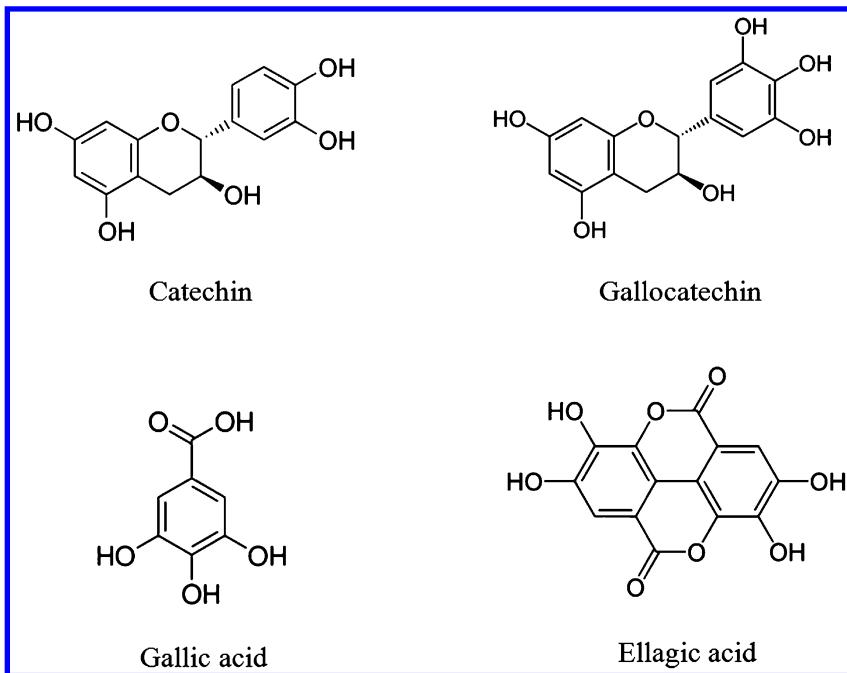


Figure 2. Condensed and hydrolysable tannins. Catechin and gallocatechin are condensed tannins; gallic acid and ellagic acid are hydrolysable tannins.

The antimicrobial effects of tannins on bacteria are variable depending on the types of tannins and bacterial species. CT of the legume sainfoin (25 to 600 µg/mL) inhibited growth and protease activity of *Butyrivibrio fibrisolvens* and *Streptococcus bovis*, but it had little effect on *Prevotella ruminicola* and *Ruminobacter amylophilus* (41). These results are consistent with a study that found decreased populations of *Streptococcus bovis*, *Butyrivibrio fibrisolvens*, *Clostridium proteoclasticum*, and *Eubacterium spp.* in the rumen of sheep fed *Lotus corniculatus* (32 g CT/kg dry matter) (42). Cellulolytic bacteria, *Fibrobacter succinogenes* and *Ruminococcus spp.*, have been inhibited in the rumen by supplementation of *Calliandra* leaves containing CT in sheep (43).

Tannins have been reported to have anti-protozoal properties, although the inhibition mechanisms are not clearly understood (36). Ruminal protozoal counts have also been suppressed by HT extracted from myrabolam and chestnut in *in vitro* studies (44). Protozoal counts were decreased by an average of 12.3 % by HT in the studies. Dietary supplementation of increasing doses of Kobe lespedeza containing CT linearly decreased protozoal numbers in wethers (45). In dairy cows, however, 150 g/d CT from quebracho trees did not affect total numbers of ruminal ciliate protozoa (46). Feeding quebracho CT in beef heifers also did not affect protozoal counts (47). The effects of tannins on ruminal protozoa depend on concentration and chemical composition of the tannins. The chemical properties

of HT and CT can be different according to numbers of galloyl and flavonoids moieties, respectively. An *in vitro* study showed the growth of *Staphylococcus aureus* was inhibited by CT extracted from different woody plant species in a dose dependent manner (48). The inhibitory effect was the highest in CT from shinnery oaks and the lowest from plums. In addition, susceptibilities of *Staphylococcus aureus* and *Escherichia coli* to different tannin monomers were tested in the same study. The inhibitory effects of tannin monomers were exhibited at the following order (from highest to lowest): catechin, ellagettannin, tannic acid, epi-catechin, and gallotannin.

Because of the symbiotic relationship, methanogens associated with protozoa may decrease in the rumen when protozoa are inhibited (49). HT (gallotannin) inclusion in ruminal culture systems decreased both methanogenic archaea and protozoal counts by 11.6 and 12.3 %, respectively (44). Methanogens of the order *Methanobacteriales* were quadratically decreased *in vitro* by increasing levels of CT extracted from *Leucaena leucocephala* (50). Protozoa population in this study was also decreased by CT. Similarly, decreased populations of methanogens and protozoa were observed as a result of supplementation dairy diets with *Vaccinium vitis idaea* containing CT (2 g/kg) (51). However, no effect of feeding tannin-rich plants was found in sheep on the methanogen populations (23). It has been suggested that CT may directly act on methanogens depending on their chemical structures and methanogen species. In pure culture systems, a polymetric fraction in CT inhibited the growth of *Methanobrevibacter ruminantium*, whereas oligomeric CT was not effective (52).

The effects of tannins on rumen fermentation and methane production have also been widely investigated. Hariadi and Santoso (53) evaluated *in vitro* the inhibitory effects of seven tropical plants containing different levels of tannins. They found a negative correlation ($r = -0.76$) between tannin content and methane production. A linear decrease in methane production was observed with increasing CT supplementation of rumen fluid from steers fed quebracho CT (54). Mixtures of HT (gallotannin) and CT (leucocyanidin) also decreased pH, total VFA concentration, and total gas and methane production in ruminal fluid *in vitro* (44). However, methane emission was not reduced by CT from quebracho trees in beef cattle even though total VFA and acetate concentrations were linearly decreased by CT supplementation (55). Dietary supplementation of *Acacia mearnsii* extract (615 mg CT/kg dry matter) in sheep decreased ruminal ammonia and acetate concentration and methane production, but did not affect total VFA concentration (56). The same CT extracted from *Acacia mearnsii* was also tested in grazing dairy cows (57). Low and high CT (163 to 326 g/d; 0.31 to 0.63 g/kg live body weight) supplementation decreased methane production in this experiment. Other studies using different sources of tannins have been reported. There was no difference in total daily methane emissions in cows grazing sulla, a CT-containing legume, compared with cows grazing perennial ryegrass (58). Gallocatechin has been found as the major CT component (50 to 66 %) in sulla leaves (59). Inclusion of *Leucaena leucocephala* containing CT in sheep diets increased ruminal ammonia and methane production, while ruminal VFA were not affected (60). An increase in methane production by HT was found in another study using sheep (61). Methane production was increased

in that study by 22% in sheep fed on a hay/concentrate-based diet supplemented with chestnut wood extract at 10.1 g HT/kg feed. Results in literature should be interpreted with caution because the chemical composition of CT and HT vary depending on their source plants as previously discussed.

Tannins are known to suppress feed intake and have negative effects on animal production (62). According to a review (63), however, forages containing moderate concentration of CT (2 to 5% dry matter) have beneficial effects on productivity in ruminants without decreasing feed intake, whereas higher concentration (6 to 12 % dry matter) depresses feed intake and productivity. This is in accordance with a sheep study that quebracho CT supplementation of up to 1.5 g/kg BW (8.3 % dry matter) did not affect feed intake in sheep (64). Higher dose of tannins (3.0 g/kg BW) decreased feed intake in this study. The inhibitory effects on feed intake were not found up to 2% CT in beef cattle (55). Cows grazing sulla had higher intake and produced more milk solids than cows grazing perennial ryegrass pasture (58). It should be noted that the nutritive value of sulla was higher than that of the ryegrass in this experiment. Beneficial effects of tannins on productivity were observed in beef cattle. The CT administration (1 or 2 % dry matter intake) into the rumen in grazing steers increased average daily gain by 15 % (54). It was hypothesized that the positive effects on productivity in this study were due to a decrease in methane production and frothy bloat by CT (54). However, CT supplementation (163 to 266 g/d; 0.31 to 0.63 g/kg live body weight) with grain in cows grazing ryegrass pasture linearly decreased milk yield due to decreased feed intake and nutrient digestibilities (57).

Compared with other PBAC, tannins have been relatively effective mitigating enteric methane emission in ruminants by inhibiting both protozoa and methanogens. However, excessive amounts of tannins are likely to have adverse effects on feed intake and productivity.

Saponins

Saponins are glucosides with foaming characteristics in which sugars are attached to polycyclic aglycones such as triterpenes (C₃₀) or steroids (C₂₇), forming triterpenoid or steroid saponins, respectively (Figure 3). It is known that triterpenoid saponins are more widely found in nature than steroid saponins (65). The foaming ability and different biological properties of saponins depend on the modifications of aglycone moieties and number of sugars (66).

Studies have reported that saponins had antimicrobial effects on bacteria, protozoa, and methanogens in the rumen or rumen fluid *in vitro* (67). Steroidal saponins extracted from *Yucca schidigera* had the antibacterial effects on cellulolytic and amylolytic bacteria *in vitro* (68). Addition of the *Yucca schidigera* extract (1% vol/vol) inhibited growth of *Butyrivibrio fibrisolvens* and *Streptococcus bovis*, and protozoa activities were decreased by the *Yucca schidigera* extract in this study. Steroidal saponins from *Yucca schidigera* also inhibited *Streptococcus bovis*, *Ruminococcus* spp., and *Fibrobacter succinogenes* (69). *Yucca schidigera* extract decreased rumen ammonia concentration and protozoal counts and increased propionate concentration in beef heifers (70).

Similarly, saponins from *Quillaja* tended to decrease ruminal protozoa numbers in heifers (47). Supplementation *Enterolobium cyclocarpum* containing triterpene saponins (0.80 mg/g dry matter) in sheep decreased protozoal numbers by an average 25% (71). It is known that reduction in rumen methanogen population is accompanied with decreased protozoal counts by saponins. Saponins extracted from *Carduus*, *Sesbania*, and *Knautia* leaves and fenugreek seeds decreased both protozoal counts and methanogen population *in vitro* (72).

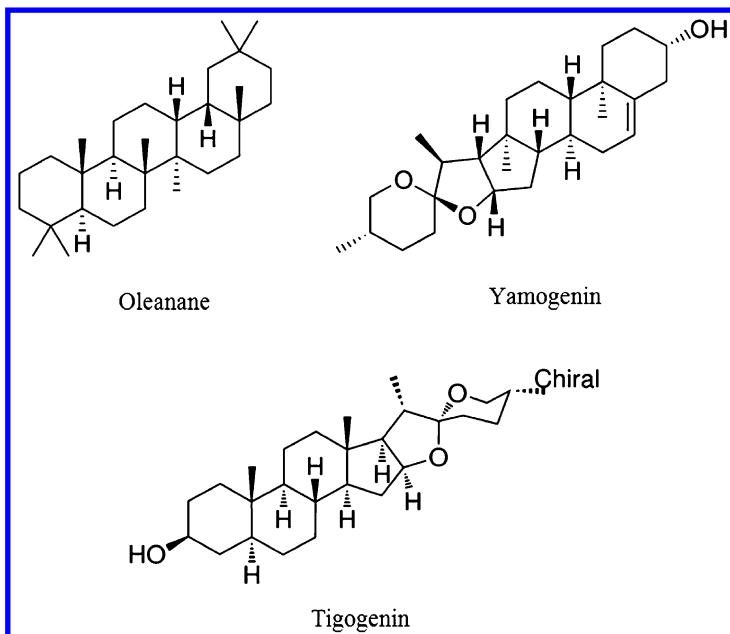


Figure 3. Triterpenoid and steroidal saponins. Oleanane is a triterpenoid; yamogenin and tigogenin are steroids.

Due to their antimicrobial properties, saponins can modify rumen fermentation and decrease methane production (26, 30, 73). A meta-analysis using 23 *in vitro* studies showed that increasing levels of saponin-rich sources linearly increased total VFA concentration and propionate proportion, and linearly decreased acetate proportion, ammonia concentration, and methane and total gas production (4). Results reported for saponins *in vitro*, however, do not always correspond to results from *in vivo* studies. Whole-plant powders from *Yucca schidigera* and *Quillaja saponaria* decreased propionate proportion, ammonia concentration, and methane production in ruminal fluid *in vitro* (74). In a follow-up study with dairy cows, however, dietary supplementation of the same saponin sources (10 g/kg dry matter) did not affect VFA and ammonia

concentrations, methane production, protozoal counts, and milk production (74). Factors that may be responsible for the observed discrepancies between *in vitro* and *in vivo* studies include composition of the basal diets, saponins application rate, and potential adaptation of the ruminal microbes. Similarly, *Yucca* powder (supplemented at 3 g/kg dry matter) or *Yucca schidigera* saponin extract did not affect methane production and milk yield in other *in vivo* studies using dairy cows (46, 75, 76).

Effects of Plant-Derived Bio-Active Compounds on Animal Immunity

Research on the effects of PBAC on immunity is relatively new area in ruminants. Immunoregulatory effects of PBAC have been widely studied in monogastric animals (77, 78), but is a relatively new area of research in ruminants. Essential oils have been shown to exhibit regulatory effects on immune cells *in vitro* and *in vivo* in monogastric animals. The immunoregulatory effects of anethol, *Capsicum* oleoresin, carvacrol, eugenol, garlic extract, cinnamaldehyde, and turmeric oleoresin was demonstrated in a comprehensive *in vitro* study using porcine macrophages (79). Oleoresins are liquids extracted from plants by solvents such as hexane, acetone, and ethanol, and their constituents are different depending on the cultivars and extraction methods (80). *Capsicum* and turmeric oleoresin are obtained from fruits of *Capsicum* plants and the rhizomes of *Curcuma longa*, and the main active compounds are capsaicin and curcumin, respectively (80, 81). All EO used in this experiment linearly decreased tumor necrosis factor (TNF)- α concentration in the culture of porcine macrophages immunologically challenged by lipopolysaccharide. The results from this study indicated that EO had anti-inflammatory effects on porcine macrophages because TNF- α is a pro-inflammatory cytokine produced by the macrophages. Interestingly, the EO, except turmeric oleoresin, increased TNF- α concentration when lipopolysaccharide was not added to the macrophages. Thus, the EO in this study modulated the pro-inflammatory cytokine depending on immune challenges (79).

The anti-inflammatory effects of EO resulted in improved productivity in pigs. Turmeric oleoresin supplementations (10 mg/kg dry matter) improved gain to feed ratio, which was accompanied with decreased pro-inflammatory responses including rectal temperature, pro-inflammatory cytokines (TNF- α and IL-1 β), and an acute phase protein (haptoglobin) in pigs challenged with porcine reproductive and respiratory syndrome virus (82). A mixture of EO (*Capsicum* oleoresin and turmeric oleoresin) facilitated cells related to the adaptive immune responses in chicken (83). Dietary supplementation of the EO mixture increased subpopulations of peripheral blood lymphocytes such as MHC class II, CD4, CD8, TCR1, and TCR2-positive cells in this study. The immunoregulatory effects of EO demonstrated in monogastric animals could be similarly exerted in ruminants, if EO bypass the rumen escaping ruminal degradation by rumen microbes. Indeed, it was reported that abomasal infusion (2 g/d; 3.7 mg/kg live body weight) of *Curcuma* oleoresin, garlic oil, and *Capsicum* oleoresin increased

CD4 positive T cells in dairy cows (5). Recently, dietary supplementation of *Capsicum* oleoresin in dairy cows linearly increased numbers of neutrophils and eosinophils in blood (Table 1) (6). In addition, phagocytic activity of neutrophils as expressed in mean fluorescence intensity tended to be quadratically increased by *Capsicum* oleoresin in this experiment. The authors concluded that dietary supplementation of *Capsicum* oleoresin could facilitate blood cells related to the acute phage immune responses in dairy cows.

Conclusion

In vitro and *in vivo* studies with ruminants indicate that PBAC such as EO, tannins, and saponins have potential to modify rumen microbial fermentation, decrease methane emission, and increase animal productivity. However, most experiments with PBAC have been conducted *in vitro* and results are inconsistent among studies. Animal diet, composition of PBAC, and application rate are important factors that need to be considered when interpreting data from studies with PBAC. In addition, differences in application units for PBAC in literature make it difficult to compare results between studies. The ability of the rumen microbes to adapt to PBAC has rarely been studied and must be addressed by future research. Apart from modification of rumen fermentation, PBAC may exhibit modulatory effects on immunity of ruminant animals as demonstrated in monogastric species. For the latter effect to take place, PBAC have to be protected from microbial degradation in the rumen, so that they exhibit physiological effects in the lower gut. Mode of action and mechanisms involved in the regulatory effects on immunity need to be elucidated and effects have to be verified in long-term *in vivo* studies.

Table 1. Effects of Dietary *Capsicum* Oleoresin on Blood Cell Counts, Neutrophil Phagocytosis, and T Cell Phenotypes in Dairy Cows^a

Item	Treatment ^b				SEM ^c	P-value ^d		
	Control	C250	C500	C1000		Con vs. T	L	Q
White blood cells, 10 ³ /µL	7.06	7.29	7.06	8.01	0.298	0.29	0.04	0.36
Neutrophils	3.59	3.75	3.62	4.56	0.252	0.19	0.01	0.33
Lymphocytes	2.99	3.00	2.91	2.85	0.076	0.47	0.16	0.89
Monocytes	0.22	0.21	0.23	0.22	0.019	0.92	0.94	0.68
Eosinophils	0.25	0.31	0.29	0.37	0.038	0.02	0.01	0.46
Basophils	0.01	0.02	0.01	0.02	0.005	0.27	0.30	0.99
As % of total								
Neutrophils	51.1	51.1	51.7	55.1	1.31	0.14	<0.01	0.43
Lymphocytes	42.3	41.6	41.0	37.0	1.20	0.04	<0.01	0.30
Monocytes	3.07	2.81	3.16	2.82	0.20	0.54	0.57	0.72
Eosinophils	3.41	4.25	4.02	4.78	0.496	0.03	0.04	0.34
Basophils	0.15	0.23	0.17	0.24	0.048	0.22	0.27	0.96
Neutrophils:lymphocytes	1.26	1.28	1.29	1.73	0.084	0.06	<0.01	0.24
Neutrophil phagocytosis								
Positive cells, %	77.8	81.2	82.3	75.9	4.09	0.48	0.78	0.20

Continued on next page.

Table 1. (Continued). Effects of Dietary *Capsicum* Oleoresin on Blood Cell Counts, Neutrophil Phagocytosis, and T Cell Phenotypes in Dairy Cows^a

Item	Treatment ^b				SEM ^c	P-value ^d		
	Control	C250	C500	C1000		Con vs. T	L	Q
MFI ^e	143	177	181	140	19.9	0.31	0.84	0.08
T cell phenotypes, %								
CD4+CD25-	9.50	8.03	6.98	7.83	1.297	0.15	0.32	0.21
CD4-CD25+	8.00	8.41	8.40	7.91	0.757	0.75	0.83	0.51
CD4+CD25+	10.8	12.5	11.9	11.3	1.32	0.19	0.93	0.15
Total CD4	20.3	20.5	18.9	19.2	1.24	0.64	0.46	0.79
Total CD25	18.8	20.9	20.3	19.2	1.95	0.31	0.95	0.20
CD8+γδ-	6.08	4.97	5.81	5.40	0.801	0.41	0.72	0.72
CD8-γδ+	8.62	9.34	9.38	8.73	0.589	0.49	0.95	0.31
CD8+γδ+	2.69	2.25	2.36	2.18	0.258	0.16	0.25	0.57
Total CD8	8.77	7.22	8.17	7.57	0.856	0.29	0.53	0.65
Total γδ	11.3	11.6	11.7	10.9	0.70	0.91	0.64	0.47

^a Data are from reference (6). ^b Control = 0 mg/d; C250 = 250 mg/d; C500 = 500 mg/d; C1000 = 1,000 mg/d of *Capsicum* oleoresin. ^c n = 32 for T cell phenotypes and neutrophil phagocytosis, n = 64 for other variables (n represents number of observations used in the statistical analysis). ^d Con vs. T = control vs. treatment; L = linear effect of *Capsicum* oleoresin; Q = quadratic effect of *Capsicum* oleoresin. ^e MFI = mean fluorescence intensity, arbitrary units.

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Chapter 12

Essential Oils as Powerful Antioxidants: Misconception or Scientific Fact?

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Many aromatic plants and spices are well-known for their various beneficial effects on human health. Recently, a considerable number of studies are focused on antioxidant activity of essential oils and aromatic plants. Considerable number of articles revealed that volatile compounds and essential oils possess strong radical scavenging capacity and ability to inhibit lipid peroxidation, especially in food and cosmetic products. However, the chemical nature of essential oils and enormous variability of oil composition made comparison of the published results very difficult. The other serious problem in interpretation of the published results is large diversity of analytical methods for determination of antioxidant capacity. These assays differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions, and in the form that the results are expressed. A single-substance/single-assay produces relative results whenever a complex mixture is involved. Therefore, a multiple-test and a simultaneous chemical characterization must be taken into account whenever assays of essential oils are performed. Furthermore, volatile compounds in essential oil, beside their protective and antioxidant activity can also act as prooxidant, by affecting the cellular redox status and damage cellular biomolecules, in the first instance proteins and DNA. All these must be taking into account when antioxidant properties of essential oils are considered. However, summing

up the large numbers of recent publications, one can conclude that essential oils of plant species like oregano, thyme, sage, lemon balm, basil and some other aromatic plants are of considerable importance as the source of natural antioxidant substances.

Introduction

The number of new pharmaceutical and dietary products based on natural raw materials has been constantly growing on the global market. Scientific research has confirmed a wide range of biological and pharmacological activities for a variety of natural products, regardless of whether they are isolated compounds or complex extracts. In the past several decades considerable interest is devoted to secondary metabolites and their antioxidant activity. This came as a result of numerous scientific studies that revealed that oxidative stress, defined as an imbalance between levels of various oxidant molecules and antioxidants, leads to many biochemical changes and, consequently, to serious disorders in the human organism. Oxidative stress can damage basic biomolecules, such as lipids, proteins and DNA, leading to cytotoxic and genotoxic effects (1, 2). Today, it is evident that mutagenicity and other adverse effects of reactive oxygen species (ROS) are involved in aging, atherosclerosis, cancer, diabetes, and several neurodegenerative diseases (3, 4). According to Halliwell and Gutteridge (5) "antioxidant is any substance that delays, prevents or removes oxidative damage to a target molecule". Antioxidant actions *in vivo* or in food may be through inhibiting generation of ROS, or by direct scavenging of free radicals (6). Good antioxidant is a molecule that reacts with ROS at low concentrations and the product of its oxidation is either stable chemical species or can be easily recycled back to an active antioxidant (7). Besides endogenous antioxidants (antioxidant enzymes, glutathione, uric acid, α -tocopherol, ascorbic acid, coenzyme Q10, bilirubin, dihydrolipoic acid, metal chelators proteins), exogenous antioxidants, especially those of natural origin, are of great importance for human health in prevention of ROS and oxidative stress damage.

While antioxidant efficacy can be demonstrated in one system, it can fail to protect other systems, or sometimes even causes damage. Typical example is one of the most abundant synthetic antioxidants, butylated hydroxyanisole (BHA), that is a powerful inhibitor of lipid peroxidation but in high doses it induces cancer of the forestomach in rats, most probably via oxidative damage of DNA (8). Therefore, its replacement with natural, non-harmful antioxidants is of great importance. Numerous natural products are already recognized and widely applied as antioxidants. The major classes are vitamins (L-ascorbic acid and α -tocopherol), carotenoids (carotenes and xanthophyll) and polyphenols (flavonoids, phenolic acids, tannins, stilbenes, lignans).

Many aromatic plants and spices are well-known for their various beneficial effects on human health. The use of aromatic plants and spices in phytotherapy is mostly related to different activities of their essential oils, such as antimicrobial, spasmolytic, carminative, hepatoprotective, antiviral, and anticarcinogenic

activities (9–11). Furthermore, essential oils and spices have great potential in the emerging nutrition industry because they are often treated as food, as well as medicine, as well, and are used in prevention and curative treatments throughout the world (12). Besides, many spices and essential oils are widely used in the food industry to improve flavor and organoleptic properties, but also to slow down the process of deterioration of foodstuffs. The latter is mainly due to their antimicrobial and antioxidant activities. According to Van de Braak et al. (13) there are more than 3000 plants used for their essential oils and more than 300 are used commercially as flavor and fragrance (14). On the other hand, although most essential oils have Generally Recognized As Safe (GRAS) status, and are generally accepted as nontoxic substances, in higher doses certain essential oils and their compounds are revealed to have numerous adverse effects to human health such as allergenic reactions, neurotoxicity, abortifacient, teratogenic (15). Nevertheless, investigations of aromatic plant spices and essential oils constantly reveal their new biological activities and commercial properties.

Current State of Research on Antioxidant Potential of Essential Oils: Facts, Speculation, Shortcomings, and Expectation

Recently, a considerable number of studies are focused on antioxidant activity of essential oils and aromatic plants. Most of them confirm the assumption that essential oils are promising natural antioxidants. However, the chemical nature of essential oils and enormous variability of oil composition made comparison of the published results very difficult. From a chemical point of view, essential oil is a complex mixture constituted of up to a hundred components. Chemical composition is influenced by genetics, physiological and various environmental factors such as geographic variation, environmental and agronomic conditions, harvest time, phenological stage of plants and extraction methods (16).

Plant extracts are complex mixtures and reports of antioxidant activities evaluated by different tests are not always concordant. Accordingly, it is realistic to assume that antioxidant activity of the entire oil is the result of the interaction of all constituents, from those present in a greater proportion to those present in minor amounts. This interaction may produce a synergistic effect, when the interaction enhances the effect of the oil, or an antagonistic effect, when the interaction negatively affects the antioxidant activity of the studied oil, which makes it very important to investigate the antioxidant properties of essential oils without considering only its major components (17). However, in order to find structure-activity relationships it is necessary to investigate antioxidant potential of the particular oil constituents. Ruberto and Barata (18) analyzed the antioxidant effectiveness of about one hundred pure components of essential oils in two model systems by measuring the formation of primary (hydroperoxydienes) and secondary (malondialdehyde) components of the oxidative process of a lipid matrix. Among all of the volatile compounds tested, two phenols thymol and carvacrol were the most active. Evidence that the presence of the phenolic group in volatile compounds is most responsible for antioxidant activity was previously confirmed by many studies (19, 20). However, in the same study,

three monocyclic aliphatic components, terpinolene, α -terpinene and ϕ -terpinene, and to a less extent, sabinene, a bicyclic one, showed a very high activity. It was proposed that the presence of strongly activated methylene groups is responsible for this behavior (18). Several papers report on antimutagenic activity of essential oils like basil, myrtle, eucalyptus, and their compounds (linalool, myrcene, eucalyptol). It was proposed that inactivation of mutagens, and subsequent prevention of DNA damage, is in accordance with their radical scavenging activities (21–24). The distribution of the oil in the cell is also very important for their anti- or pro-activity (25).

Volatile compounds in essential oil, beside their protective and antioxidant activity can also act as prooxidants, by affecting the cellular redox status and damage cellular biomolecules, in the first instance proteins and DNA (25). Conversion of antioxidant into prooxidant is concentration dependent. It was found that some terpenoids such as thymol, carvacrol and γ -terpinene when present in low concentration protected DNA from strand breakage, whereas when present in high concentration increased DNA damage (26).

The other serious problem in interpretation of the published results is large diversity of analytical methods for determination of antioxidant capacity. These assays differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions, and in the form that the results are expressed. Even when only one of these assays is considered, different antioxidant standard compounds, solvents, reaction time and pH are frequently applied (27). A single-substance/single-assay produces relative results whenever a complex mixture is involved. Therefore, a multiple-test and a simultaneous chemical characterization must be taken into account whenever assays of essential oils are performed (28).

Current Methodology Procedures

Most commonly, antioxidant activity of essential oils is assessed in regard to their ability to neutralize and scavenge ROS/NOS and inhibit lipid peroxidation in different *in vitro* and *in vivo* systems. According to Prior et al. (29) when selecting methods the following requirements/criteria should be followed: (i) utilization of biologically relevant molecules; (ii) technically simple; (iii) with a defined endpoint and chemical mechanism; (iv) readily available instrumentation; (v) good repeatability and reproducibility; (vi) adaptable for assay of both hydrophilic and lipophilic antioxidants; (vii) and adaptable to high-throughput analysis.

Considering radical scavenging capacity, two categories according to the type of the oxidant species are recommended: (i) scavenging capacity assays against specific ROS/RNS; (ii) scavenging capacity assays against stable, non-biological radicals and evaluation of total reduction capacity (27).

Scavenging capacity assays against specific ROS/RNS include: (i) *Peroxyl radical (ROO[•]) scavenging capacity assays*. The oxygen radical absorbance capacity - ORAC; (ii) *superoxide radical anion (O₂^{•-}) scavenging capacity assays*; (iii) *hydrogen peroxide (H₂O₂) scavenging capacity assay*; (iv) *hydroxyl radical (HO[•]) scavenging capacity assays*; (v) *hypochlorous acid (HOCl) scavenging capacity*; (vi) *singlet oxygen (¹O₂) scavenging capacity assay*; (vii) *nitric oxide radical (NO[•]) scavenging capacity assays*.

Scavenging capacity assays against stable, non-biological radicals and evaluation of total reduction capacity include: (i) *Scavenging of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (ABTS⁺) or Trolox equivalent antioxidant capacity (TEAC)*; (ii) *Scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[·] assay)*; (iii) *Ferric reducing antioxidant power (FRAP assay)*; (iv) *Folin-Ciocalteu reducing capacity (FC assay)*; (v) *cupric ion reducing antioxidant capacity (CUPRAC)*.

Depending on the mechanism of the antioxidant action, these methods may be classified as electron transfer (ET) and hydrogen atom transfer (HAT)-based assays. ET assay includes the ABTS/TEAC, CUPRAC, DPPH, Folin-Ciocalteu and FRAP, whereas HAT assay includes ORAC assay, radical-trapping antioxidant parameter (TRAP assay), crocin bleaching assay using AAPH as radical generator, and β-carotene bleaching assay (30).

A very important criterion in the evaluation of antioxidant efficacy, especially in the food and organic media, is the extent of inhibition of peroxidation of polyunsaturated fatty acids (PUFAs) in membrane lipids. There are several measurement strategies and techniques. Most commonly, they are based on formation of either primary or secondary oxidation products. Hydroperoxides are considered as primary, whereas simple volatile molecules (aldehydes, ketones, alcohols, short chain carboxylic acids and hydrocarbons), derived mainly from hydroperoxide decomposition, are considered to be the secondary products. The latter is suitable for studying the model lipid systems (liposomes), as well as lipids isolated from their natural environment (microsomes, LDLs) (31). The most convenient methods for assay lipid peroxidation (LP) are based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA). Formation of the red-coloured MDA-TBA adducts is measured at 532 nm. However, this method has been criticized primarily because of low specificity of TBA towards various carbonyl compounds. MDA quantity can be measured by UV spectrophotometric methods, but also by HPLC with DAD or fluorimetric detectors and by GC-FID after derivatization (32).

Recent Knowledge and Our Experiences in Antioxidant Potential of Aromatic Plants and Essential Oils

Although essential oil is produced by plants that belong to over 60 plant families, some of them are distinguished as the most promising source of essential oils with strongest antioxidant potential. Some recent and comprehensive studies showed that plants belonging to Lamiaceae, Myrtaceae, Apiaceae, Cupressaceae, and Asteraceae families are of special significance (33–35). Therefore, this chapter summarizes articles related to antioxidant properties of some plants belonging to those families, with special emphasis on our own experiences.

Lamiaceae Family

The family of Lamiaceae consists of about 230 genera and 7100 species worldwide (36). Many species of the Lamiaceae family are considered of high

importance because of their uses in medicine, culinary, and cosmetics (36). In addition, many plants are considered as important sources of compounds with antioxidant activity (37, 38). However, emphasis was given mainly to their solvent extracts, but not volatile constituents (39, 40). Two spice plants classified in this family were first recognized as the most powerful natural antioxidants: sage (*Salvia officinalis* L.) and rosemary (*Rosmarinus officinalis* L.) (41–43). In rosemary extract, several phenolic diterpenes and other phenolic acids were identified as major antioxidants: rosmanol, rosmarinidiphenol, carnosol, rosmarinic and carnosic acid (44). In many other Lamiaceae species (thyme, oregano, savory, mint) flavonoids, alone or together with other phenolic compounds, have been found to contribute to the overall antioxidant ability of the essential oil (45, 46).

Although the phenolic compounds are recognized as being responsible for the antioxidant ability, recent studies showed that volatile compounds could also, individually or in mixtures (essential oil), contribute to the whole antioxidant ability. Oregano, a characteristic ingredient of the Mediterranean cookery, has increasingly gained the interest of many research groups as a source of novel natural antioxidants. Recent studies (47–49) showed that its volatile oil is valuable antioxidants considering their ROS scavenging activity and reducing power. High antioxidant activity of oregano was attributed to the high content of the aromatic terpenoids carvacrol and thymol. The essential oil of different *Thymus* L. species, chemically very close to oregano and basil (*Ocimum basilicum* L.), possesses similar antioxidant activity as evaluated by various chemical and physicochemical assays and in different biological model systems (50, 51).

In the scope of our extensive study of alternative antioxidants from natural sources, we have also investigated the most important aromatic plants of the Lamiaceae family collected from the Balkan area. In our early study (52) we examined the antioxidant activity of the essential oil of the three *Mentha* L. species: *M. aquatica*, *M. longifolia* and *M. piperita*. Antioxidant capacity was evaluated by measuring radical scavenging capacity (RSC) towards DPPH[•] and hydroxyl (HO[•]) radicals. In both tests, *M. piperita* exhibited the highest activity and *M. aquatica* the lowest one. Scavenging capacity of the peppermint oil was within the range of the one showed by commercial antioxidant BHT. By using the combined DPPH/TLC test (dot-blot), C-3 monoterpane ketones (menthone, isomenthone and pulegone) were proposed as the most active compounds. Similar results on the high ROS scavenging capacity, particularly showed by L-menthone, were previously reported (53, 54). However, numerous *in vitro* and *in vivo* studies show that the main antioxidants in mints are phenolic compounds, mainly flavonoids and phenylpropanoid derivatives (46). Lemon balm (*Melissa officinalis* L.) is also very well known medicinal and spice plant. The essential oil of this plant is known to possess strong antibacterial and antifungal activity and also has mild antidepressant and spasmolytic properties. In our previous study, we examined the antioxidant capacity of the essential oil of lemon balm, by measuring its DPPH and OH radicals scavenging activities, as well as its ability to inhibit Fe²⁺/ascorbate and Fe²⁺/H₂O₂, induced LP in liposomes as oxidizable substrates. In all of the systems applied, lemon balm oil exhibited the higher antioxidant activity than the synthetic antioxidant, BHT. GC-MS analysis showed

that the main compounds in the oil were citronellal at 13.7%, nerol at 16.5% and geranial at 23.4% (55). Our further studies were focused on the most popular spice plants as basil (*Ocimum basilicum* L.), oregano (*Origanum vulgare* L.) and thyme (*Thymus vulgaris* L.). The same antioxidant tests as in the above mentioned studies were performed. The highest DPPH[•] scavenging activity was expressed by oregano and thyme oils, while a somewhat lower activity was shown by the basil oils. However, all of the examined essential oils were found to be more potent radical scavengers than BHT. Similar results were obtained for HO[•], where the thyme oil was the most active, but neither the essential oils nor BHT achieved 50% of inhibition of HO[•] generation from 2-deoxyribose. All the three samples strongly inhibited LP in liposome, regardless of the fact that it was induced by Fe²⁺/ascorbate or Fe²⁺/H₂O₂. In the first system, oregano and basil exhibited similar and the highest activity, while in the second system the thyme oil showed a somewhat higher activity. Considering the results of GC-MS analysis and DPPH-TLC (dot-blot) assay, one can conclude that oxygenated monoterpenes (carvacrol, thymol and methyl chavicol) were most responsible for the high activities of the essential oils obtained from these three spice plants (56). Very similar results were obtained with rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) essential oils. Both oils exhibited moderate antioxidant capacity. It was proposed that the most active compounds were bicycle oxygenated monoterpenes: α - and β -thujone, camphor, and bornyl acetate (57). In addition, we found that nepetalactone the main constituent of essential oil of *Nepeta nuda* L., ssp. *nuda*, is mostly responsible for DPPH[•] scavenging activity and inhibition of LP (58). Close relationship between antioxidant efficiency and the chemical composition of the essential oil is proved by several studies. As expected, the phenolic hydroxyl groups, such as in thymol, carvacrol and methyl chavicol is the structural feature mostly responsible for the antioxidant activity of the essential oil.

Apiaceae Family

Apiaceae family consists of over 3700 species, distributed over more than 400 genera (the classification is still a matter of debate). Numerous plants from Apiaceae family are commonly used as vegetables or spices due to high content of essential oils with desirable flavor. The most common examples include carrot (*Daucus carota*), parsley (*Petroselinum crispum*), parsnip (*Pastinaca sativa*), dill (*Aethum graveolens*), anise (*Pimpinella anisum*), fennel (*Foeniculum vulgare*), caraway (*Carum carvi*), celery (*Apium graveolens*), chervil (*Anthriscus cerefolium*), coriander (*Coriandrum sativum*), and cumin (*Cuminum cyminum*) among others. In addition to culinary usage, some species have found their place in traditional and, sometimes, official medicine, due to the presence of a wide range of natural products, some of which are well known as strong antioxidants. Here we tried to summarize the results concerning antioxidant capacity of essential oils obtained from some Apiaceae plants. Most data came from comparative studies undertaken by Wei and Shibamoto (2007) (59), Kiralan and associates (2012) (60), and Farag and El-Khawas (1998) (61).

Angelica (*Angelica archangelica* L.). Numerous studies confirmed strong antioxidant and protective properties of angelica extracts, while only several studies investigated the essential oil. It was reported that nor *Angelica archangelica* nor *Angelica sinensis* (Oliv.) expressed significant antioxidant and radical scavenging activity (59, 62).

Anise (*Pimpinella anisum* L.). Kiralan and associates (2012) reported on chemical composition and antioxidant activity of essential oils isolated from six plants from Iraq, including anise (60). The main component of the oil was *trans*-anethole, amounting up to 93.5%, and it was accompanied by traces of other phenolic derivatives (the most abundant being estragole at 1.7 %) and terpenoids (with γ -himachalene as the most abundant at 1.8 %). The authors demonstrated anti-radical activity (as determined by DPPH assay) of the oil, however it was not compared to any reference compound.

Celery (*Apium graveolens* L.). Nagella and co-workers (2012) (63) evaluated antioxidant activity of the essential oil from celery leaves, obtained by hydrodistillation. The oil was able to neutralize DPPH radical, although to a lesser extent than the reference substance α -tocopherol. Kiralan and co-workers (60) demonstrated the ability of celery seed essential oil to scavenge DPPH radicals (although activity was not compared to any reference antioxidant). The investigated oil consisted predominantly of limonene at 76.6%), accompanied by 11.1% of β -selinene and minute amounts of other terpenoids. Although essential oil obtained from celery did not exhibit considerable antioxidant activity, organic solvent extracts showed strong antioxidant and hepatoprotective activity both in *in vivo* and *in vitro* studies (64).

Parsley (*Petroselinum crispum* (Mill.) Hill. In comparative study by Wei and Shibamoto (59), ability of the parsley seed oil to scavenge DPPH radical, to prevent oxidation of hexanal, and to prevent lipid peroxidation of squalene, was investigated. The studied oil was dominated by phenylpropanoids myristicin at 44.0% and apiole at 12.1%), followed by monoterpenes α - and β -pinene at 15.5% and 11.7%, respectively. Of the 13 investigated oils, parsley oil was among the most potent regarding the ability to prevent hexanal oxidation. The concentration of 500 μ g/mL provided nearly 100% inhibition over the course of 40 days, comparable to α -tocopherol. The ability to scavenge DPPH radical was less pronounced, reaching only about 50% inhibition at 200 μ g/mL. Finally, it showed inhibition of UV-initiated lipid peroxidation of squalene in a dose-dependent manner, exhibiting the strongest ability among the investigated oils. The observed activity is ascribed to phenylpropanoids, as pinene-rich *Juniperus* essential oil was much weaker antioxidant. Kiralan and co-workers (61) also investigated antioxidant properties of parsley seed oil. Under the experimental conditions, the oil was able to scavenge about 59% of DPPH radical. The investigated oil represented mainly a mixture of α - and β -pinene at 22.9% and 19.2%, respectively, limonene at 11.3%, 1,2,3,4-tetramethoxy-5-(2-propenyl) benzene at 13.6% and apiole at 13.8%.

Wild carrot (*Daucus carota* L.). Ksouri and co-workers (2015) (65) compared the chemical composition and antioxidant effects of wild carrot fruits and leaves essential oil and methanolic extracts. The chemical composition of fruits and leaves essential oil differed significantly. Leaf oil represented a mixture of several

dominant terpenoids α -pinene at 27.4%, β -pinene at 25.3%, germacrene at 16.3%, alongside a number of minor components of terpenoid origin. On the other hand, fruit oil was dominated by geranyl acetate at 52.4%, accompanied by cedrone S at 14.0%, (E)-asarone at 11.4%, β -bisabolene at 4.8% and numerous minor components. While both oils were able to neutralize DPPH $^{\bullet}$ in dose-dependent manner, IC₅₀ values were extremely high (by four orders of magnitude higher than IC₅₀ of ascorbic acid). At the same time, methanolic extracts performed much better indicating that, in this species, the main antioxidants are non-volatile compounds such as phenols. Both oils (especially seed oil) were also able to neutralize products of lipid peroxidation, to a somewhat lesser extent than α -tocopherol (65).

Caraway (*Carum carvi* L.). Samojlik et al. (2010) (66) investigated antioxidant activity of caraway fruit essential oil by DPPH and H₂O₂ scavenging assays, and lipid peroxidation inhibition assay. The oil, obtained in a high yield of 5.8%, was found to consist predominantly of oxygenated monoterpenes, with carvone at 78.8% as the most abundant compound. While the results of H₂O₂ scavenging assays were limited, the essential oil did manage to scavenge DPPH radical, although the IC₅₀ value of 4.1 μ L/mL was high. The most active components were identified by TLC-DPPH to be *trans*-anethole, carveole isomers, carvone and menthol. Caraway oil was also found to inhibit Fe²⁺/ascorbate-induced lipid peroxidation, with IC₅₀ < 2.5 μ L/mL. Farag and El-Khawas (61) studied the ability of several Apiaceae essential oils to inhibit peroxidation of sunflower oil during storage. Caraway essential oils isolated from untreated, γ -irradiated or microwaved fruits were able to slow down rancidification to a significant extent – time required to reach peroxide value of 15 meq/kg was prolonged by about 30%, regardless of the fruit treatment. The effect was comparable to that of BHT+BHA mixture applied at the same concentration.

Coriander (*Coriandrum sativum* L.). In aforementioned study by Samojlik et al. (66), the chemical composition and antioxidant activity of coriander fruit essential oil were also investigated. It was found that the oil consisted almost entirely of oxygenated monoterpenes, with linalool as the single most abundant component at 74.6%, followed by camphor at 5.9% and geranyl acetate at 4.6%. Coriander oil was less active than caraway oil towards DPPH radical, with high IC₅₀ of 54 μ L/mL, while the effects on lipid peroxidation were markedly pro-oxidant (66).

Cumin (*Cuminum cyminum* L.). Topal and coworkers (2008) (67) investigated the ability of a number of essential oils obtained by steam distillation and supercritical CO₂ extraction, to scavenge DPPH radicals. Cumin extract was among the most potent oils, inhibiting 92.0% of present DPPH $^{\bullet}$ (the reference antioxidant BHT inhibited 91.4% of present DPPH $^{\bullet}$), while hydrodistilled oil inhibited less than 60% of present DPPH $^{\bullet}$. Kapoor and co-workers (2010) (68) studied black cumin or black caraway (*Carum bulbocastanum* Koch. syn. *Bunium bulbocastanum* L.), an Asian species closely related to cumin, that is used as a carminative and spice. It was able to protect linseed oil from formation of peroxides (measured as peroxide value) more efficient than synthetic antioxidants BHT and BHA, and comparable to PG. The formation of malondialdehyde (measured by TBA reagent) and 2-alkenals (as determined by anisidine value)

was also slowed down, with effect comparable to that of BHT and BHA, but weaker than PG (propyl galate). The oil was also able to scavenge DPPH radicals and to reduce ferricyanide in both cases, better than BHT and BHA, but worse than PG.

Fennel (*Foeniculum vulgare* Mill.). In comparative study by Farag and El-Khawas (61), fennel essential oil appeared to be the most potent antioxidant among the investigated oils. In other comparative study by Topal and coworkers (67), fennel essential oils isolated by hydrodistillation and supercritical CO₂ extraction was able to scavenge DPPH radicals. As with the other two Apiaceae species – cumin and anise, supercritical extract performed better, with about 80% inhibition, while hydrodistillate neutralized less than 50% of DPPH[•], making it the least active of the studied Apiacae species. The hydrodistilled oil was dominated by phenolic derivatives as anethole at 90.0% and estragole at 5.9%, with only traces of other compounds. The main component of supercritical extract was also anethole at 67.0%, but it was accompanied by monoterpenes of which limonene was the most abundant at 5.4%, the phenolic derivatives of estragole at 4.0%, *p*-anisaldehyde at 3.0%, anisyl alcohol at 2.6% and carboxylic acids as linoleic acid at 6.0%, and mandelic acid at 2.2%. Antioxidant properties of fennel essential oil were also investigated by Kiralan and co-workers (60). Under the applied experimental conditions, the oil consisting mostly of 67.0% *trans*-anethole, 12.5% of estragole and 7.3% of limonene was able to neutralize DPPH radicals, thus demonstrating radical-scavenging ability.

Safety Issue

It should be noted that, while numerous Apiaceae species represent rich sources of secondary biomolecules with potential or proven health benefits, including antioxidant activity, their use is not without risks. This is due to constituents exhibiting adverse effects, from mild irritation to potentially fatal intoxication. This factor is especially important nowadays, when general population considers medicinal plants and herbs to be "natural" and, thus, intrinsically safe. Apiole is a volatile phenylpropanoid (1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene) found in parsley and celery. While the normal dietary intake is insufficient for adverse effects, prolonged exposure to higher amounts can result in irritation, liver and kidney damage, haemolytic anaemia, and abortion (69, 70). Related compounds such as estragole (1-allyl-4-methoxybenzene), characteristic for chervil, fennel, anise and tarragon, and methyl eugenol (1-allyl-3,4-dimethoxybenzene), present in numerous Apiaceae species, are believed to be genotoxic and carcinogenic (69–71), although further research is needed. Another phenylpropanoid such as myristicin (1-allyl-3-methoxy-3,4-methylenedioxybenzene) is a hallucinogen, and also exhibits carcinogenicity and hepatotoxicity (72). Furanocoumarins are also present in Apiaceae species, such as parsley, parsnip and celery. These compounds are phototoxic, and intake by food, followed by sunlight or exposure to UVA light can be sufficient to cause rash or burns.

Table 1. Antioxidant Activity of Essential Oils of *Juniperus* Species

species	<i>IC₅₀</i> values ($\mu\text{L}/\text{mL}$ for essential oils; $\mu\text{g}/\text{mL}$ for standards)			
	<i>DPPH</i> [•]		<i>LP</i>	
	leaves	seed cones	leaves	seed cones
<i>J. communis</i>	8.1 ± 0.5	9.0 ± 0.1	(10.0 ± 0.56) × 10 ⁻³	(22.8 ± 1.47) × 10 ⁻³
<i>J. macrocarpa</i>	6.9 ± 0.6	12.1 ± 0.76	(57.3 ± 0.54) × 10 ⁻³	0.16 ± 0.01
<i>J. excelsa</i>	16.1 ± 1.01	20.4 ± 2.62	(43.9 ± 5.10) × 10 ⁻³	(59.8 ± 9.00) × 10 ⁻³
<i>J. foetidissima</i>	11.2 ± 0.44	>33	0.33 ± 0.00	0.22 ± 0.01
<i>J. oxycedrus</i>	25.5 ± 1.94	11.9 ± 0.81	1.57 ± 0.08	0.60 ± 0.05
<i>J. sabina</i>	27.0 ± 0.06	>33	0.38 ± 0.01	(68.2 ± 2.64) × 10 ⁻³
<i>J. phoenicea</i>	16.3 ± 0.64	25.2 ± 1.90	0.29 ± 0.05	0.86 ± 0.6
standards				
BHT		8.8 ± 0.9		19.3 ± 2.08
BHA		12.39 ± 1.15		24.1 ± 2.52

Asteraceae Family

This family is the largest family of flowering plants (Angiospermae), with more than 23 000 species. Most of the members of this family are economically important aromatic plants, known for their various biological and pharmacological properties and uses. Aromatic plants in the Asteraceae family mostly originate from the three genera: *Achillea*, *Artemisia* and *Matricaria*. These plants are commercially used as herbs and in the production of herbal teas and beverages. The most important plants from this group are yarrow (*Achillea millefolium* L.), wormwood (*Artemisia absinthium* L.) and chamomile (*Matricaria chamomilla* L.). These three aromatic plants contain azulenes, which give dark blue color to their essential oils, after yielding chamazulene during the hydrodistillation. It is found that the high content of chamazulene ensures good antioxidant activity of the essential oils (73–76).

Yarrow (*Achillea millefolium* L.) is an important medicinal plant with many different pharmaceutical uses (77–79). The pharmacological effects of yarrow are mainly due to the essential oil, proazulenes and other sesquiterpene lactones (80). Principal components of the essential oil are eucalyptol, camphor, α -terpineol, β -pinene, and borneol (81, 82). The yarrow essential oil has good antioxidant activity, manifested by reducing DPPH $^{\bullet}$ ($IC_{50} = 1.56 \mu\text{g/mL}$), scavenging HO $^{\bullet}$ ($IC_{50} = 2.7 \mu\text{g/mL}$), and inhibiting the lipid peroxidation of rat liver homogenate ($IC_{50} = 13.5 \mu\text{g/mL}$). However, the antioxidant activity of the main essential oil components e.g. eucalyptol, camphor, β -pinene, borneol, terpinen-4-ol, α -pinene was also tested, and none of them exhibited antioxidative properties. This can implicate that the main components in the total oil show synergism of antioxidant activity (81, 83, 84). It also has a protective effect against H₂O₂-induced oxidative damage in human erythrocytes and leucocytes (85).

Wormwood (*Artemisia absinthium* L.) is also a well-known medicinal and aromatic plant. Free radical scavenging and antioxidant activity of *Artemisia absinthium* has been reported both *in vitro* and *in vivo* (86, 87). The major components are chamazulene, β -thujone, camphor, 1-terpinen-4-ol, and bornyl acetate (87–89). The wormwood essential oil exhibits moderate antioxidant activity by showing ability to scavenge stable DPPH $^{\bullet}$ and reactive HO $^{\bullet}$, as well as by preventing the linoleic acid oxidation (87–90). Antioxidant activity is mostly attributed to the presence of chamazulene and camphor (91).

Chamomile (*Matricaria chamomilla* L. (synonyms *Matricaria recutita* L. and *Chamomilla recutita* (L.) Rauschert) is widely used as a food supplement and as herbal tea. Its pharmacological activity is especially associated with the essential oil components, such as α -bisabolol and chamazulene (92–95). In addition to the medicinal uses, the oil is extensively used in perfumery, cosmetics, aromatherapy and the food industry (96).

Over 120 compounds have been identified in the chamomile essential oil, with the main constituents being terpenoids such as α -bisabolol and its oxides at ~78%, farnasene at 12 to 28%, spathulenol, spiroethers including the *cis/trans*-en-yn-dicycloethers at 8 to 20%, and azulenes including chamazulene at 1 to 15% (96, 97). Although chamazulene is an artefactual component, formed from matricin during the hydrodistillation process, it is one of the most bioactive

components of the chamomile essential oil (93, 98). The quality of the essential oil is determined by its blue color, as it serves as the chemical marker for the presence of azulenes (95, 96). DPPH analysis has shown that chamomile flower essential oil has moderate antioxidant activity. On the other hand, FRAP test demonstrated that the essential oil, although modestly active in scavenging of free radicals, had a significant ferric reducing/antioxidant power at ~640 $\mu\text{mol TE}/100\text{g}$ dry weight (91, 96, 99, 100). Chamomile oil also inhibits the linoleic acid oxidation, thus showing high total antioxidant capacity (101). Chamazulene may be responsible for part of the effect, as it is one of the major components of the oil with a reduction potential in an iron free system (98, 102, 103). Although showing weak potential in DPPH test, chamazulene showed high free radical scavenging activity in the ABTS test with an IC_{50} value of 3.7 $\mu\text{g/mL}$ (101). Chamazulene was also shown to inhibit Fe^{2+} /ascorbate-induced lipid peroxidation, in a concentration and time-dependent manner (50 % inhibition with 18 μM). It also inhibited the autoxidation of DMSO by 76% (89, 102, 103). The α -bisabolol also improves the antioxidant network and restores the redox balance by antagonizing oxidative stress (104, 105). However, the antioxidant activity of the chamomile essential oil cannot be attributed to the chamazulene or α -bisabolol only. Nevertheless, it is a result of the synergistic action and contribution of all of the essential oil components (101).

Cupresaceae Family - The Genus *Juniperus*

Genus *Juniperus* L. comprises 67 species and 34 varieties . In spite of the widespread use in food preparation and beverage manufacture and the great healing power of the *Juniperus* species, there are a very few reports concerning antioxidant activity of their essential oils (59, 106–113). It should be noted that plants that are regularly used in food preparation processes, such as spices (i.e. common juniper), are particularly interesting as candidates for new food preservatives with significant antioxidant properties. In this Chapter an overview of the antioxidant capacity of the essential oils of 7 *Juniperus* species cones and leaves is presented (111). Species introduced within are widely used in cooking and traditional medicine, i.e. *J. communis* and *J. oxycedrus*, as well as species that are less utilized but could potentially be new food supplements, i.e. *J. macrocarpa*, *J. excelsa*, *J. foetidissima*, *J. sabina* and *J. phoenicea*.

Antioxidant activity of essential oils were evaluated toward inhibition of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH \cdot) and lipid peroxidation (LP; Table 1.). Results were compared with the synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Overall, results demonstrated that both essential oils, from leaves and seed cones, of *Juniperus* species showed significant antioxidant activity, with leaves expressing greater activity than seed cones. Interestingly, essential oils showed a significantly better capacity in inhibition of LP, compared with DPPH \cdot , which is probably due to the non-polar nature of the substrates and emulgators used in the assay. It is noteworthy to emphasize that the potency of the essential oils to inhibit LP is comparable with the standards, what is considered as great activity. In addition, essential oils of *J. communis* showed even better inhibition potential toward LP

compared with the standards. The analysis of the results presented herein, shows that the antioxidant activity of the essential oils of the tested species of the genus *Juniperus*, descent in the order: *J. communis* > *J. excelsa* > *J. macrocarpa* > *J. oxycedrus* > *J. foetidissima* > *J. Phoenicea* (III). In particular, the ability to inhibit LP correlates to the high percentage of sabinene in the essential oils and is up to 54%, which is known to be an effective antioxidant (18). It is known that terpenes whose structure contains activated methylene and hydroxyl groups show a pronounced capacity to neutralize radical reactions, which are the main part of the LP process (18). Presence of that category of terpenes, such as β -myrcene, limonene and terpinen-4-ol, is confirmed in significant amounts in the examined essential oils (III).

Conclusion

In this Chapter, an overview of investigations of antioxidant potential of the essential oils conducted during the last two decades, is presented. Special attention is given to the plants belonging to the Lamiaceae, Apiaceae, Asteraceae and Cupressaceae families.

The chemical nature of the essential oils, as well as their enormous variability in composition, made the comparison of the published results very difficult. Furthermore, a great diversity of the analytical methods applied and the form in which the results are expressed, is another serious obstacle in obtaining the right judgement (assessment) of the oxidative status of particular essential oils. Besides, it seems very difficult to reveal the exact mechanism of antioxidant activity of essential oils, because the overall activity is the result of the synergistic effect of many compounds with different structural properties. The presence of numerous compounds with different molecule structures and performances, makes the identification of the leading antioxidant compound very difficult. A single-substance/single-assay produces relative results whenever a complex mixture is involved. Therefore, a multiple-test and a simultaneous chemical characterization must be taken into account whenever assays of essential oils are performed.

The lipophilic character of the essential oils also obstructs and limits application of some common antioxidant tests. However, the majority of authors agree that terpenoids with phenolic groups such as carvacrol, methyl chavicol, thymol, eugenol, are the most active antioxidant principles in essential oils.

Future Prospects

Finally, although less effective than other extracts containing phenolic compounds (flavonoids and phenolic acids) obtained from aromatic plants, essential oils certainly deserve the attention of the pharmaceutical, foodstuff and cosmetic industries as promising antioxidant substances. Furthermore, due to their lipophilic character, the essential oils can very easily penetrate through the cell membranes and reach the target molecules. However, the clear evidence of their effectiveness is required to be proven in various *in vivo* biological systems.

Therefore, besides standardization of the existing methods, essential oils must also be evaluated by *in vivo* tests prior to their application in preventive medicine and food processing industry.

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Chapter 13

Breeding of German Chamomile, *Chamomila recutita* L., with the Highest Content of /-/ α - Bisabolol

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Chamomile (*Chamomila recutita* L.) is characterized by considerable variability in the amount of biologically active compounds in the essential oil such as /-/ α -bisabolol oxide A, /-/ α -bisabolol oxide B, /-/ α -bisabolol, spiro ether and chamazulene. Pharmacological properties include anti-inflammatory, antiseptic, carminative, healing, sedative, and spasmolytic activity. The world market currently has chamomile drugs of various origins and therapeutic values. The content of /-/ α -bisabolol in the flower has become an important indicator of flower quality and value. The present chapter discusses developments in the breeding process of new chamomile variety in Slovakia. Four varieties Bona, Novbona, Lutea, and Goral were bred with different characteristics in previous times. Breeding efforts during the development of variety Lianka focused on a high content of /-/ α -bisabolol and low content of /-/ α -bisabololoxides A and B. The emphasis was put on a high yield of flower inflorescences as well as the uniformity and stability of plant morphological characteristics. Seed material was isolated for testing seed maturation. TLC and GC analyzes were carried out on individual plants and in tufts. The fourth season of breeding work confirmed all contractually defined parameters of the newly bred variety which was registered in 2013 as a new variety in Slovakia. The methods of individual plant selection and “the middle seedbed” were used.

Chamomila recutita L.

Chamomile is one of the oldest plants which is gathered, bred, and used in folk medicine and is also included in the production of different phytotherapeutic and cosmetic preparations (1). Chamomile essential oil contains more than 120 compounds, with various pharmacological effects. This plant is known for its curative properties and was therefore included in the *Pharmacopoeia* in 26 countries. Pharmacological properties include anti-inflammatory, antiseptic, carminative, healing, sedative, and spasmolytic activities. One constituent, γ - α -bisabolol, is regularly recommended in the treatment of ulcers induced by alcohol and X-ray burns (2). Terpenoids, γ - α - bisabolol including their oxides, azulenes, and chamazulene are the most important compounds of the chamomile essential oil. These components are mainly extracted from flower inflorescences. Therefore, chamomile, *Chamomila recutita* L., can be considered as a star between numerous medicinal species (2).

The chamomile flowers are the most important parts used for production of natural products. The flowers are formed at the end of the branches (30 to 100 mm long) individually, but sometimes numerous and up to 200 on a single plant (Figure 1). An average diameter of one flower is 10-25 mm, target diameter 6-8 mm. Involucrum is half hollow spheres composed of 20 to 30 almost single row layout, oblong, blunt and green bracts that have a narrow, brownish membranous margin.

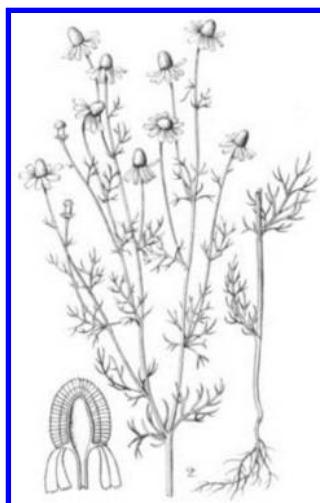


Figure 1. Flowers and morphology of chamomile.

The bell-shaped flowers on a target are ambiguous, with a five-pointed crown, golden yellow. Linguistically white flowers, of about 15, 69 mm long and 2-3 mm wide, are longer than involucrum nearly the bottom of bending. Bed attire is 1-2 mm thick, initially flat, tapered later and then extended pointed conical and hollow, without husk. Charts on Figure 2 present differences between flower from target and lingulaceous flower in which chamomile belongs to.

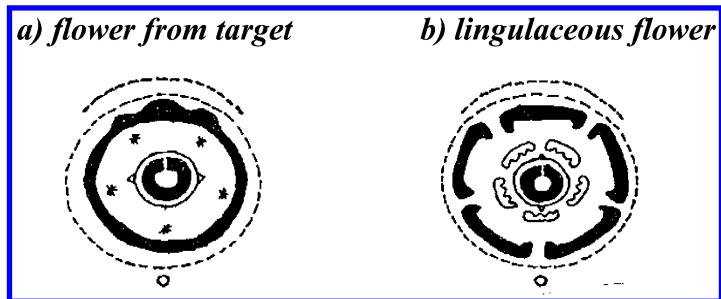


Figure 2. Flower chart of chamomile.

Flowering begins in May and continues during the growing season until the end of September. The pollen grains are more or less spherical in shape with pores. The number and shape of the pores are different in diploid and tetraploid plants. The number of chromosomes in diploid plants is $2n = 18$, while tetraploids have $2n = 36$ chromosomes. In general, diploids plants are smaller than tetraploids.

Pollination occurs mostly as entomophily by the insect species of the genus *Syrphidae* (*Diptera*) or *Erycinidae* (*Lepidoptera*) and anemophily cannot be ruled out.

Achenes are developed from the lower testis after pollination. At the time of ripening the achenes are longitudinally curved, 1-2 mm long, inside with five grooves and outside with the glandular spotted on top truncate. Weight of 1000 seeds of chamomile ranges from 0.03 to 0.09 g.

The germination capacity of the seeds (diaspores) is usually maintained for 5 to 6 years. Diaspores of chamomile are spread mainly by inadvertent transport of seeds by humans in farming or as part of organic waste from fields and gardens. More often small seeds adhere to various objects such as parts of clothing, animal fur or are transmitted by running water after rain. The germination of the seeds require enough water and light although germination is also possible in the dark.

Short History of Chamomile Large Scale Cultivation in Slovakia

The introduction of chamomile to large-scale cultivation in Slovakia has started in the late fifties of the 20th century. The permanent enlargement of chamomile cultivation up to 300 ha occurred in the second half of the 80s. The main influence of this process was to solve the array of problems involved in the large-scale cultivation and production of this medicinal plant. Its active components met quantity and quality requirements and were stable enough for pharmaceutical purposes. Mechanization for the harvest (collector) of the flowers (anthodia) and post-harvest processing were developed. At present, the annual area of arable lands of chamomile production is in the range from 350 to 500

hectares. An examination of possible profits indicates the value of chamomile in Slovakia. The sale price of dry flowers is approximately \$8 - \$10 per kg. An average yield of dry inflorescence is 400 kg/ha (350 lb/acre), resulting in a product worth \$3,200 - \$4,000/ha. Under optimal condition, yield of chamomile can reach 1,000 kg/ha (3).

Four different chemotypes of the chamomile essential oil (EO) (4) are recognized:

- A type: β - α -bisabolol oxide B > β - α -bisabolol oxide A > β - α -bisabolol
- B type: β - α -bisabolol oxide A > β - α -bisabolol oxide B > β - α -bisabolol
- C type: β - α -bisabolol > β - α -bisabolol oxide B > β - α -bisabolol oxide A
- D type: β - α -bisabolol oxide B ~ β - α -bisabolol oxide A ~ β - α -bisabolol

The active components in essential oil from chamomile grown under natural conditions in the East Slovakia lowland have reached 49.8 % of β - α -bisabolol oxide A, 7.4 % of chamazulene and only 5 % of the most therapeutically important β - α -bisabolol, which belonged to chemotype B. Chamomile EO with desired higher content of β - α -bisabolol was the reason to start breeding new varieties with different composition. The breeding process was done between the years 1975 and 1995. The diploid varieties Bona and Novbona and tetraploid varieties Goral and Lutea were developed (5). Varieties Bona and Novbona are characterized with high EO content and high amount of β - α -bisabolol. The variety Lutea contains 0.2 % higher EO amount than the previous two varieties. Dominant EO components are chamazulene and β - α -bisabolol. The mentioned bred varieties have better parameters than diploid variety Bohemia, which was the only certified variety in Czechoslovakia from 1952 (Slovakia was part of the Czechoslovakia until 1993). The main qualitative-quantitative characteristics of chamomile EO isolated from different varieties from large-scale cultivation in Slovakia are mentioned in Table 1. Essential oil content ranges from 0.6 to 1.1 %. This amount is at standard level as compared with different varieties grown abroad and in some cases can reach even higher amount.

Varieties bred in Slovakia are top rated in different counties because of their high content of β - α -bisabolol and chamazulene. Evaluation of gas chromatograph parameters for the identification and quantification of EO constituents and its development within 15 years are presented in Table 2. As an example the variety Lutea was selected. This mentioned variety is characterized by high content of β - α -bisabolol at 38.80 to 48.20 % and chamazulene at 25.00 to 27.50 %. Based on the EO dominant components, variety Lutea belongs to chemotype C (6).

Qualitative characteristics of variety Lutea are genetically determined and the variability depends on interactions between the plant and its environment. Essential oil content and main components β - α -bisabolol and chamazulene were compared with the amount determined in varietal testing.

Three international projects allowed to test stability of determined parameters of this Slovak variety of chamomile. The aim of the cooperation was the comparison of the qualitative and quantitative characteristics of the Slovak origin chamomile experimentally grown in different environmental conditions.

Table 1. Content and Main Components of Chamomile Essential Oil (EO) in Four Cultivars Bred in Slovakia

EO components	Chamomile varieties			
	Bona	Novbona	Lutea	Goral
	-----diploid-----		-----tetraploid-----	
----- % -----				
trans- β -farnesene	4.0 ± 2.0	9.0 ± 3.0	5.0 ± 3.0	9.0 ± 3.0
/-/ α -bisabololoxide B	3.0 ± 0.5	2.0 ± 0.5	2.0 ± 0.5	9.0 ± 2.0
/-/ α -bisabolonoxide A	0.5 ± 0.2	0.7 ± 0.2	0.6 ± 0.1	0.5 ± 0.2
/-/ α -bisabolol	42.0 ± 2.0	39.0 ± 5.0	48.0 ± 2.0	30.0 ± 3.0
chamazulene	20.0 ± 1.0	12.0 ± 3.0	16.0 ± 2.0	19.0 ± 2.0
/-/ α -bisabololoxid A	2.0 ± 1.0	1.0 ± 0.5	0.5 ± 0.5	16.6 ± 4.0
trans/cis-dicycloethers	0.1 ± 0.05	0.1 ± 0.05	0.8 ± 0.1	1.3 ± 0.3
EO content	0.6 ± 0.02	0.82 ± 0.04	0.95 ± 0.02	1.1 ± 0.05

Table 2. Percent Variability of the Main EO Components in the Variety Lutea during the Period of 1990 to 2005

EO components	1990	1995	2000	2005
	----- % -----			
farnesene	16.90	6.00	5.00	11.2
/-/ α -bisabololoxid B	0.50	1.10	2.40	2.70
/-/ α -bisabololoxid A	0.20	0.50	2.30	1.20
/-/ α -bisabolol	46.90	45.00	38.80	48.20
chamazulene	27.50	25.00	26.00	26.40
EO content	1.00	1.20	1.10	0.95

Seeds of Bona variety were sown and plants grown in environmental conditions in Montana (USA), Scotland (United Kingdom) and Tasmania (Australia). After harvesting the plant material, dried chamomile flowers were evaluated for differences in essential oil quantity and quality. Climate in Montana was characterized as temperate dry with average annual precipitation of 350 to 500 mm and average temperature in July of 23 °C. Scotland has temperate seaside climate with higher average annual precipitation of 700 to 2000 mm and lower average temperature in July of 14.4 °C. Tasmania in Australia presented temperate humid climate with the highest precipitation of 1800 to 3500 mm.

The amount of essential oils from plants from the different growing areas was very different. The differences depended on different soil and climatic conditions, harvest time, development stage of inflorescence and probably different ways of the sorting and drying temperature. The percentage of oil content in the plant material was within the range of 0.36 to 1.02 %.

The main quantitative and qualitative characteristics of chamomile essential oil are presented in Table 3 (7). Dominant components were identified as trans- β -farnezene (7.37 – 46.98 %), /-/ α -bisabolol (4.91 – 41.50 %), and chamazulene (2.67 – 19.20 %). The content of all /-/ α bisabololoxides was suppressed and ranged from 0.29 to 8.33 % (7).

Table 3. Content and Components of Chamomile Essential Oil (EO) from Variety Bona Harvested in Three Different Localities in the World

EO components	Location					
	Montana		Scotland		Tasmania	
	1	2	1	2	1	2
----- % -----						
trans- β -farnesene	7.37	46.98	24.34	24.34	7.71	10.0
/-/ α -bisabololoxide B	1.09	2.44	2.11	0.55	0.32	1.14
/-/ α -bisabolonoxide A	2.22	8.33	2.28	1.4	0.84	1.31
/-/ α -bisabolol	44.88	4.91	31.9	11.2	37.2	41.5
chamazulene	10.35	3.80	5.38	2.67	19.2	15.3
/-/ α -bisabololoxid A	1.81	2.67	0.99	0.29	0.50	0.54
cis-dicycloethers	12.57	3.40	4.00	1.56	20.20	15.30
trans-dicycloethers	4.17	0.76	0.72	0.18	0.90	1.24
EO content	0.41	-	0.65	-	1.2	0.88

As evident from the results, there were differences in EO compositions. High amount of trans- β -farnezene (24.34 and 46.98 %) were identified in two samples from different localities in Montana and in Tasmania. The presence of the high content of trans- β -farnezene presupposes a high amount of green plant parts in the distillation samples.

Contents of /-/ α -bisabolol and /-/ α -bisabololoxides in the chamomile drug flowers can vary widely depending on the origin of raw material and its chemotype. This fact makes the differences in the pharmacological efficacy of proprietary herbal medicine and cosmetics.

Development of secondary constituents (metabolites) in chamomile flowers depends on endogenous and exogenous factors. Qualitative characteristics of

chamomile essential oil of different origins are genetically determined, but their differences also depend on the interaction between plants and the environment. For example, the essential oil content in flowers collected in Montana was low due to the extremely dry season. The amount of $/-\alpha$ -bisabolol in the essential oil was comparable to the other analyzed samples, while on the other hand, chamazulene content varied considerably.

Plant Population Ideotype of New Variety

The properties, characteristics and features of plant ideotype are defined as follow:

Characterization of EO from the flowers without stems:

content of $/-\alpha$ -bisabolol [Bo]: from 55 to 65 % (noncalibrated result), or over 400 mg/100 g dried plant biomass (calibrated result);

content of chamazulene [Ch]: from 20 to 25 % (noncalibrated result),

or from 180 to 200 mg/100 g dried plant biomass (calibrated result);

content of en-in-dicycleters [*cis*-Dc, *trans*-Dc]: up to 10 %,

content of farnezene [Fa]: up to 5 %,

content of $/-\alpha$ -bisabololoxides A and B [BoA a BoB]: below 1 %,

content of $/-\alpha$ -bisabolonoxide A [BnA]: below 1 %,

content of EO in dried flowers: over 1 %,

Plant architecture and yield.

Nowadays, the certified varieties are characterized by only few morphological characteristics: plant height, diameter of 1 flower and its weight.

It is very difficult to determine the exact habitus of the ideal chamomile plant with the highest yield. However, there are some rules, which are important for large-scale cultivation of chamomile. These rules are:

- higher root biomass influences better regeneration after plants harvest,
- higher number of leaves on the plant is able to regenerate higher number of flowers after harvest,
- after different seeds in sowing, the number of the plants in population and its architecture will stabilize,
- when mechanized harvest is used, flowers should be in the range of 0.40 – 0.60 m aboveground. It is well known, that after harvest and regeneration, the zone of developing new flowers is higher.

Breeding of the New Chamomile Variety Lianka in Slovakia

As it was mentioned above, the breeding of chamomile started in Slovakia in 1976. Until 1995, there were four registered varieties: two diploid forms Bona and Novbona, and two tetraploid forms Lutea and Goral. The first three varieties are bisabolol type and Goral is bisabolol oxide type. Diploid forms have the ability to accumulate, in terms of their essential oil content, 45 to 52 % $/-\alpha$ -bisabolol, 18 to 22 % chamazulene and 0.1 to 1.0 % $/-\alpha$ -bisabolol oxides. In Slovakia

Novbona and Lutea varieties were registered in 1995. Unfortunately, the current varieties do not yield essential oils that contain acceptable levels of biologically active compounds. Diploid varieties Bona and Novbona have approximately 40 % of β - α -bisabolol but on a large-scale cultivation the content is even less than the declared amount. On the other hand, an increase of β - α -bisabolol oxide A and B from 9 to 12 % was observed. This resulted in a new breeding program in Slovakia focused on a high content of β - α -bisabolol and a low content of β - α -bisabolol oxides.

The Aims of the Breeding and Varieties Ideotypes

Breeding goals:

- high content of essential oil,
- high content of β - α bisabolol,
- low content of β - α bisabolol oxides A and B,
- high content of chamazulene,
- high yield of inflorescences,
- good health condition,
- distinctness, uniformity and stability evaluation according to UPOV (International Union for the Protection of New Varieties of Plants) TG 152/4.

One prerequisite for the success of plant breeding is genetic diversity. Breeders use plants natural diversity. When the natural diversity disappears, it is necessary to create new plant varieties. The use of natural variability in the chamomile selections focused on high capacity of β - α bisabolol and low bisabololoxides A and B chemotypes was not possible in Slovakia, because of natural occurrence of populations which belong to chemotypes β - α bisabololoxides. The variety Bona was then used as a resource for selection of individual plants.

Success was achieved by selection and evaluation of offsprings of selected plants by method of “the middle seedbed”. In the case of individual selection it is necessary to take into account that it is the population of cross-fertilized heterozygotes. Repeated and longtime lasting selection of extreme individuals causes progressive decay of primary population to families. Father plants (pollinators) also influenced their fertility. Selection objectivity and also their effectiveness is affected by different father partners and their different genetic basis. Based on this fact, the selection was realized so than these negative influences were reduced as much as possible.

The most suitable method of the middle seedbed was chosen for the offspring estimation of selected individuals (Figure 3). Principle consists of using checked pollination of selected fathers individuals. It does not require any space isolation.

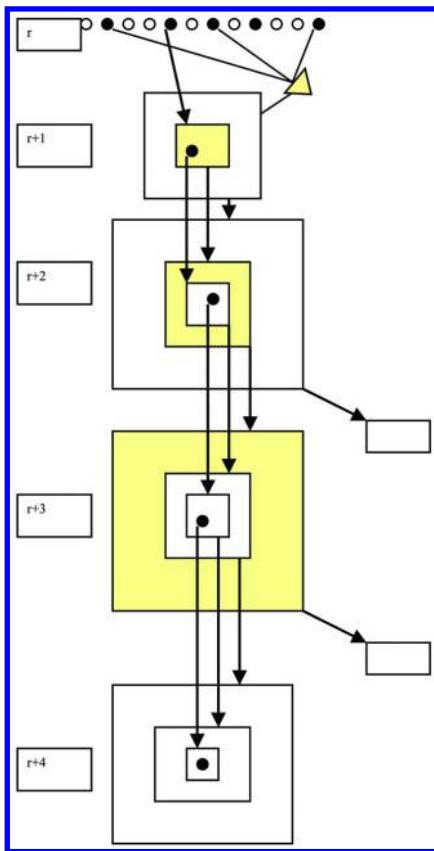


Figure 3. Scheme - method of “the middle seedbed” (8).

Offsprings of the best selected plants were seeded in the following dimension: they were surrounded from each side by the nearest relative plants, by offsprings of the same primer population. Outer lines consisted of individuals selected from father population in the previous year. This double surrounding is necessary for the protection of the best plants against unwanted cross-fertilization.

Method for the Breeding Maintenance of New Variety

Estimated plot allocated for the maintenance breeding must not be less than 100 m². Storing the seed is done by the method of halves, i.e. it is always necessary to use as much seed in order to maintain the half of relevant emergency or loss of the crop. The 100 m² cropped area is compactly distributed as follows:

0.5 m borders around as a protective zone,

the remaining areas are divided into 9 equal squares.

When plants begin flowering it is necessary to eliminate large flowers by subjective assessment. Five random samples from the nine squares have to be chosen at the beginning of flowering for chemical analysis. Selected flowers

require homogenization and chemical analysis for determination of the content of components by gas chromatography. The contents of /-/ - α -bisabolol and chamazulene are determined. Plant material at experimental plots, which reached values below the average required numbers had to be plucked.

Such a procedure should be sufficient to maintain the required parameters in the plant population. The areas and quantities of seeds shall be determined in its sole discretion. In a similar way, it is possible to produce elite seeds.

Aims of the Breedings of New Chamomile Variety in Slovakia

To achieve the goals, the standardized method of breeding was used:
selection of individuals according to requirements,
evaluation of the offsprings of selected individuals by the method of middle plot (8),
repetitive selections of required genotypes and evaluation of its offsprings by the method of middle plot.

Phases of the Breeding Process

Introduction

The introduction phase consisted of the study of chamomile biodiversity and selection of individual plants in regard to the following objectives: stabilized oil content in inflorescence, high content of /-/ - α bisabolol and chamazulene in the essential oil, high yield of dried plant material. In the case of breeding of the new variety Lianka, the prime material was obtained from a population of the diploid variety Bona.

Evaluation of the New Variety by the Method of Middle Seedbed

The breeding process in the second phase focuses on establishing the breeding plots by the special middle seedbed method. This method was developed to protect the best chosen individual plants with the required properties.

Homogenization of Breeding Material and Preparation of Technical Documentation

In this breeding phase the evaluation of the best properties continued. Elimination of the oxidized chemotypes, evaluation of production potential of inflorescence was done.

Testing of New Bred Variety and Registration by the State Institution

After few years of homogenization of selected population, seed material was subjected to experimental testing in Central Control and Testing Institute of Agriculture. Successful testing resulted in registration of the new bred variety Lianka.

Law 50/2007 Z.z. for the registration of varieties of cultivated plants, regulates the conditions for registration of plant varieties in Slovakia. Testing of new varieties and their registration ensures Central Control and Testing Institute of Agriculture (hereinafter referred to as „Control Institute“). The decision on the registration of plant cultivation (the “variety”) is proceeded only after the official trials (the "varietal test") and when it is ascertained that the variety:

- a) is different
- b) is sufficiently uniform
- c) is stable
- d) it could have satisfactory economical value – in the case of registration it is not required
- e) has a name which satisfies the requirements of a special regulation
- f) ensures the maintenance breeding of the variety
- g) does not have adverse effect to the health of humans, animals and the environment
- h) is genetically modified and meets the criteria under the special regulation in the case of material derived from it, which is intended for use as food or animal feed, and in the case of varieties of vegetable species as a food or food ingredient.

Examination of the value is not required in the case of varieties of ornamental plants, medicinal plants, and aromatic plants. Registration of these varieties in the Slovak Republic is voluntary.

Primer Material for the Breeding Process

The breeding program started in 2004. The variety Bona was included in the experimental plan for a comparison with breeding plants in 2011.

Breeding Method: Middle Seedbed

The chosen conventional breeding method for the selection and examination of posterity of selected plants was done by the method of “middle seedbed”. The purpose of the selected method was to achieve high content of /-/ α -bisabolol and low content of /-/ α - bisabolol oxides A and B.

Selected seeds from the best plants (with a very high content of /-/ α -bisabolol) were seeded in the middle. The seeds of the other good, but not the best plants, were seeded around. Similar procedure was used during the next years. After this procedure, breeding material was obtained with required parameters according to variety ideotype and was planted for the purpose of homogenization and stabilization, as well as assessment of the flower crop (Table 4).

Table 4. Ideotype of New Bred Variety of Chamomile

Parameters	Primers material	New bred variety ideotype
----- m -----		
Plant height	0.2 - 0.4	0.3 - 0.5
----- mm -----		
Inflorescence diameter	15	20
----- g -----		
Inflorescence weight	20	30
----- % -----		
Amount of EO	0.5 - 0.6	0.8 - 1.2
Chamazulene	15.00	18.00 - 22.00
Bisabolol oxides	1.6 and more	below 1.0
/-/α bisabolol	40.00 - 42.00	45.00 - 55.00

The most productive plants from the evaluated groups were grown in replication on ground-plots of 1.2 m² (Figure 4 and Figure 5). Plant material was evaluated for the quality of its inflorescent, as well as for essential oil content and composition. Overall the selection process was conducted following test guide UPOV 152/4.



Figure 4. Experimental plots – University of Pressov, Slovakia. (Photograph by Jozef Fejér; used with permission.)



Figure 5. Breeding nursery established in Experimental field at University of Presov, Slovakia (seeding on September 2009, selection in 2010). (Photograph by Jozef Fejér; used with permission.)

Evaluation of the Main Characteristics

Evaluation of morphological characteristics was performed according to testing guide UPOV 152/4 (The International Union for the Plant Protection of Plants New Varieties). Selected individual plants were evaluated according to their offsprings. The selective breeding nursery was established from the best offsprings with required parameters.

The selection of individual plants for the purpose of evaluation flower yield and elimination of β - α -bisabolol oxides types was done at the beginning of flowering. Flower samples were collected from selected plants which were marked as presented in Figure 6 and Figure 7.



Figure 6. Individual plant selections and marking for TLC analysis. (Photograph by Jozef Fejér; used with permission.)



Figure 7. Collection of the selected individuals after elimination of oxidized chemotypes.

For detection of β - α -bisabolol oxides the method of thin-layer chromatography (TLC) was used (Figure 8). Plants which contained this compound of essential oil were omitted from further evaluation.



Figure 8. TLC analysis. (Photograph by Jozef Fejér; used with permission.)

Yield of biomass was evaluated by the method of zonal analysis (9). Fundamentals of choosing for subsequent breeding process were harvest of flowers and identification of required content and composition of essential oil.

Based on the weight of flowers the most productive plants from the evaluated group were selected. These plants were cultivated in two repetitions in ground-plots of 1.2 m² for the purpose of fertility verification of their offsprings and appreciating of essential oil content. The seed reproductions of selected plant material were also tested. There were two harvests of flowers which were weighted from each ground-plot. Average flower samples from both replications were used for the followed analysis.

TLC Analysis

Thin-layer chromatography (TLC) was used for the first identification of /-/α-bisabolol oxides types (10). Extract from flower samples (Figure 9) was spread on silica gel plates ALUGRAM SIL G/UV₂₅₄. The /-/α-bisabolol oxides create yellow spots on the gel plate. Plants, which contained this essential oil compound, were omitted from further evaluation. The biomass yield was evaluated by the method of zonal analysis (9).

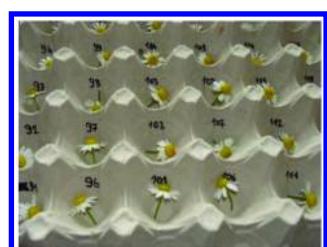


Figure 9. Selected flowers samples for chemical analysis. (Photograph by Jozef Fejér; used with permission.)

TLC Protocol:

1. Selected flowers were macerated in N-hexane to obtain the extracts.
2. Extracts were filtrated and 2-3 µl were placed in start position on TLC plate ALUGRAM SIL G/UV₂₅₄. The plate was left at room temperature to dry.
3. Mobile phase was prepared from benzene and ethyl acetate in 95:5 ratio.
4. TLC plate with the extract was placed in chromatographic chamber and lasted until 12 centimetre of the column moved.
5. TLC plate was taken off and dried at room temperature
6. The presence of the required components was visualized by the reagent (1 % of the solution of vaniline and sulphuric acid).
7. Identification of components was done according to color in the plate: bisabolol oxides – yellow spot, spathulenol, bisabolol and farnezene – violet spots, chamazulene blue spot and en-in-dicycloethers – brown spot.

Extraction of Essential Oil

Fifteen grams of each chamomile sample were grounded in a blender and then subjected to hydrodistillation in a Clevenger-type apparatus for 3 h in order to extract the essential oil. The oils were solubilized in *n*-hexane and stored under N₂ at 4 °C in dark until analysis. The plant materials yielded blue oils. Samples for analysis by gas chromatography–mass spectrometry (GC-MS) were diluted 1:1000 in *n*-hexane.

GC/MS Analysis

The identification of components of chamomile essential oil was carried out by HEWLETT – PACKARD 5890/5970 GC/MSD system, with split-splitless system for injection, MSD detector, column BPX – 5 (SGE Ltd., Melbourne), fused capillary column, 50 m long x 0.25 mm i.d., film thickness 0.25 mm. The following experimental conditions were observed: carrier gas UHP helium, column pressure 21 p.s.i (flow velocity 250 mm/s¹), injection temperature 240 °C, detector temperature 290 °C, oven temperature was programmed at 50 to 250 °C at 3 °C·min⁻¹, and then held for 15 minutes. Sample sizes were 1.0 µl with manual type of injection. Components were identified by comparison of their mass spectra with those stored in NIST 02 (software library) or with mass spectra from the literature (11, 12) and a home-made library, as well as by comparison of their retention indices with standards and calculated Kovats indices.

Results and Discussion

Through several years of selection and testing of plant offsprings, seeds with high content of β - α -bisabolol and low content of β - α -bisabolol oxides A and B were obtained. Breeding nurseries were started from seeds selected from the primers variety Bona. Generated material was evaluated in terms of flower yield, morphological characteristics and homogenisation of breeding material, according to its phenotype and chemotype. More than five hundred individual plants were selected from the breeding plots in 2010, while 359 plants were deemed not suitable for further study (Table 5). Researchers (9) recommend the method of zonal analysis to be used for a yield selection. This method can assess whole variability of characteristics together with comparison of selected material in accordance with genetic principles. The next step of the breeding work consisted of selecting 16 individual plants, which were used for further testing and evaluation in 2011.

Inflorescences Yield

On the basis of the TLC analyses and evaluation of the yield of flowers, we selected 16 of the most yielded plants ($x + 2s = 3.86 \text{ g} + 2 \times 2.04\text{g}$), in which the presence of α bisabolol oxide A and B were not detected.

The most productive plants from the evaluated groups were grown in replication in ground-plots of 1.2 m^2 . Plant material was evaluated for the quality of its inflorescent, as well as for essential oil content and composition. Overall the selection process was conducted to test guide UPOV 152/4.

The ability of chamomile offsprings for flower production was evaluated during two repetitions. All new breeding variants showed higher yields of inflorescences, as compared with the control variety Bona. The average weight of fresh inflorescences of the control variety was 680 g/m^2 , while the average weight of fresh inflorescences from the new breeding plants ranged from 736 g/m^2 to 886 g/m^2 .

From the above mentioned experiment we evaluated the yield and the content of essential oil and its composition. Three experimental variants were selected from the initially selected 16 plants, their seeds were mixed and developed into a new source for the quality evaluation by the Central Control and Testing Institute in Agriculture (CCTIA).

Morphological Characteristics

Morphological characteristics were evaluated according to the test guide UPOV 152/4 in the growing seasons of 2010 and 2011. Three plant morphological characteristics of the newly-bred variety and the control are shown in Table 6.

Table 5. Evaluation of Select Single-Plants and Their Standard Deviation in 2010

<i>Characteristics</i>	<i>Plant high</i>	<i>Number of stems</i>	<i>Weight of plant</i>	<i>Weight of stems with leaves</i>	<i>Weight of root</i>	<i>Weight of inflorescences</i>
-- mm --			----- g -----			
Average	588.9	5.92	17.51	13.65	0.90	3.86
Standard deviation	59.9	3.06	7.92	6.15	0.45	2.04
Number of cases	359.0	359.0	359.0	359.0	359.0	359.0
Average mean error	3.2	0.16	0.42	0.32	0.02	0.11
Minimum	400.0	1.00	3.99	2.62	0.21	0.78
Maximum	740.0	22.00	50.80	35.81	3.06	15.35

Table 6. Evaluation of Morphological Characteristics of a Newly-Bred Variety and the Control Variety According to UPOV TG/152/4

Number of descriptor	Descriptor	Degree of manifestation	
		Bona	Plant Breeding Material
5.	Stem: anthocyanins coloration	Medium 5	Weak 3
7.	Leaf: intensity of green color	Medium 2	Light 1
12.	Flower head: content of /-/α bisabolol in essential oil	High 3 (39 - 40 %)	High 3 (52 - 55%)

Table 7. Average Contents of Essential Oil Components in the Newly-Bred Variety Compared with the Control Variety in 2010 and 2011 Analyzed in Dry Inflorescences by GC/FID and GC/MS

Essential oil components	Year 2010				Year 2011				Average			
	Control variety Bona		Plant Breeding Material		Control variety Bona		Plant Breeding Material		Control variety Bona		Plant Breeding Material	
	GC/ FID	GC/ MS	GC/ FID	GC/ MS	GC/ FID	GC/ MS	GC/ FID	GC/ MS	GC/ FID	GC/ MS	GC/ FID	GC/ MS
----- % -----												
/-/- α Bisabolol	38.0	40.0	53.5	58.5	42.2	40.0	52.3	52.2	40.1	40.0	52.9	55.4
Chamazulene	17.0	13.0	19.0	17.0	23.5	17.0	19.1	18.9	20.3	15.0	19.1	18.0
Bisabolol oxide - A	14.0	17.0	3.5	3.5	6.1	7.6	1.7	1.7	10.1	12.3	2.6	2.6
Bisabolol oxide - B	6.5	7.5	3.5	4.5	10.1	12.0	1.9	2.1	8.3	9.8	2.7	3.3
Cis - spiroter	8.5	11.0	9.5	11.0	7.3	12.0	10.8	15.0	7.9	11.5	10.2	13.0
Farnesen	2.5	4.5	2.5	2.5	1.9	1.7	5.9	4.9	2.2	3.1	4.2	3.7

Table 8. Essential Oil Content and Composition during 2011 – 2013, Average Values from GC/FID and GC/MS Analyses

Year	<i>Essential oil content</i>	<i>/-/α-bisabolol</i>	<i>chamazulene</i>	<i>/-/α-bisabolol oxide A</i>	<i>/-/α-bisabolol oxide B</i>	<i>cis spiroeter</i>	<i>farnezene</i>
%							
2011	0.66	52.3	19.0	1.7	2.0	9.7	4.5
2012	0.80	61.5	13.5	1.0	2.0	11.0	2.5
2013	0.65	51.5	17.0	2.0	3.0	17.0	9.0
Average	0.70	55.1	16.5	1.6	2.3	12.6	5.3

Essential Oil Content and Composition

Bertnáth (13) considered to search of inter- and intra-specific environmental conditions of breeding work with exact determination of active compounds. Last two years of experiments (years 2010 and 2011) were focused on plant selection based on yield of flowers, content and composition of essential oil (low content of β - α -bisabolol oxides). Other breeders (14) evaluated a contamination of β - α -bisabolol types of tetraploid chamomile varieties by β - α -bisabolol oxides types. These were transferred by dominant genes O/o. According to the presented results, changes in quality of plant source material were predictable. The important part of this work was the elimination of bisabololoxides chemotype individuals. The bisabolol chemotype was selected by using the right breeding process and analytical methods. Thin-layer chromatography (TLC) was used to confirm or negate inheritance of the gene that led to the synthesis of this compound.

Content of essential oil in inflorescences of chamomile changed during ontogenesis, and ranged from 0.3 to 1.5% before full flowering (15). This experiment resulted in plant material that contained, over the two-year period an average of 0.67 % of essential oil, which is comparable with the control variety Bona. Essential oil amount of new bred variety ranged from 0.45 % to 0.95 %, while in control variety Bona it was from 0.50 % to 0.85 %. The essential oil content variation in the newly bred variety within this 2 year breeding process is presented in Table 7.

The average content of the most valuable pharmacological compound β - α -bisabolol, in the new variety Lianka ranged from 52.9 % to 55.4 % and the content of β - α bisabolol oxides A and B did not exceed 3.0 % in the plants from our experiments during the years 2010 and 2011. On the other hand, the important compound chamazulene ranged from 18.0 % to 19.1 %. Based on the results of the mentioned experiments, the newly bred variety was logged into the state variety experiments named PO-MATRI_REC-1. Along with the experiments established by CCTIA, the experiments at Presov University continued. The results from three experimental years are presented in Table 8.

The evaluation of essential oil content and composition during 2012 and 2013 continued. Content of essential oil of the new bred chamomile variety Lianka was 0.70 %. The β - α -bisabolol reached 55.1 %, chamazulene 16.5%, and the β - α -bisabolol oxides A and B were 1.6 % and 2.3 %, respectively.

Summary

The result of the first breeding season was the selection of mother plants from the chamomile diploid variety Bona. These plants became the primers for the breeding process. Next season confirmed the accuracy of the selected method and enough plant material of new offsprings (new bred variety) was obtained. Breeding process in mentioned season had few working specifics, which wideness selections. New qualitative-quantitative parameters of EO were established and previous breeding plots have been extended to the test performance. Furthermore, the work continued with the selection of individual plants, but also began with

selection of bunches. It has become crucial bonitation of newly bred material in the plots of test performances. Seed material was isolated for testing seed maturation. TLC and GC analyzes were carried out of individual plants and in tufts. The qualitative and quantitative characteristics of essential oil were important for its core components. The fourth season of breeding work confirmed all contractually defined parameters of the newly bred variety.

Table 9. Breeding of Chamomile in 2005 – 2008

<i>Parameters</i>	<i>Bona</i>	<i>Offsprings of selected mother plants</i>	
	<i>2005/2006</i>	<i>2006/2007</i>	<i>2007/2008</i>
----- % -----			
EO	0.63	0.93	0.83
/ -/- α -bisabolol	47.00	66.78	59.74
chamazulene	12.15	15.50	11.78
----- /m ² -----			
plant density	1183	395	258
number of offshooted plants	55	101	117
number of flowers	4105	6682	3947
----- g/m ² -----			
total plant biomass (R+S)	777.8	910.00	490.7
underground biomass (R)	41.5	57.3	28.6
above-ground biomass (S)	736.7	853.3	462.00
flowers biomass	69.0	137.6	77.65
----- g -----			
weight of individual flower	0.0166	0.0200	0.0196
----- R/S ratio -----			
R/S ratio	0.0562	0.0671	0.0620

It is important to mention, that along with the breeding process in selecting of the plant material, the variety Bona (as a primer plant material) was cultivated in parallel. The main characteristics of the bred population were as follow: average EO content was 0.68 %, average amount of /-/- α -bisabolol was 41.8 %, chamazulene 16.85 %, plant density of 266 plants per square meter, the number of offshooted plants was 100 per m², total plant biomass (R+S) 296.4 g/m²,

underground biomass (R) 15.9 g/m², above-ground biomass (S) 280.5 g/m², R/S ratio 0.0567, flowers weight 50.82 g/m², number of flowers was 3028 per m², average weight of 1 flower was 0.0167 g. Table 9 presents main characteristics of the breeding process in three consecutive seasons (2005 – 2008).

All together 290 individual plants were analyzed by TLC, selected from the middle plots S*1 and S*2 (Table 10). Fifteen plants were determined as oxide chemotype.

Table 10. TLC Analysis of Selected Offsprings in Middle Plots S*1 and S*2

	<i>N+</i>	<i>N</i>	<i>O</i>
S1/2,1.....S1/2,15	8	7	0
S1/3,1.....S1/3,30	19	9	2
S1/4,1.....S1/4,55	32	20	3
Total	59	36	5
in %	59.00	36.00	5.00
<hr/>			
S2/2,1.....S2/2,20	14	4	2
S2/3,1.....S2/3,30	20	9	1
S2/4,1.....S2/4,80	48	26	6
S2/5,1.....S2/5,60	38	21	1
Total	120	60	10
in %	63.16	31.58	5.26

N+ - present higher intensity of patch coloring, *N* – present low intensity of patch coloring, *O* – present low intensity of patch coloring.

There were plants with the low intensity of patch color (*O*). There were no individuals found with high intensity of patch color. Patch color intensity for the */-/α*-bisabolol in TLC were non+oxidated individuals divided into groups: *N* – low intensity of coloring (96 individuals), *N+* higher intensity of coloring (179 individuals). In the middle plot were found 5.13 % of oxidated chemotype individuals and 94.87 % nonoxidated chemotype individuals. *N* for bisabololoxides were 33.79 % and *N+* 61.08 %. Summary of the selected oxidated and nonoxidated individuals in covered plots and in middle plots are presented in Table 11. Total amount in TLC determination of oxidated and nonoxidated chemotypes in vegetation season were 5.95 % and 94.05 %.

Table 11. Evaluation of the Selected Oxidized and Nonoxidized Chemotypes Individuals in Covered and Middle Plots

	<i>N+</i>	<i>N</i>	<i>O</i>	<i>O+</i>
number of individuals	321	605	59	5
in %	32.45	61.60	5.45	0.5
Total	94.05		5.95	

N+ - higher intensity of patch coloring, *N* – low intensity of patch coloring, *O* - low intensity of patch coloring, *O+* higher intensity of patch coloring.

Conclusion

Experimental results confirmed that breeding of the new cultivar was successful in years 2010-2013. The main goals of this study consisted of producing plants with high */-/α* bisabolol and chamazulene contents, but with low */-/α*- bisabolol oxides A and B contents. The registration of medicinal, aromatic and spice plants is voluntary in the Slovak Republic. The condition for a new variety is its uniformity and stability, and does not require evaluation of flower yield. The important part of this work was the evaluation of morphological characteristics and confirmation of differences compared with the control variety. An important parameter for large-scale production is production ability. By the individual selection method, this team was successful at selecting the best plants with a high production potential. In the final stage of the breeding process offspring were tested for their yield performance. On the basis of the obtained results, we have selected the material, which was registered as a new variety at the Central Control and Testing Institute of Agriculture in Bratislava, Slovakia. After a successful evaluation (years 2012 – 2013), this chamomile was registered as a new variety under the name Lianka.

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Chapter 14

Phytochemistry of *Schizandra Chinensis* (Turcz.) Baill Cultivated in Bulgaria

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The chemical composition of the oil fruits from of *Schizandra chinensis* (Turcz.) Baill, cultivated in Bulgaria were analyzed using GC and GC/MS. The major constituents (over 3%) of the essential oil (1.65%) were found to be α -himachalene (10.83%), α -ylangene (10.50%), β -himachalene (9.02%), schisandrin (8.23%), β -chamigrene (6.44%) and γ -muurolene (5.52%). The essential oil studied demonstrated antimicrobial activity against Gram-positive and Gram-negative bacteria and yeasts. Oleic acid (80.8%), stearic acid (14.5%) and palmitic acid (4.7%) were the main components in the triacylglycerol fraction (24.6%). In the tocopherol fraction α -tocopherol (96.7 %) predominated, and in sterol fraction: β -sitosterol (91.0%) and campesterol (5.1%).

Keywords: *Schizandra chinensis* (Turcz.) Baill ; essential oil ; antimicrobial activity ; lipid fraction

Introduction

The genus *Schizandra* (also known as *Schizandra*) includes 25 species of deciduous vines belonging to the Schizandraceae family (Magnolia vine family). All but one is native to the forests of Northern China, the Russian Far East, Korea and Japan. The Chinese name for *Schizandra* is *wu-wei-zi*, which means “five taste-fruits” or “five flavor herb” and alludes to the fact that the fruits contain all five flavors: sweet, sour, bitter, pungent and salty. Sucking on a dry fruit is an interesting experience because of its various flavors. *Schizandra chinensis* (Turcz.) Baill vines prefer some shade and well-drained, deeply cultivated sandy soil with plenty of moisture, rich compost and cold temperatures. Like most vines they need to grow on an arbor, wall or fence. They usually begin bearing fruit after the second or third year. *Schizandra* is propagated by seed, cuttings or layering (1). *Schizandra* is being used by western herbalists as an overall tonic and recovery herb for various deficiencies and weak body conditions. It can be used in combination with other herbs for chronic stress, chronic fatigue, insomnia, poor memory. Some clinical studies have shown that the berries improve brain efficiency while at the same time calming the central nervous system. When taken over several weeks as an adaptogen (a substance that helps the body adapt to stresses), *Schizandra* helps improve energy levels, reduces tiredness, and improves the immune system’s response, thus being a valuable tonic for many people (2, 3). Pharmacological studies on animals have shown that *Schizandra* increases physical working capacity and delivers a stress-protective effect against a broad spectrum of harmful factors including heat shock, skin burn, cooling, frostbite, immobilisation, swimming under load in an atmosphere with decreased air pressure, aseptic inflammation, irradiation, and heavy metal intoxication. The phytoadaptogen provides a beneficial effect on the central nervous, sympathetic, endocrine, immune, respiratory, cardiovascular and gastrointestinal systems, on the development of experimental atherosclerosis, on blood sugar and acid-base balance, and on uterus myotonic activity (4). The main active principles present in all parts of the plant are dibenzo cyclooctadiene lignans (5, 6).

Krotova and Efremov (7) investigated the chemical composition of *Schizandra* fruits. They established that the content of essential oil was 1.65 % and lipids were 40.3%. The volatile components of various species of the plant were studied by many authors (6, 8–10). The major compounds determined by Deng et al. (11) through steam distillation of the *Schizandra chinensis* fruits from China were: α -santalene (28.51%), 2,4 α , 5,6,7,8-hexahydro-3,5,5,9-tetramethyl-1H-Benzocycloheptene (13.31%), ζ -cadinene (13.25 %), 4-isopropylidene-1-vinylmenth-8-ene (7.71%); by Wang et al. (12): (-)-1,7-dimethyl-7-(4-methyl-3-pentenyl)-tricyclo [2.2.1.0(2,6)] heptane (18.06%), (1 alpha, 4 beta, 8 alpha)-1,2,3,4,4 α ,5,6,8 α -octahydro-7-menthyl-4-methylene-1-(1-methylethyl)-naphthalene (15.58%), 2,4 α ,5,6,7,8-hexahydro-3,5,5,9-tetramethyl-1H-benzocycloheptene (11.45%), 1-methyl-4-(1-methylethyl)-1,3-cyclohexadiene (9.90%); 1-methyl-4-(1,2,2-trimethylcyclopentyl)-benzene (9.13%); while the fruits from Russia examined by Krotova and Evremov showed the following composition (7): α - and β -chamigrenal (26.5%), α - and β -chamigrene (19.5%), sesquicarene (10.5%). The

essential oil had antimicrobial activity against Gram-positive and Gram-negative bacteria and proved to be a weaker antioxidant compared to BHT (12). The essential oil, ethanol extract and various fractions (petroleum, ethyl acetate and n-butanol of ethanol extract) demonstrated a strong antioxidant activity (10).

Bulgaria is a relatively small country in South-East Europe, which occupies 111000 km² and is located in the centre of the Balkan Peninsula, sharing borders with Romania, Serbia, the Republic of Macedonia, Greece, Turkey and the Black Sea. Besides being very picturesque and rich in history and culture, Bulgaria possesses a highly varied topography and a range of micro-climatic areas: mountains, plains, rivers and seacoast valleys. The climate ranges from moderate continental in the northern part to Mediterranean/subtropical in southwest and southeast regions. As a result of these favorable climatic conditions, soils and other natural factors, Bulgaria has been the perfect place for growing of medicinal plants and therefore a producer of essential oils for over 400 years. Bulgaria has been long renowned as one of the two major global suppliers of rose oil and, also, as a source of many other essential oils, including lavender, dill, pine, clary sage, basil, bigroot geranium and milfoil. Shizandra is a new plant for Bulgaria introduced as a crop in the late 90s of the twentieth century. Nowadays it is grown in the region of Pleven - Northern part. The harvested fruits are used in the food industry for preparing various products - jams, juices and more, as well as in traditional medicine.

The aim of present study is the production of the essential and vegetable oils from Schizandra fruits grown in Bulgaria and determination of their chemical composition and characteristics for possible application in natural cosmetics, pharmaceuticals and food products.

Materials and Methods

Sample. The fruits were harvested in 2014 in the vicinity of the town of Pleven, Bulgaria. Pleven's climate is temperate continental. The average annual temperature is around 13°C. The region is characterized by the fertile black earth soils known as chernozems.

The moisture of the aerial fruits (12.5%) was determined by drying to 105°C, according to Russian Pharmacopoeia (13).

Isolation of essential oil: The air-dried fruits were ground in a laboratory mill to a size of 0.7-1cm. The oil was prepared through hydro distillation for 5h in a laboratory glass apparatus as per British Pharmacopoeia, modified by Balinova and Diakov (14). The oil obtained was dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4°C until analyzed.

Isolation of fruit oil: The ground fruits were extracted with n-hexane in Soxhlet apparatus for 18h. The solvent was partly removed in a rotary vacuum evaporator, the residue was transferred to a pre-weighed glass vessel and the remaining solvent was removed under stream of nitrogen to a constant weight, in order to determine the oil content (15).

Chemical composition of the essential oil: GC analysis was performed using Agilent 7890A gas chromatograph; column HP-5 ms (30m x 250 μ m x 0.25 μ m); temperature: 35°C/3 min, 5°C/min to 250°C for 3min, total 49min; carrier gas: helium 1ml/min constant speed; split ratio: 30:1.

GC/MS analysis was carried out on a mass Agilent 5975C spectrometer, carrier gas: helium, column and temperature the same as of the GC analysis.

The identification of chemical compounds was performed by comparing their relative retention time to library data. The components identified were arranged in accordance with their retention time and the percent.

Fatty acids: The total fatty acid composition of the oil was determined by GC after transmethylation of the respective sample with 2N methanolic KOH at 50°C according to Christie (16). Fatty acid methyl esters (FAME) were purified by TLC on 20cm x 20cm glass plates covered with 0.2mm Silica gel 60 G layer (Merck, Darmstadt, Germany) with mobile phase n-hexane:acetone, 100:8 (by volume). Determination was performed on a gas chromatograph equipped with a 30m x 0.25mm x 25 μ m (I.D.) capillary EC 30-Wax column (Hewlett Packard GmbH, Vienna, Austria) and a flame ionization detector. The column temperature was programmed from 130°C (hold 4min), at 15°C/min to 240°C (hold 5min); injector and detector temperatures were 250°C. Hydrogen was the carrier gas at a flow rate 0.8ml/min; split was 50:1. Identification was performed by comparison of the retention times with those of a standard mixture of FAME subjected to GC under identical experimental conditions (17).

Sterols: Unsaponifiables were determined by weight after saponification of the lipid fraction and extraction with hexane (18). The unsaponifiable matters (100 mg, precisely measured) were applied on 20cm x 20cm glass plates (ca. 1 mm thick Silica gel G layer) and developed with n-hexane:acetone, 100:8 (by volume). Free sterols ($R_f = 0.4$) were detected under UV light by spraying the edges of each plate with 2',7'-dichlorofluorescein, they were then scraped, transferred to small glass columns and eluted with diethyl ether. The solvent was evaporated under a stream of nitrogen and the residue was weighed in small glass containers to a constant weight. Sterol composition was determined by GC using HP 5890 gas chromatograph (Hewlett Packard GmbH, Vienna, Austria) equipped with a 25m x 0.25mm DB – 5 capillary column (Agilent Technologies, Santa Clara CA, USA) and a flame ionization detector. Temperature gradient was from 90°C (hold 2min) up to 290°C at a rate 15°C/min and then up to 310°C at a rate of 4°C/min (hold 10min); the injector temperature was 300°C and the detector temperature was 320°C. Hydrogen was used as carrier gas at a flow rate of 0.8ml/min; split 50:1. Identification was confirmed by comparison of retention times with those of a standard mixture of sterols (19).

Tocopherols: Tocopherols were determined directly in the oil by high performance liquid chromatography (HPLC) by a Merck-Hitachi (Merck, Darmstadt, Germany) unit equipped with a 250mm x 4mm Nucleosil Si 50-5 column (Merck, Darmstadt, Germany) and a fluorescent detector Merck-Hitachi F 1000. The operating conditions were as follows: mobile phase n-hexane:dioxan, 96:4 (by volume), flow rate 1.0 ml/min, excitation 295nm, emission 330nm. 20 μ l 1% solution of crude oil were injected. Tocopherols were identified by comparing the retention times to those of authentic individual pure tocopherols.

The tocopherol content was calculated on the base of tocopherol peak areas in the sample vs. tocopherol peak area of the standard tocopherol solution (20).

Determination of antimicrobial activity: The antimicrobial effect of the essential oil was tested against Gram-positive bacteria *Bacillus cereus* (food spoilage isolate), two strains of *Staphylococcus aureus* (ATCC 6538 and one food spoilage isolate) and *Listeria monocytogenes* (food spoilage isolate), as well as the following Gram-negative bacteria: two strains of *Escherichia coli* (ATCC 25922 and one clinical isolate), two strains of *Salmonella abony* (ATCC 6017 and one clinical isolate), three strains of *Pseudomonas aeruginosa* (ATCC 27853, one clinical isolate and one food spoilage isolate) and *Pseudomonas fluorescens* (food spoilage isolate), sources given in Table 2. Additionally antimicrobial testing against two strains of *Candida albicans* (ATCC 10231 and one clinical isolate) was performed. All strains were deposited in the Microbial Culture Collection of the Department of Biochemistry and Microbiology (University of Plovdiv, Bulgaria). The bacterial strains were stored on Nutrient Agar (NA, HiMedia Ltd., India) and the yeasts strains were stored on Sabouraud Dextrose Agar with chloramphenicol (SDA, HiMedia Ltd.).

Stock solutions of the samples for antimicrobial testing were prepared by dissolving the respective compound in 2% DMSO (Sigma-Aldrich Co.). The antibacterial activity evaluation of the studied compounds was performed according to Clinical Laboratory Standard Institute (21) reference method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. The anticandidal activity of the studied compounds was tested according to CSLI (22) reference method for broth dilution antifungal susceptibility testing of yeasts. Briefly stock solutions were added to the RPMI1640 broth medium buffered to pH 7.0 with 0.165mol/L MOPS buffer (3-N-morpholinopropanesulfonic acid, Sigma-Aldrcih,Co) to reach dilutions with final sample concentrations, after inoculation with microbial test suspension, between 1024 μ g/mL and 4 μ g/mL. Controls consisting of inoculated medium without tested sample and without DMSO, as well as with DMSO were also prepared. The DMSO concentration in the broth dilution assay was low to keep the effect on microbial growth to a minimum. Antimicrobial activity determined by broth microdilution tests was expressed as Minimal Inhibitory Concentration (MIC) in μ g/mL. MIC was defined as the lowest concentration of the tested compound at which total inhibition of microbial growth was detected. All tests were performed in triplicate.

Results and Discussions

The content of the essential oil was 1.65% and was comparable with the data from the literature (7). The essential oil was light yellow, with a specific odor. The chemical composition of the essential oil was given in Table 1. Thirty two components representing 91.81% of the total content were identified. Twenty two of them were in concentrations over 1% and the rest 11 constituents were in concentrations under 1%. The major constituents (over 3%) of the oil were as follows: α -himachalene (10.83%), α -ylangene (10.50%), β -himachalene (9.02%), schisandrin (8.23%), β -chamigrene (6.44%) and γ -muurolene (5.52%).

The difference in the quantities of the chemical composition of the essential oil extracted in our laboratories and the reported data may be due to the environmental conditions under which the plant has grown as well as to the variation in conditions of analysis.

Table 1. Chemical Composition of Essential Oil from *Schizandra chinensis*

No.	Compounds	RI	%
1.	α -Pinene	939	1.13
2.	Camphene	954	1.66
3.	β -Pinene	979	0.34
4.	Myrcene	990	0.39
5.	α -Phellandrene	1003	0.10
6.	α -Terpinene	1018	0.58
7.	<i>p</i> -Cymene	1025	1.08
8.	Limonene	1029	0.91
9.	γ -Terpinene	1060	2.50
10.	α -Terpinolene	1088	0.33
11.	Terpinene-4-ol	1179	0.61
12.	3-Methoxy- <i>p</i> -cymene	1215	1.35
13.	Bornyl acetate	1288	2.41
14.	α -Ylangene	1375	10.50
15.	β -Bourbonene	1388	0.26
16.	β -Elemene	1396	1.13
17.	β -Caryophyllene	1419	2.69
18.	(<i>Z</i>)- β -Farnesene	1448	1.21
19.	α -Humulene	1455	0.43
20.	β -Chamigrene	1468	6.44
21.	β -Eudesmene	1477	0.45
22.	β -Himachalene	1486	9.02
23.	α -Himachalene	1491	10.83
24.	γ -Muurolene	1496	5.52
25.	Alloaromadendrene	1499	1.50
26.	β -Humulene	1512	2.27

Continued on next page.

Table 1. (Continued). Chemical Composition of Essential Oil from *Schizandra chinensis*

No.	Compounds	RI	%
27.	α -Cubebene	1525	2.87
28.	Dehydroaromadendrene	1577	0.74
29.	Longipinocarvone	1748	3.72
30.	Gomisin F	2380	1.69
31.	Schisandrin	2420	8.23
32.	Gomisin A	2480	1.22

The distribution of major groups of aroma substances in the essential oil was shown in Figure 1. Sesquiterpene hydrocarbons were the dominant group in the oil (69.67%), followed by monoterpene hydrocarbons (8.54%) and oxygenated sesquiterpenes (4.05%). The results were similar to those in the literature.

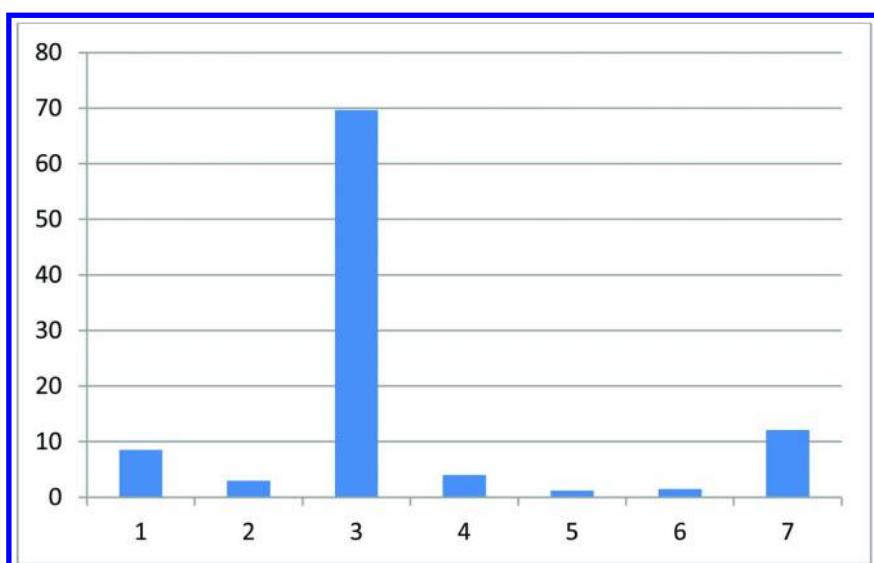


Figure 1. Groups of components in the oil, %. 1 - Monoterpene hydrocarbons; 2 – Oxygenated monoterpenes; 3 – Sesquiterpene hydrocarbons; 4 – Oxygenated sesquiterpenes ; 5 - Phenyl propanoid hydrocarbons; 6 – Oxygenated phenyl propanoids ; 7 – Other compounds.

The results of antimicrobial testing were shown in Table 2. The summary results of the oil studied demonstrated its antimicrobial activity against the tested microbial strains. Gram-positive bacteria were the most sensitive microbes, followed by yeasts and Gram-negative bacteria. The less sensitive were three strains of *P. aeruginosa*. The ability of *P. aeruginosa* to grow in a form of biofilm and the production of extracellular polysaccharide increased antimicrobial resistance of these bacteria mainly through permeability barrier. These strains also produced two types of soluble pigments, pyoverdin and pyocyanin, which probably participate in cell defense against antimicrobials. The oil demonstrated equal antimicrobial activity against Gram-negative bacteria *P. fluorescens* and both strains of yeasts belonging to species *C. albicans*. The results obtained are comparable with the results published for the same essential oil by other authors.

Table 2. Antimicrobial Activity of the Essential Oil

<i>Test microorganisms</i>	<i>Origin</i>	<i>MIC, %</i>
<i>B. cereus</i>	Minced meat	50
<i>S. aureus</i>	ATCC 6538	128
<i>S. aureus</i>	Pork fillet	128
<i>L. monocytogenes</i>	Chicken breasts	128
<i>E. coli</i>	ATCC 25922	256
<i>E. coli</i>	Clinical isolate	256
<i>S. abony</i>	ATCC 6017	256
<i>S. abony</i>	Clinical isolate	256
<i>P. aeruginosa</i>	ATCC 27853	1024
<i>P. aeruginosa</i>	Clinical isolate	1024
<i>P. aeruginosa</i>	Minced meat	1024
<i>P. fluorescens</i>	Chicken breasts	512
<i>C. albicans</i>	ATCC10231	512
<i>C. albicans</i>	Clinical isolate	512

The triacylglycerol fraction was 24.6%, and not corresponding with the data found in the literature (7). The fatty acid composition was presented in Table 3. Data show that three fatty acids were determined, constituting 100% of the total oil content. The fatty acids found in the triacylglycerol fraction were oleic acid (80.8%), stearic acid (14.5%) and palmitic acid (4.7%). Regarding the individual presence of oleic acid, the oil was similar to the oils from other nontraditional

materials such as grape seeds, watermelon, tobacco and poppy seeds (23, 24). Schizandra fruits oil was found to contain very high amounts of the saturated stearic and palmitic acids, which came close to the levels in other oils, like olive oil and corn oil (25).

Table 3. Fatty Acid Composition

No.	Fatty acids	Content, % (w/w)
1	C _{16:0} Palmitic	4.7
2	C _{18:0} Stearic	14.5
3	C _{18:1} Oleic	80.8

Phytosterols, more commonly known as plant sterols are currently approved by the U.S. Food and Drug Administration for use as a food additive; however, there is some concern that they may block absorption not only of cholesterol but of other important nutrients as well.

Sterols were present in the so called non-saponified part from the lipid fraction. The total content in the oil was found to be 0.8%. The individual sterol composition was given in Table 4. β -sitosterol (91.0%) and campesterol (5.1%) predominated in the sterol fraction. The data obtained made obvious that regarding its sterol content and composition, Schizandra fruits results were similar to the findings for cotton seed oil (26).

Table 4. Sterol Composition

No.	Sterols	Content, % (w/w)
1.	Cholesterol	0.2
2.	Campesterol	5.1
3.	Stigmasterol	0.3
4.	β - Sitosterol	91.0
5.	Δ^5 - Avenasterol	2.2
6.	Δ^7 - Stigmasterol	0.9
7.	$\Delta^{7,25}$ - Stigmastadiol	0.3

Tocopherols are a class of organic chemical compounds, mostly with vitamin E activity. α -Tocopherol is the main source found in supplements and in European diet, where the main dietary sources are olive and sunflower oils, while γ -tocopherol is the most common form in the American diet due to a higher intake of soybean and corn oil.

The total content of tocopherols in the lipid fraction was comparatively higher – 660mg/kg. The tocopherol composition was presented in Table 5. The α -tocopherol predominated in the oil, followed by β -tocopherol. The oil with higher content of α -tocopherol proved superior to a number of common food oils, thus showing similarity to some non-traditional oils (23, 24).

Table 5. Tocopherol Composition

No.	Tocopherols	Content, % (w/w)
1.	α -Tocopherol	96.7
2.	β -Tocopherol	3.3

Conclusion

Schizandra fruits (*Schizandra chinensis* (Turcz.) Baill) can be used as a non-traditional material for production of oil rich in biologically active substances as essential oil, lipid fraction (sterols and tocopherols) for nutritive purposes, as well as for an additive in fodder mixtures to enrich them with valuable nutrients.

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Chapter 15

Essential Oils for the Prevention and Treatment of Human Opportunistic Fungal Diseases

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Human infectious diseases have significantly increased during the past decade, especially among immunocompromised patients. As high as 10% of hospital acquired systemic infections are caused by fungi. Among animal and human pathogens, the dermatomycetes group is the main cause of dermatomycoses, which are chronic but not life threatening infections that bring about considerable morbidity. Under certain circumstances *Candida spp.* commensals (i.e., *C. albicans* and some non-*albicans* species) can become pathogenic, which causes amucous membrane infection to turn into a life-threatening systemic disease, particularly in patients with a weakened immune system. In addition to increased resistance of human pathogens to current commercial drugs,

conventional treatments have many adverse side effects, thus drugs may become insufficient for treatment and this presents a serious medical problem. Hence, the development of more effective and less toxic antifungal agents, including natural products, is vitally important. Due to their biologically active secondary metabolites, many plants have been traditionally used in ethnomedicine for their therapeutic antifungal potential, with some recent experimental compounds proven to be promising. Essential oils and/or their individual constituents play an important role as potential therapeutic agents. The main advantage is their lipophilic nature, which allows the compounds to easily penetrate the plasma membrane, in addition to the ability to cure opportunistic fungal diseases without harmful effects on human and animal tissues. With growing interest in the use of essential oils in the pharmaceutical industry, systematic examination of their preventive and therapeutic properties has become increasingly important. This paper reviews the antifungal efficacy of essential oils belonging to various plant families for the prevention and treatment of common opportunistic diseases in humans (i.e., dermatomycosis and candidosis), specifically those infections caused by fungi with primary entry routes through skin and mucosa.

Introduction

Fungal infections remain a therapeutic problem in many fields despite the availability of a number of treatments. Resistance problems are pressing the pharmaceutical industry to find new solutions for disease control (1). Natural products derived from plants have traditionally been used in ethnomedicine. Some of the most investigated and applied plant secondary metabolites for the treatment of infectious diseases are essential oils.

Although pathogenic microorganisms have traditionally been treated by chemical means, the use of synthetic compounds is limited due to undesirable characteristics, which include carcinogenicity, teratogenicity, acute toxicity and lengthy degradation time, which can lead to the consequent development of environmental pollution problems (2).

In addition, there are barriers to the effective reduction of mycoses: a) fungi are difficult to eliminate because they are wide spread in the nature; b) mycoses are not easy to diagnose because they have various clinical manifestations that are host dependent; c) a proper therapy is difficult to prescribe because a number of available drugs are restricted access; and d) treatments are available for a limited number of fungi and some treatments are only for animal species.

Human pathogens can cause serious infections, and almost any fungus can potentially cause an infection. Although these diseases are uncommon,

a higher incidence of fungal diseases has been seen. This trend is due to the increased number of transplants that are being performed and the rise in the number of people afflicted with AIDS. For transplant recipients, they must take immunosuppressant drugs so the transplanted organ will not be rejected by their body: while the immune systems of individuals afflicted with AIDS have been comprised by the nature of the disease. With an increase in patients with compromised immune systems, there has been a rise in the number of people that have succumbed to fungal diseases (3). The frequency of dermatomycoses is greater in communities with low socioeconomic status: crowded living conditions provide multiple opportunities for skin-to-skin contact and close proximity to animals, while hygiene may be suboptimal (4).

Fungal diseases are often more difficult to treat than bacterial infections. Often topical and oral treatments are long term and may only be partially successful in controlling the fungus or not effective at all. Many infections are chronic with the possibility of recurrence of the disease. In addition, anti-fungal drugs may cause harmful side effects due to similar cellular functions between fungal and human eukaryotic cells by which the drugs may target.

Opportunistic Mycoses

There are several types of fungi, including yeasts and molds, which may cause diseases in humans. The majority of human pathogenic fungi appear to be soil-inhabiting species that live as saprobes. However, under the appropriate conditions (i.e., if a person is not healthy, has an open wound present, is directly injected with a fungus or at risk due to life-style factors or AIDS), fungi may aggressively attack people. Thus, many fungal infections may be due to opportunistic fungi (i.e., facultative parasites) rather than fungi that specifically cause human diseases (5).

Although fungi are common and pose a low risk to healthy populations, they can also lead to serious and even deadly complications in people with compromised immune systems. In recent years, fungal infections have become more of a problem due to the rising number of people with compromised immune systems (e.g., people with AIDS, organ transplants, diabetes and undergoing treatments for various forms of cancer). An important factor contributing to the increase in the incidence of opportunistic infections is the rise in the number of immunocompromised patients who are susceptible hosts. Fungi that are most frequently isolated from such patients are either saprophytic (i.e., from the environment) or endogenous (i.e., a commensal). However, when the immune system of the host is weakened either by disease or medications, the fungi may grow out of control and cause serious health problems.

Opportunistic diseases in humans induced by fungi that have primary entry routes via the skin and mucous membranes (Table 1), are termed dermatomycosis and candidiasis, respectively.

Table 1. Main Fungi That Cause Dermatomycosis and Candidiasis Infections in Humans

<i>Primary route of entry</i>	<i>Common distribution</i>	<i>Disease</i>	<i>Causal agent</i>
Skin	Keratinized human tissues	Dermatomycoses <i>ringworm</i> <i>tinea</i>	<i>Trichophyton</i> spp. <i>Microsporum</i> spp. <i>Epidermophyton</i> spp.
Mucosa	Commensal human micobiota	Candidiasis - oral and vulvo-vaginal thrush - stomatitis	<i>Candida albicans</i> several non- <i>albicans</i> species

Main Causal Agents of Dermatomycoses Whose Primary Route of Entry Is the Skin

Dermatomycetes are pathogenic fungi that cause dermatomycoses, which are superficial infections of the skin, hair, and/or nails/claws tissues in animals and humans. They grow in a zone just above the area where the protein keratin is deposited (6) and have the ability to degrade keratin in order to utilize it as a food source (7). Disseminated infections due to any of the dermatomycetes are very unlikely due to the restriction of the infection to keratinized tissues. A skin infection occurs when the fungus contaminates or colonizes the epidermis or hair follicles. Clinical changes in the skin may not always be obvious. Lesions caused by a fungus depend on the location and the structure of the place where it is induced to grow (e.g., superficial layer of the cutis, hair, or nails).

Dermatomycetes are non-invasive saprotrophs of the skin and related appendages but the growth causes irritation and inflammation of the underlying epithelial cells, causing a reaction that may result in the cells' death. Although dermatomycoses do not jeopardize life, the diseases can be chronic and bring about significant morbidity. In many cases, therapy is limited by the toxicity of the medications and associated side effects (e.g., nausea, abdominal pain, and itching) (2).

In human medicine, dermatomycosis accounts for 6-9% of all pathological changes of the skin (8). Yet, mechanism between the host and fungus that contributes to incidence of a disease is not well understood. The most significant aspect with regard to dermatomycosis is related to broadening the knowledge of all factors involved in pathogenesis (e.g., proteases, secretory enzymes, adhesion mechanisms and ability to modulate defense mechanisms of the host) (8).

The spectrum of dermatomycetes is not static; booming mass tourism, international sports activities and increasing migrations mean that less common or forgotten species are being imported and disseminated (4). Dermatomycetes

are widespread in nature and their classification depends on the habitat and presence in various ecological niches. Daecon (9) and Chabassea and Piheta (10) classified fungi into several categories: zoophilic (i.e., infect animals primarily but can readily be transmitted to people), geophilic (i.e., occur naturally in soil, presumably as a saprobe, but capable of infecting animals and people as facultative parasites), and anthropophilic (i.e., infect people but cannot be transferred to animals). Among all dermatomycetes, the zoonoses gain the most attention because they commonly infect both people and animals.

Three genera (*Epidermophyton*, *Microsporum*, and *Trichophyton*) encompass the dermatomycetes (Table 2). Representatives of these three genera with regard to virulence and significance to human health are explained in the text that follows.

Epidermophyton spp.

This genus has two species of which the pathogenic one is *Epidermophyton floccosum* (Harz) Langeron et Milošević.

- *E. floccosum* – is an anthropophilic species that infects the body, feet and nails, while the hairs do not get infected. The fungus is distributed worldwide (11). The infection is restricted to the nonliving cornified layers of the epidermis because it lacks the ability to penetrate the viable tissues of immunocompetent hosts (12). The fungus is communicable and usually transmitted by contact, particularly in public showers and gym facilities. Terbinafine, itraconazole, and ketoconazole are in common use for treatment infections caused by *E. floccosum* (13).

Microsporum spp.

This genus encompasses 19 species but only 9 are involved in infections (9). *Microsporum* species have the ability to degrade keratin and thus can reside on skin and its appendages, while remaining noninvasive. The enzymes produced by the fungi including keratinase, proteinases, and elastases may act as virulence factors.

Except for *M. persicolor* that does not infect hair, *Microsporum* spp. mostly infect the hair and skin, while nail infections are very rare. Immunocompromised patients are easily infected (14), and asymptomatic carriage of the fungi is also observed. The pathogenesis of the infection depends on the natural reservoir of the fungal species: acquired via contact with the soil (geophilic), via an infected animal (zoophilic), or via direct or indirect (e.g., fomites) human-to-human transmission (anthropophilic). With regard to pathogenicity, the most clinically important *Microsporum* species are *M. canis* Bodin and *M. gypseum* Bodin.

Table 2. Major Dermatophytes That Infect People

	<i>Microsporum</i> <i>spp.</i>	<i>Trichophyton</i> <i>spp.</i>	<i>Epidermophyton</i> <i>spp.</i>
Anthropophilic	<i>M. audouinii</i>	<i>T. mentagrophytes</i> var. <i>interdigitale</i>	<i>E. floccosum</i>
	<i>M. ferrugineum</i>	<i>T. rubrum</i>	
		<i>T. tonsurans</i>	
Zoophilic	<i>M. canis</i> (dogs, cats)	<i>T. equinum</i> (horses)	
	<i>M. equinum</i> (horses)	<i>T. mentagrophytes</i> var. <i>mentagrophytes</i> (mice, rodents)	
	<i>M. nanum</i> (pigs)	<i>T. verrucosum</i> (cattle)	
	<i>M. persicolor</i> (rodents)		
Geophilic	<i>M. gypseum</i>	<i>T. terrestrae</i>	

- *M. canis* – is a widely distributed (11) zoophilic species, which is the most common source of ringworm in people. This fungus infects the body of adults and the skin of the scalp in children but rarely attacks the nails. Animals, such as dogs, cats (in rare occasions monkeys, pigs, horses, mice, cows, and rabbits) are carriers of ringworm, but these animals do not necessarily show outward signs of the disease. Symptomless animals, and people as well, are carriers of the disease. The infections are spread mainly by spores, but mycelial fragments in the skin and hair can presumably also occur. Long-living spores can remain viable for years in blankets, clothing, bedding, combs, and other grooming tools.
- *M. gypseum* – is a geophilic species that also infects animals, mostly dogs, and it infects the skin and hair of humans (15). Griseofulvin was a commonly used drug for treatment of infections caused by *Microsporum* spp., however, terbinafine and itraconazole are the preferred drugs, as they are more available and widely used (14).

Trichophyton spp.

This genus encompasses 22 species (9), and the most pathogenic species are *Trichophyton mentagrophytes* (Robin) Blaochard, *Trichophyton rubrum* (Castell.) Sabouraud and *Trichophyton tonsurans* Sabouraud.

- *T. mentagrophytes* – is anthropophilic and zoophilic (i.e., rodents and mammals) species; found in the soil; and it infects the feet, body, nails, beard, scalp, arm, and groin areas. The anthropophilic species do not infect hair. Most of the *Trichophyton* species have teleomorphic forms, and these teleomorphs are classified in the genus *Arthroderma*. These fungal species are widely distributed (11).
- *T. rubrum* – is the most frequently isolated dermatomycete. It is an anthropophilic species that is also isolated from animals but never from the soil. It infects the feet, nails, body, groin, and rarely the scalp. This fungus is widely distributed (16).
- *T. tonsurans* – is anthropophilic, and it infects the scalp, skin, and nails. This fungus is widely distributed (16). Similar to the other two genera, *Trichophyton* is a keratinophilic filamentous fungus. It has the ability to invade keratinized tissues and possesses several enzymes (e.g., acid proteinases, elastase, keratinases, and other proteinases), which are the major virulence factor of this fungus (17).

Ketoconazole, clotrimazole, itraconazole, terbinafine, naftifine, and amorolfine have all proven to be active against *Trichophyton* spp. under *in vitro* conditions; while terbinafine usually appears to be the most effective agent. In general, isolates of *T. rubrum* are more susceptible to antifungal agents compared to *T. mentagrophytes*. The azole derivatives are also active *in vitro* against *Trichophyton* spp. (18).

Human Dermatomycoses

Tinea Diseases

Infections caused by dermatomycetes are called dermatomycoses (i.e., ringworm). Ringworm can affect the human skin at different locations (e.g., beard, body, feet, groin area, scalp). The fungus that causes ringworm thrives in warm, moist areas. An infection is more likely to occur under wet (e.g., sweating) conditions and from minor injuries to the skin, scalp, or nails. The naming convention for these infections includes naming the location of the lesion followed after the Latin word *tinea*. Common human dermatomycetes causing *tinea* diseases are explained in the text that follows.

- *Tinea corporis* – dermatomycosis that appears on exposed skin, shoulders, legs, and may also appear on the face. It is commonly called “ringworm of the body”. Clinical signs could be severe with clearly limited erythematous vesicular spots. The main causal agents are *Trichophyton rubrum*, *T. verrucosum* and *Microsporum canis*.
- *Tinea cruris* – infection of the crotch, perianal and perineal regions. It is also called “jock itch”. It appears mostly in older male adults and most often in the inguinal region. The symptoms are flushing with dry dandruff. The main causal agents are *Trichophyton rubrum*, *Epidermophyton floccosum* (17) and *T. mentagrophytes* var. *interdigitale*.
- *Tinea faciei* – is present on the glabrous skin of the human face, and the main causal agent is *Trichophyton rubrum*.
- *Tinea pedis* – is present on the soles of feet and toes. It is also called “athlete’s foot”. It could be chronic with squamous epithelia, hyperkeratosis, redness, and inflammation. The main causal agents are *Trichophyton rubrum*, *T. mentagrophytes* var. *interdigitale*, and *Epidermophyton floccosum* (17).
- *Tinea manuum* – lesions are found on the palms and interdigital areas of hands. Hyperkeratosis and cracking of the skin are also present. The main causal agent is *Trichophyton rubrum* (17).
- *Tinea capitis* – is also called “scalp ringworm” and appears on the head that is covered with hair but can also be found on eyebrows and eyelashes. The disease could be subclinical with erythema or severe folliculitis, alopecia, and sometimes with lymphadenopathy (19). The main causal agents are *Trichophyton* spp. and *Microsporum* spp.
- *Tinea barbae* – stands for the infection of the chin, which could be superficial or deep with severe inflammatory pustular folliculitis. It is caused by zoophilic dermatomycetes (20). The main causal agents are *T. verrucosum* and *T. mentagrophytes*.
- *Tinea unguium* – attacks toenails and appears under the nails or superficially. The main causal agents are *Trichophyton rubrum* and *T. mentagrophytes* var. *interdigitale*.

- *Tinea favus* – causes lesions on the head that appear as prominent yellow scabs and dry dandruff. It has been recorded mostly in Euro Asia and Africa (21).
- *Tinea imbricate* – a chronic infection that appears in places where the skin folds, and the causative agent is *T. concentricum*, which is a strictly anthropophilic dermatomycete. It is found in Asia, North and South America and Oceania (21).

Candidiasis

Members of the genus *Candida* are representative of several yeast species that are considered to be commensally oral microbiota.

Candidiasis is the most common opportunistic yeast infection in the world. A variety of *Candida* species lives on humans and homoeothermic animals. Some species colonize in the first few days after birth, primarily in the mucosa of the gastrointestinal tract (40 - 50%) as well as in the upper respiratory passages, mouth, pharynx, and larynx. They are found in up to 10% of the male population on the genitalia; in the transitional zone between the mucous membrane and the skin; and in 5-30% of nonpregnant women, depending on hygiene, oral contraceptive use, and use of intrauterine devices for birth control. Among pregnant women, *Candida* species colonization increases to 30 - 40% due to an altered immune response with increased glycogen levels and changed pH levels.

The source of the infection is usually pathogen colonization on the body with predisposing factors contributing to the infection. Infections may occasionally be transmitted from another source (e.g., contaminated catheters, non-sterilized needles among drug users, or sexual partners infected with *Candida balanitis*). Candidiasis can affect patients of any age. In infants, the infection may occur after inoculation in the birth canal; and in older children and adults, infection may occur in conjunction with immunosuppression.

Predisposing factors include: changed pH levels (especially in the vagina and mouth), pregnancy, premature birth, diabetes, malnourishment, primary immune deficiency, acquired immune deficiency (e.g., patients with leukemia, lymphoma, HIV/AIDS, or long-term use of antibiotics, corticosteroids, and cytostatic agents), oral contraceptive use, surgery, catheter placement, and prior inflammatory skin diseases (e.g., contact dermatitis or diaper dermatitis), nail trauma, persistent moisture on the hands, poor teeth and ill-fitting prostheses (22).

- *Candida albicans* – is the most commonly isolated from the human oral cavity (23) and can be easily grown and indentified on Sabourad Dextrose Agar (Figure 1a) or Chromagar (Figure 1b). The non-*albicans* species (e.g., *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. dubliniensis*) are generally isolated less frequently although there are indications showing that the non-*albicans* species recovered from cases of oral candidiasis have begun to increase (24). Under certain circumstances, *Candida* spp. commensals become pathogenic and cause infections that range from mucous membrane infections (25) such as pseudomembranous

candidiasis (26) and denture-induced stomatitis (27), to life-threatening systemic diseases (28), particularly in immunocompromised patients with AIDS, cancer and diabetes mellitus (29). Oral candidiasis is an opportunistic infection usually accompanied by various symptoms, including burning, painful sensation, change of taste and swallowing difficulty, but it can be also asymptomatic. The fungal infection can be treated with various synthetic antifungal agents, although they possess disadvantages, such as high toxicity to the host tissues, emergence of drug-resistant species, and high cost (30). There are a number of case reports describing the colonization and infection of immunocompromised patients or denture wearers who have been subjected to a long term regimens of oral antifungal agents, resulting in less drug responsiveness or resistance (31–36). Even cross-resistant *Candida* spp. (37) have been recovered. In addition, recurrences of oral candidiasis are commonly observed in these patients (38).

Candida albicans is an opportunistic pathogen that normally inhibits the gastro-intestinal tract of humans (23), but under altered conditions it can cause severe mucous membrane infections (39), particularly in immunocompromised patients (40). In addition, it is apparent that many oral conditions, including various forms of human periodontal diseases, may involve mixed-species infections, mainly caused by *C. albicans* and bacteria (41). Recent reports by researchers indicate that the presence of yeasts in the oral cavity has also been linked with oral carcinogenesis (42, 43). According to the literature, more than 70% of all cancer deaths occurred in low- and middle-income countries, and these countries are also associated with poor oral hygiene (44). Cancer-related deaths are predicted to increase to ca. 11.5 million deaths in the year 2030, and approximately 27 million new cancer cases and 17.5 million cancer deaths are projected to occur globally by the year 2050 (45). This growing trend indicates that there is a crucial need for highly efficient antifungal agents with minimal side effects and at an affordable cost. The vast structural diversity of natural compounds of plant origin provides a unique opportunity for the discovery of such new drugs.

Testing the susceptibilities of *Candida* spp. enhances the selection of the most promising treatments because a number of species have already developed resistance against widely used antifungal drugs. The increased resistance of these species to mycotics, in combination with the fact that many drugs can be toxic and cause side effects, renders the development of new antifungal agents a subject of great interest.

Essential Oils as Active Antifungal Substances

Urge To Discover Novel Antifungal Drugs of Natural Origin

In the past few decades, an increase in the incidence of fungal infections has been observed as well as a rise in the resistance of some fungal species to various fungicides used in medicinal practice. Fungi seem to belong to a class of neglected

pathogens, as demonstrated by the fact that Amphotericin B, a polyene antifungal discovered several decades ago, is still used as the standard for antifungal therapy to treat systemic mycosis and other fungal infections that do not respond to other drugs. Azole drugs are also widely used, but these only inhibit fungal growth and do not kill the fungus.

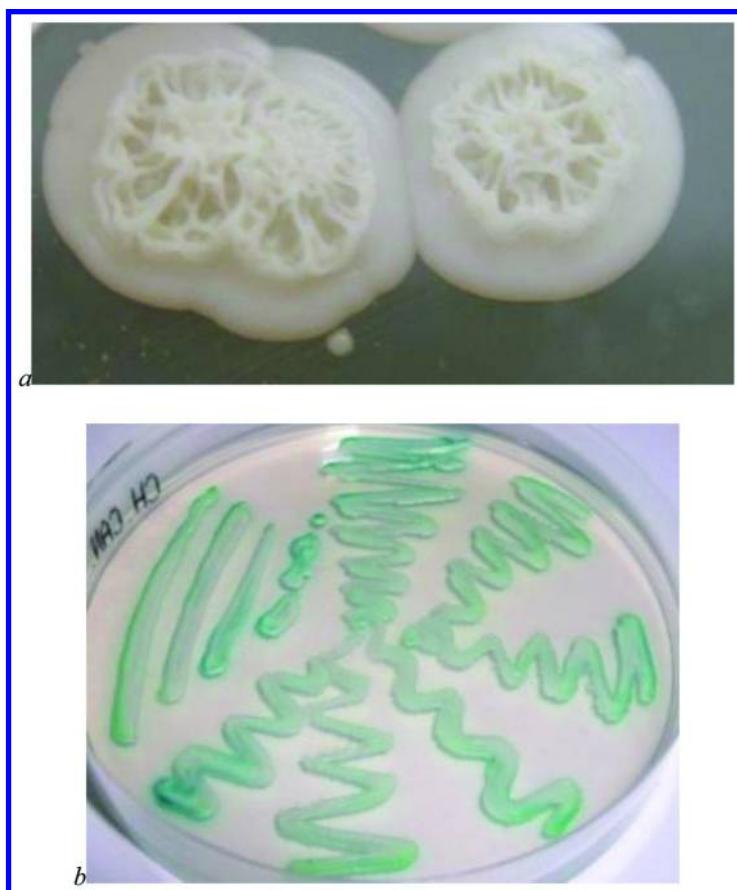


Figure 1. (a) *Candida albicans* colonies on Sabourad Dextrose Agar (top). *(b)* *Candida albicans* colonies on Chromagar (bottom)

Most chemical treatments are also toxic to humans as well as the fungus. In addition to toxicity, the majority of clinically used antifungals have other drawbacks, including low efficacy and high cost. The frequent use of antifungals has also brought about the emergence of drug-resistant strains.

In recent years, public pressure to reduce the use of synthetic fungicides in agriculture has increased, and concerns have been raised regarding both the environmental impact and the potential health risk of using these compounds.

Consequently, there is a demand for novel antifungals that belong to a wide range of structural classes, selectively act on new targets, and possess fewer side effects. One possible approach is to focus on natural products of plant origin and to test whether plants that have been traditionally used for their antifungal activity can serve as a potential source of new antifungal agents. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug development due to a unparalleled availability of chemical diversity.

Despite advancements in science and technology, the development of novel and efficient antifungal drugs is still lagging behind. Because fungi are also eukaryotic with cellular mechanisms similar to human beings, it is a challenge to develop an antifungal agent specific to fungi that will not affect human cells.

Several factors must be considered for the selection of appropriate antifungal treatment: the site of infection, etiological agent, and antifungal drug penetration ability. Because the dermatomycetes reside in the stratum corneum within the keratinocytes, the antifungal agents must possess a good penetrating ability through tissues (46). In addition for the successful treatment of infections, more effective and less toxic antifungal agents are required. Essential oils seem promising with regard to these criteria to achieve strong antifungal activity: the majority have good penetration potential and the lipophilic properties of their constituents may help to penetrate the plasma membrane. When applied in active concentrations (e.g., MIC and MLC) these plant-derived drugs should not be harmful for animals and humans.

This review aims to examine the recent efforts towards discovering novel antifungal drugs of natural origin. This information has been organized into easily accessible and comparable sections with quick reference to the essential oils or single oil constituents studied.

Essential Oils and Active Constituents with Antifungal Activity

The increasing resistance to antifungal compounds and a reduced number of available drugs due to side effects are major reasons why many researchers are studying therapeutic alternatives from aromatic plants and the essential oil constituents. In recent years, studies on antifungal properties of natural products have mainly involved aromatic plant families, and these studies have demonstrated that many essential oils are with antifungal effects and beneficial to humans (47, 48). However, only limited information exists about the activities against the specific fungal pathogens; and thus the information that follows will review recent research data with regard to this perspective.

Many aromatic plants that belong to **Lamiaceae family** have been tested for activity against various fungi. The antifungal effects of the oils from *Satureja montana* L., *Lavandula angustifolia* Mill., *Lavandula hybrida* Reverchon, *Origanum vulgare* L., *Rosmarinus officinalis* L., and six chemotypes of *Thymus vulgaris* L. against *C. albicans* growth have been studied (49). The greatest efficacy was obtained with the thymol chemotype of the *T. vulgaris* essential oil ($IC_{50} 0.016 \mu\text{g/mL}$).

The antifungal activities of the essential oil of *L. angustifolia* and its main components (i.e., linalool and linalyl acetate) were investigated against 50 clinical isolates of *C. albicans* (oropharyngeal and vaginal strains). Linalool alone was more effective than the essential oil, while linalyl acetate was nearly ineffective (50).

The essential oil of *O. gratissimum* showed *in vitro* fungicidal activity against all of the *Candida* spp. studied (i.e., *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*). Analysis of the ultrastructure of the yeast cells revealed changes in the cell wall and in the morphology of some subcellular organelles (51).

The screening of 20 essential oils against a number of human pathogens, including *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton rubrum*, and *Candida albicans*, concluded that the oils of *Ocimum gratissimum* (Lamiaceae family) and *Trachyspermum ammi* (Apiaceae family) were the most effective with fungicidal properties at 50 ppm. The ointments of two oils, which were prepared in PEG (polyethylene glycol) to enhance skin penetration, blocked dermatomycoses induced by *E. floccosum* and *M. gypseum* in 5- and 8-months-old guinea pigs, indicating their potential efficacy as herbal treatments. The ointment made with *O. gratissimum* oil cured the disease in the guinea pigs in 11 and 9 days, respectively, while the ointment with *T. ammi* oil worked in 13 and 11 days (52).

The antifungal activity of the essential oil of *Nepeta crista* Willd., an endemic plant species from Iran exhibited a noticeable antifungal activity against four fungi, including *C. albicans* ATCC5027 and *M. gypsum* ATCC5070. The main oil constituents were 1,8-cineole (47.9%) and 4aa,7a, 7a β -nepetalactone (20.3%) (53).

Plant essential oils from the genus *Thymus*, showed potential in anti-*Candida* activity screening tests. The antifungal activities of *Thymus pulegioides* (thymol/carvacrol chemotype) oil and its major components (thymol, carvacrol, p-cymene, and γ -terpinene) were evaluated against several clinical strains of *Candida* spp. isolated from recurrent cases of vulvovaginal candidosis (*C. albicans*, *C. krusei*, *C. tropicalis*, *C. guillermondi*, and *C. glabrata*), four *Candida* spp. ATCC strains, and dermatomycetes clinical strains isolated from nails and skin (*Microsporum canis*, *M. gypseum*, *Trichophyton rubrum*, *T. mentagrophytes*, and *Epidermophyton floccosum*). The MIC and MLC values of the oil for all the *Candida* spp. ranged from 0.32 to 0.64 μ g/mL while for the dermatomycetes these values were lower and ranged from 0.16 to 0.32 μ g/mL. When thymol was applied alone, a 0.16 μ g/mL concentration was lethal for almost all tested fungi. When thymol was applied in combination with carvacrol, for the *Candida* spp. a lethal dose up to 0.32 μ g/mL was required, while for the dermatomycetes a lower concentration of 0.16 μ g/mL was required. The authors concluded that the essential oil from *T. pulegioides* has potential as a topical antifungal agent against the tested fungi that are pathogenic to humans, and they proposed that the mechanism of the oil activity was a cytoplasmic membrane lesion it creates. They also suggested the need for further toxicity studies with an emphasis on improving formulations and determination of optimal concentrations for clinical applications. In addition, the researchers suggested comparative studies with currently used drugs to test the therapeutic efficacy of essential oils to control

mucocutaneous infections (54). A recent study from the authors of this paper with essential oils from three thyme species (*T. serpyllum*, *T. algeriensis*, and *T. vulgaris*) found that the growth of *Candida* spp. was inhibited in a range between 1 and 10 µg/mL, which confirmed the strong anti-*Candidal* activity of these oils (55).

Essential oils from the plant species *Thymbra capitata* (L.) Cav., which are rich in carvacrol (60.0 - 65.8%), possessed antifungal activity against all clinical isolates and ATTC strains of the *Candida* spp. studied and all clinical dermatomycetes strains with IC₅₀ values ranging from 0.08 to 0.32 µg/mL. The authors also concluded that the fungicidal effect was mainly due to a direct lesion of the membrane (56).

The *in vitro* antifungal activities of the essential oils and their major constituents from *Origanum vulgare* subsp. *hirtum*, *Mentha spicata*, *Lavandula angustifolia*, and *Salvia fruticosa* indicate that they could be used as effective antifungal agents against the human pathogens *Trichophyton rubrum* and *Trichosporon beigelii*. In the Ames test, the oils did not exhibit mutagenic activity. The *in vivo* studies showed promising results of the therapeutic efficacy of oregano oil on rats experimentally infected with *T. rubrum*. With regard to the testing of the major oil components, carvacrol and thymol exhibited the highest levels of antifungal activity (57). There is an apparent relationship between the strong antifungal activity of oils rich in phenolic components, particularly thymol and carvacrol, and the strong activity of the oils containing them because they appear to be responsible for the entire effect, although the other oil constituents may enhance activity (58).

The oils from another five Lamiaceae species, including *M. piperita*, *M. pulegium*, *L. angustifolia*, *S. montana*, and *S. lavandulifolia*, were tested against 58 oral clinical isolates of *Candida* spp., and these oils expressed strong inhibition with the oil of *S. montana* as the most potent (MIC 0.9-1.0 µg/mL and MFC 1.0-3.0 µg/mL) (56).

Four chemotypes (geraniol, linalool, thymol, and carvacrol) of *Thymus zygis* subsp. *sylvestris* from Portugal were tested against two dermatomycete strains isolated from nails and skin (*Trichophyton mentagrophytes* FF7 and *Microsporum canis* FF1), two standard dermatomycetes (*T. rubrum* CECT 2794 and *M. gypseum* CECT 2908), two *Candida* strains isolated from recurrent cases of vulvovaginal candidosis (*C. krusei* H9 and *Candida guillermondi* MAT23), and three standard *Candida* strains (*C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, and *C. parapsilosis* ATCC 90018). The most effective against the dermatophyte strains proved to be the carvacrol chemotype of the oil with MIC of 0.16 µL/mL, and this same concentration also proved to lack cytotoxicity in mouse skin dendritic cells. The authors suggested further investigation in order to evaluate the suitability of the antifungal property of this oil in practical applications for fungal diseases involving mucosal and cutaneous infections and as an alternative to synthetic fungicides (59).

The *in vivo* evaluation of the antifungal activities of several Lamiaceae essential oils and the major components have been conducted for the therapeutic potency against experimentally induced dermatomycoses in 2-month old male Wistar rats caused by the most frequent dermatomycetes (*Trichophyton*

mentagrophytes, *T. rubrum*, and *T. tonsurans*). The study showed the following results: *Lavandula angustifolia* oil rich in linalool (27.2%) and linalyl acetate (27.5%) exhibited therapeutic activity within 13 days following the treatment; while rats treated with *Ocimum basilicum* oil with linalool as a major component (69.3%) were cured in 25 days. The shortest period of curing (12 days) was observed at animals treated with *Salvia officinalis* oil (Figure 2), while the longest period (45 days) was observed in treatment with *Citrus aurantium* and *Citrus limon* oils, both rich in limonene (71.8% and 59.7%, respectively). The main essential oil components were also tested as potential antifungal agents: camphor exhibited therapeutic and antifungal activity in 14 days and linalool in 32 days; while the rats treated with 1,8-cineole and limonene were cured in 40 and 50 days, respectively (60).

The *in vivo* testing of the essential oil of *Mentha x piperita* and its major constituent menthol showed the following results: the oil completely cured the Wistar rats infected with *T. metagrophytes* in 15 days, *T. tonsurans* in 29 days, and *T. rubrum* in 30 days; while menthol expressed the highest therapeutic and antifungal activities and cured the animals in 10 days, which was more effective than the imidazole antifungal drug bifonazole (61).

The antifungal activity of the essential oil from *Lavandula angustifolia* showed therapeutic and antifungal potential during the 13-day observation period and completely cured the rats (62).

The essential oil of *Thymus vulgaris* L. and its major component thymol was also tested for therapeutic potency *in vivo*. This oil completely cured 2-month-old male Wistar rats infected with *T. metagrophytes*, *T. rubrum* and *T. tonsurans* in 24, 37, and 32 days, respectively; while those rats treated with the commercial drug bifonazole were cured in 14–15 days. Moreover, thymol had a higher therapeutic and antifungal activity in comparison to the whole oil and cured the animals in 14 days (63).

In research by the authors of this paper, investigations on the antifungal activities of essential oils from the *Lamiaceae* family and their components yielded the most promising oils as *S. officinalis* (Figures 2a and 2b) followed by the oil of *L. angustifolia*, and menthol as the single oil component with good therapeutic and antifungal effects under *in vivo* conditions (64). These oils and the component may contribute to the field of medical mycology as fast and reliable alternatives in control of skin infections caused by fungal pathogens.

Here follows a few reports on the activity of essential oils from the members of the *Apiaceae family*. Tavares and colleagues (65) investigated the chemical constituents and antifungal effects of the oil samples from Iberian endemic plant species, traditionally used in the treatment of contact dermatitis and skin infections. The oil of *Distichoselinum tenuifolium* (rich in myrcene at 47.7–84.6%) was tested against dermatomycetes (*Microsporum canis* FF1, *Trichophyton rubrum* CECT 2794, *Microsporum gypseum* CECT 2905, and *Epidermophyton floccosum* FF9) and *Candida* spp. (*C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, *C. krusei* H9, *C. guillermondii* MAT23, and *C. parapsilosis* ATCC 90018). The authors concluded that the superior antifungal activity of the *D. tenuifolium* oil compared to that of myrcene alone is due to the synergistic effect among the different compounds present in the oil. *D.*

tenuifolium oil showed more effective antifungal activity against dermatomycete strains (MIC and MLC values ranging from 0.64 to 1.25 $\mu\text{L}/\text{mL}$) than that of *Candida* spp. (MIC of 2.5 to 5.0 $\mu\text{L}/\text{mL}$ and MLC of 2.5 to 10 $\mu\text{L}/\text{mL}$). The higher susceptibility of dermatomycetes has also been reported for other essential oils (66, 67).

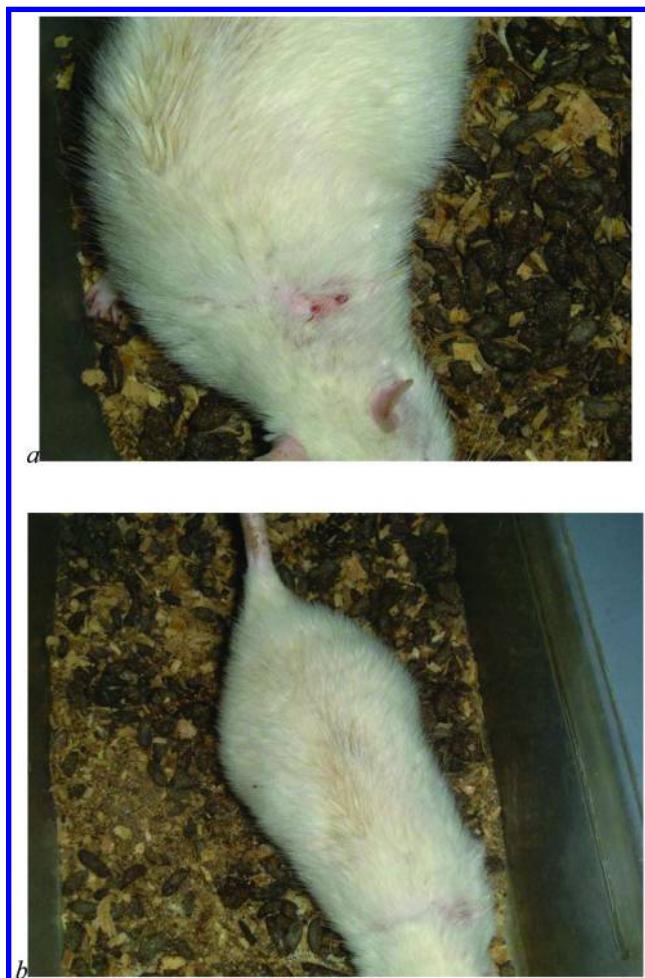


Figure 2. (a) Experimentally induced dermatomycoses in rats by *T. mentagrophytes* - first symptoms, day 3 (top). (b) Experimentally induced dermatomycoses in rats by *T. mentagrophytes* - completely cured with *S. officinalis* oil, day 12 (bottom)

Reports on the activity of essential oils from plants in the *Asteraceae family* against fungi that cause dermatomycoses in humans could also be found in the literature.

The *in vitro* antifungal assays of the leaf oils of five endemic *Psiadia* species from Mauritius revealed that only *Psiadia lithospermifolia* Lam. oil, with major constituent of (E)-isoasarone (51.5%), significantly inhibited the growth of *Candida pseudotropicalis* (68).

Ageratum houstonianum leaf essential oil showed potent efficacy in the control of human nails infestation, *Tinea unguium*, by the most frequent causal agents *Candida albicans* and *Trichophyton rubrum*; the MIC values for both fungi were 400 ppm, while at 500 ppm the oil was lethal. The minimum killing times were 30 sec and 40 sec, respectively. In addition, the oil was stable at temperatures up to 100°C and during storage up to 180 days (69).

The effect of the *Matricaria recutita* flower essential oil (rich in chamazulene at 61.3%) was evaluated against medically important dermatomycetes and opportunistic saprophytes. Percent growth inhibition for the dermatomycetes exposed to serial two-fold concentrations of the oil (2.5 to 80 µg/mL) ranged as follows: *Microsporum gypseum* at 3.2 - 68.2%, *M. canis* at 24.5 to 100%, *Trichophyton mentagrophytes* at 11.4 to 96.7%, *T. rubrum* at 27.8 to 100%, and *T. tonsurans* at 45.7 to 100%. The authors concluded that the oil of *M. recutita* may be considered as a potential candidate for designing effective antifungal formulations suitable for treatment of dermatomycoses (70).

The essential oil extracted from *Spilanthes acmella* Murr was studied for efficacy against *Trichophyton mentagrophytes* MTCC 7687 infections induced on the skin of *Mus musculus*. Mice with skin lesions were subjected to topical applications of the oil at a concentration of 1 µl/mL (v/v, oil/emulsifier), and terbinafine (5 mg/mL, W/V) was administered as a control drug. The *S. acmella* essential oil (1 µl) had a comparable therapeutic activity with regard to the terbinafine against the test fungus. While terbinafine showed absolute growth inhibition at day 8, the oil had a 75% inhibitory effect at day 11. Thus, the authors conclude that the oil, as a natural plant product, should be considered as a topically preferred drug in patients with hypersensitivity where the use of terbinafine is contraindicated (71).

Other authors reported the antifungal activities of essential oils from aromatic plants that belong to the *Verbenaceae family*. The *in vitro* testing of the essential oils of the aerial parts of *Lippia graveolens* and *Lantana achyranthifolia* (both oils rich in carvacrol at 37.8% and 30.6%, respectively) against *Trichophyton mentagrophytes* showed that both oils had antifungal activity (IC_{50} values were 110 and 20 µg/mL, respectively). This study confirmed the use of the oils from both species in the folk medicinal treatments of dermatological diseases (72).

The *in vitro* testing of two chemotypes, citral (geranal-neral) and carvone, of the *Lippia alba* oil were conducted. The MIC values of the citral chemotype against *Trichophyton rubrum* ATCC 28188 and *T. mentagrophytes* ATCC 24198 were 31.3 and 125 µg/mL, respectively, while MIC values for the carvone chemotype were higher (73). In another study, the carvone chemotype of the *L. alba* oil proved to possess good antifungal properties against *Candida krusei* and *C. parasilopsis* (74).

Results obtained in the study of *Lippia gracilis* essential oil (thymol and two carvacrol chemotypes) against *Trichophyton rubrum* showed that the fungus is highly susceptible to the oil, which exhibited antifungal activities similar to

those values seen for the commercial antifungal drug fluconazole. The thymol chemotype of the oil (61.8%) presented better activity in comparison to the carvacrol chemotypes of the oil (54.5% and 48.9%), and the authors suggested that thymol contributes substantially to the antifungal activity of this oil (75).

Recently the authors of this review tested the sensitivity of common *Candida* spp. (ATCC strains and clinical isolates) recovered from human oral cavities (*C. albicans*, *C. krusei*, and *C. glabrata*) to four commercial essential oils from the ***Burseraceae family***. The oils included two *Boswellia carterii* Flueck oils, *Canarium luzonicum* (Blume) A. Gray oil, and *Commiphora myrrha* (Nees) Engl. The *B. carterii* oil (sample 2), characterized by a high α -pinene content (31.8%), presented the strongest activity. The average MIC and MFC values ranged from 1.25 - 1.34 mg/mL and 2.50 - 3.75 mg/mL, respectively, depending on the fungus tested. This study supports the possible use of essential oils from the *Burseraceae* family in the reduction and elimination of *Candida* spp. infections in patients (76).

There are a few reports on the antifungal essential oils from the ***Cupressaceae family***. Out of three *Juniperus* spp. oils, *J. oxycedrus* ssp. *oxycedrus* L. leaf oil, rich in alpha-pinene (65.5%) and delta-3-carene (5.7%), proved to be the most effective against the dermatomycete strains of *E. floccosum* FF9, *T. mentagrophytes* FF7, *M. canis* FF1, and *M. gypseum* FF3 (MIC and MLC values ranging from 0.08 to 0.32 μ L/mL) and the *Candida* strains of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* (MIC and MLC values ranging from 0.32 to 2.5 μ L/mL). The authors concluded that the oil proved to be an emergent alternative as an antifungal agent against the tested dermatophyte strains with delta-3-carene being fundamental for the activity (77).

A study on the antifungal potential of *Metasequoia glyptostroboides* Miki ex Hu. essential oil against different strains of common dermatomycetes, including *Trichophyton rubrum* (KCTC 6345, KCTC 6375, and KCTC 6352), *T. mentagrophytes* (KCTC 6085, KCTC 6077, and KCTC 6316), and *Microsporum canis* (KCTC 6591, KCTC 6348, and KCTC 634), revealed the following: the most resistant strains to the oil were *T. rubrum* KCTC 6352 and *M. canis* KCTC 6349 with an MIC value of 500 μ g/mL, but the range of MIC values for the remaining strains of the dermatomycetes was from 62.5 to 125 μ g/mL. The authors concluded that the oil could be used as a source of natural antioxidants and as a novel antidermatophytic agent to control superficial human fungal infections (78).

Reports on the antifungal activity of essential oils from aromatic plants belonging to other botanical families have also been found in the literature. These are usually oils from specific plant species for which the authors already have some information on their use in traditional medicine for fungal skin disorders.

The essential oils within the ***Lauraceae family***, from eight *Cinnamomum* spp. (*C. pubescens* Kochummen, *C. impressicostatum* Kosterm, *C. microphyllum* Ridl., *C. scorchedinii* Gamb., *C. rhyncophyllum* Miq., *C. cordatum* Kosterm, *C. zeylanicum* Blume, and *C. mollissimum* Hook f.) and the major oil constituents were examined for antifungal activity against six dermatophytes (*Trichophyton rubrum*, *T. mentagrophytes*, *T. tonsurans*, *Microsporum canis*, *M. gypseum*, and *M. audouini*) and four yeasts (*Candida albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*). Most of the oils showed moderate to strong activity against

the fungi: the leaf and bark oils of *C. zeylanicum* showed the highest activity against all the fungi tested (MIC values of 0.04 - 0.63 µg/µL), while the other strong inhibitors included the leaf oil of *C. cordatum* and the bark and twig oils of *C. pubescens* and *C. impressicostatum*. Cinnamaldehyde, the major constituent of bark oil from *C. zeylanicum*, showed the strongest activity against all the fungi studied; for all tested *Candida* spp. the MIC value was 0.08 µg/µL, except for *C. glabrata* at 0.16 µg/µL, and for all dermatomycetes the MIC value was <0.04 µg/µL. The following constituents had the lowest MIC values at <0.04 µg/µL: benzyl salicylate against *T. rubrum*, *T. mentagrophytes*, *M. gypseum*, and *M. audouini*; benzyl benzoate against *T. rubrum* and *T. mentagrophytes*; and geraniol against *T. rubrum* and *M. audouini*. Based on the results, the authors concluded that high levels of cinnamaldehyde, eugenol, geraniol, benzyl benzoate, and methyl cinnamate in the oils and in combination with the minor components, are responsible for the high antifungal activity (79).

The essential oil of *G. procumbens* from the *Ericaceae family* showed inhibition of a planktonic cell culture of *C. albicans* and anti-*Candida* activity against a biofilm structure (80). Because biofilm structures are known for their resistance and serve as a reservoir for infection and cross-contaminations, the prevention of *Candida* adhesion and biofilm formation by plant essential oils is highlighted.

The essential oil obtained from *Melaleuca alternifolia*, which is a tree or tall shrub in the *Myrtaceae family*, is one of the most studied essential oils for activity against *Candida* spp. This oil has been used in several preparations for its bioactive properties. Numerous studies confirm its potent anti-*Candida* activity (81, 82) and recommend its use because the oil is efficient, well tolerated and, apart from its antimicrobial efficacy, it also has good antioxidant activity (82).

Other two oils obtained from plants of the same family, *Leptospermum petersonii* Bailey and *Syzygium aromaticum* L. Merr. Et Perry also proved to be good natural agents against *Microsporum canis* (KCTC 6591), *Trichophyton mentagrophytes* (KCTC 6077), *T. rubrum* (KCCM 60443), *Epidermophyton floccosum* (KCCM 11667), and *Microsporum gypseum*. The antifungal activity of *S. aromaticum* oil applied at 0.2 mg/mL exceeded 60% and eugenol proved to be the most effective constituent against *T. mentagrophytes* and *M. canis*. Essential oil of *L. petersonii* applied at 0.2 mg/mL, was even more effective against all tested fungi (above 90%), except for *T. rubrum*, and geranal proved to be the strongest antifungal oil constituent (83).

In a screening study, the antifungal activity of an essential oil isolated from *Cymbopogon winterianus* in the *Poaceae family* against 16 strains of *T. rubrum*, was investigated. The pure essential oil inhibited all of the strains with zones of inhibition ranging from 24 to 28 mm in diameter. The MIC₅₀ and MIC₉₀ values were 312 µg/mL, which inhibited 93.8% of *T. rubrum*, while the MFC₅₀ and MFC₉₀ values were about eight times higher. All tested essential oil concentrations strongly inhibited the mycelium development. The main morphological changes caused by the oil included loss of conidiation, alterations in form and pigmentation of hyphae. The authors concluded that *C. winterianus* oil could be used as a potential antifungal agent, particularly in prevention against *T. rubrum* infection (84).

The essential oil from another member of the Poaceae family, *Cymbopogon martini* (rich in *trans*-geraniol at 60.9%) exhibited stronger inhibitory activity in comparison to the oil of *Chenopodium ambrosioides* from ***Chenopodiaceae family***, rich in m-cymene at 43.9%, against the tested dermatomycetes. The MICs of the *C. martini* oil against *M. gypseum* and *T. rubrum* were 200 and 150 ppm, while the MLCs were 750 and 500 ppm, respectively. At 200 ppm, the tested oils inhibited growth of *T. mentagrophytes* at 82% and 80%, respectively. The authors concluded that these two oils and the combination of the oils may be future alternatives to synthetic antifungal drugs for the treatment of *Tinea corporis* (ringworm) and other superficial mycoses in humans (85).

In an attempt to develop stable and natural antifungal agents, the activity of essential oils from the ***Liliaceae family***, including *A. sativum* var. *pekinense*, *Allium fistulosum* L., and *A. cepa*, were investigated for activity against three *Trichophyton* species (*T. rubrum*, *T. erinacei*, and *T. soudanense*) responsible for severe mycoses in humans. The oil of *A. sativum* exhibited the strongest growth inhibition for all three *Trichophyton* spp., with an MIC at 64 µg/mL. The activity of this oil was 12.5–25% of the activity of ketoconazole, and the authors considered it as a remarkable level of activity for a natural product, particularly considering the side effects and toxicity associated with ketoconazole. Additionally, the oil also showed significant synergistic antifungal activity when combined with ketoconazole (86).

Among several essential oils derived from various plant families, the leaf oil of *Pelargonium graveolens* L'Herit ex Aiton from the ***Geraniaceae family***, and its major constituents, citronellol and geraniol alone, and in combination with ketoconazole, were tested and generally exhibited the highest activities against all tested KCCM strains of the *Trichophyton* spp. (*T. erinacei*, *T. mentagrophytes*, *T. rubrum*, *T. schoenleinii*, *T. soudanense*, and *T. tonsurans*). The MIC and MFC values ranged from 0.2–0.5 mg/mL and 0.5–1 mg/mL, respectively. The MIC and MFC values for geraniol were 0.25–1 mg/mL and 0.5–2 mg/mL, respectively; while for citronellol the MIC and MFC values were somewhat higher at 0.5–2 mg/mL and 1–4 mg/mL, respectively. The combination of the oil and its components with ketoconazole exhibited especially strong synergistic inhibition against the lesser-known African dermatomycetes *T. schoenleinii* and *T. soudanense* (2).

The essential oil of seeds from *Nigella sativa* in the ***Ranunculaceae family*** and its major constituent thymoquinone (42.4%) were tested against *Trichophyton mentagrophytes*, *Microsporum canis*, and *M. gypseum*. This study presents a first step in the search for new anti-dermatomycete drugs, supporting the use of *N. sativa* seeds in traditional medicine for dermatophytic infections (87).

Different Modes of Action of Essential Oils and Their Constituents

From previously presented data it is obvious that a great number of essential oils and their selected constituents, tested *in vitro* or *in vivo* experiments, do express beneficial activity on dermatomycetes. After reviewing the results of the antifungal activities of essential oils and individual constituents from the *in vivo* experiments, the composition of essential oils and the proportion of

the individual constituents may explain the differences between their activities. For instance, menthol and thymol showed better antifungal activity than the essential oils of *Mentha spicata* and *Thymus vulgaris*, respectively, that contain them as major constituents. Because the individual essential oils showed lower antifungal activities than the tested single major oil constituent (88), it is evident that the activity can be attributed to the individual constituents. Apart from a well known synergistic effects of the oil constituents, mentioned many times in the literature, antagonistic effects between the oil constituents are also possible; some oil constituents can diminish or completely block each other activities. This certainly means that the effectiveness of the tested oils and the single oil constituents towards different microorganisms has to be analyzed and interpreted with attention. The essential oil of Sweet basil, which is known for its beneficial activity on the skin for healing wounds, is also used to treat fungal infections. The oil showed better antifungal activity *in vivo* than lemon and bitter orange oils. The dominant constituent in these oils was linalool. Tested alone, linalool proved to be a good antifungal agent, although weaker in comparison to limonene. Lemon and bitter orange oils showed lower antifungal potential, and limonene although present in a high percentage in the oils, is suspected for the diminishing efficacy of the entire oils (88).

The essential oils of *S. officinalis* and *L. angustifolia* have proved to be the most effective in the treatment of experimentally induced dermatomycoses. If compared, the results obtained during the investigation of the antifungal activities of the essential oils *in vitro*, with the results generated *in vivo*, it is obvious that the oil of *S. officinalis* and *L. angustifolia* expressed lower potentials *in vitro*. In contrast, in the *in vivo* experiments, these two oils proved to be very effective and thus possess better therapeutic activities than many other tested oils. In addition, it is known that sage and lavender have traditionally been used to treat various skin diseases and are included in cosmetic products for skin care (89). The efficacy of an essential oil can be explained by interactions of the individual components, which in the case of the lavender oil mainly refer to linalyl acetate and linalool (90). The essential oils and/or their constituents certainly represent alternatives for the treatment of animals and humans infected by dermatomycetes, however, available data, obtained from *in vivo* experiments on the antifungal activities of essential oils towards human fungal pathogens, are still limited (64).

It can be seen that the growth of the tested fungi responded diversely to the essential oils and components, which indicates that different components may have different modes of action or that the metabolisms of some fungi are able to overcome the effects of or adapt to the oil. Terpenic compounds inhibit electron transport, proton translocation, phosphorylation steps, and other enzyme-dependent reactions, or these compounds may act on the cell membrane. The antifungal activity depends on the chemical composition of the cell wall and on the structure of the terpenoid molecules. Terpenic hydrocarbons are water insoluble and reveal poor activity; among the water-soluble compounds, vanilin, piperonal, and camphor were not remarkably active; whereas, the non-aromatic ester borneol acetate showed antiseptic effects. Aliphatic alcohols, such as linalool or citronellol, and ketones, such as pipertone or carvone, exhibited antifungal properties. Phenol compounds showed very strong antifungal activities

in spite of their relatively low capacity to dissolve in water (91). The most active terpenoids were found among the phenols, followed by aldehydes, ketones, alcohols, and hydrocarbons. Thymol and carvacrol were the most effective compounds, causing total inhibition of oxidative phosphorylation. The ability of terpenoids to inhibit the reactions described is due to both the lipophilic properties, which enables the compounds to dissolve in the cytoplasmic membrane, and from the functional groups, which interfere with enzyme structure (91–93). Studies of the antimicrobial activities of essential oils and their components showed that terpene acetates and hydrocarbons tended to be relatively inactive, regardless of structural type, and that this inactivity appears to be closely related to limited hydrogen-bonding capacity and water solubility. Ketones, aldehydes, and alcohols showed activity but with differing specificities that were not always defined by the functional group present but associated with the hydrogen-bonding parameters in all cases (94). The results from the authors of this review concerning the antifungal activity of many essential oils and the components indicate differing efficacies. Also, the modes of action of essential oils differ among fungal species. The strong antifungal activities of the oils of *Mentha* spp., *Thymus vulgaris*, and *Origanum vulgare* can be explained by a high percentage of the major oil components: menthol, thymol, and carvacrol, respectively. For the remaining oils, no significant correlation between antifungal activity and relative amounts of the major components has been found. This finding suggests that the oil components present in large proportions are not necessarily responsible for a greater share of the total activity. A different antifungal activity exhibited by an oil compared to the activities of its major components can be explained by either a synergistic effect of the diverse components in the oil and/or by the presence of other components that may be active in small concentrations (94). With increasing acceptance that the chemical diversity of natural products is well suited to provide core scaffolds for future antimicrobial agents, there will be increased development in the use of novel natural products and chemical libraries based on these natural products (95). The methodology employed is another point that needs to be considered in depth. For non-polar extracts, the use of diffusion techniques is probably inadequate, although many reports employing these techniques have been published. Solid dilution techniques are suitable for studying plant extracts or nonpolar compounds, and only when the amount of available sample is small is the use of diffusion techniques probably more appropriate (60, 94, 96–100). Based on the experience from the authors of this review, it is proposed to use the microdilution method, carried out in microtiter trays, which involves a low workload for a larger number of replicates and the use of small volumes of both the test substance and growth medium (101).

With regard to therapeutic use of natural products, considerable changes in legislation have been made and there are increasing consumer trends for natural alternatives to chemical fungicides (102). The use of essential oils is particularly advisable because herbs and spices are common plant additives. Among the natural antimicrobials that were tested in the laboratory by the authors of this review, the essential oils of *Origanum vulgare* and *Thymus vulgaris* (Figure 3), as well as the oil main constituents carvacrol and thymol, were identified as the most promising antifungal candidates (103).

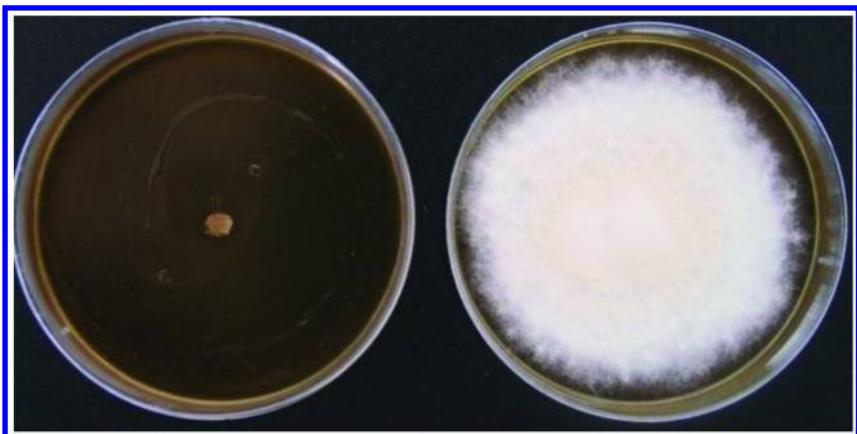


Figure 3. Antifungal activity of the essential oil from *Thymus vulgaris* against *Trichophyton mentagrophytes* (left) vs. a control colony (right).

The addition of various plant-derived antimicrobials applied in combination should improve both the spectrum of activity and the extent of inhibition due to synergistic effects. Thus, the combination of these compounds might have even higher potential. The use of essential oils is limited, and possible reasons for this may be the strong smell and taste when used at effective dosage levels (104, 105). Although the majority of essential oils are classified as Generally Recognized As Safe (GRAS) (106), the use in foods as preservatives is often limited due to flavor considerations, as the effective antimicrobial dosage levels may exceed the organoleptically acceptable levels. Therefore, there is an increasing demand for accurate knowledge of the minimum inhibitory (effective) concentrations (MIC) of essential oils to enable a balance between sensory acceptability and antimicrobial efficacy (107).

Conclusion

From previously reported results in the review authors' laboratory and results reviewed here, it can be concluded that the essential oils and the individual constituents tested possessed beneficiary antifungal and therapeutic effects both *in vitro* and *in vivo*. Therefore, these natural compounds could be further tested and possibly employed as alternatives for the treatment of patients infected by dermatomycetes. Due to a number of side effects observed with application of commercial fungicides, including pathogen's resistance to many synthetic mycotics, preparations with natural products have advantages in the treatment of fungal caused diseases. Based on the reviewed data, it can be concluded that products based on secondary metabolites (i.e. essential oils extracted from aromatic plants) proved to be effective antifungal and therapeutic agents with limited harmful effects on humans and animals. Therefore, the future of fungicide management will likely be influenced by research on natural products. Modern instrumentation has simplified the isolation and identification of lead compounds

from which fungicides can be composed of. The reviewed studies clearly demonstrate that natural products from plants present great potential for medicinal purposes and for use in the food, cosmetic, agricultural, and pharmaceutical industries.

Particularly desirable is the discovery of novel prototypes of antifungal agents that represent new chemical classes and modes of action compared to existing antifungal agents, thus reducing the resistance of pathogens to currently employed chemicals. The goals of future studies are to develop safe, effective, and inexpensive formulations and processes based on natural products to reduce infections by pathogenic fungi in humans.

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Editor's Biographies

Dr. Valtcho D. Jeliazkov (Zheljazkov)

Valtcho D. Jeliazkov (Zheljazkov) received his Ph.D. in Plant and Soil Sciences from the University of Massachusetts in Amherst under the direction of Dr. Lyle E. Craker. Previously, he obtained a Ph.D. in Agronomy from the Agricultural University in Plovdiv, Bulgaria under the guidance of Prof. Venelin D. Topalov. Dr. Jeliazkov is currently a tenured associate professor at Oregon State University. Previously, Dr. Jeliazkov has worked as a professor in his native Bulgaria (Agricultural University in Plovdiv), in Canada (Nova Scotia Agricultural College/Dalhousie University), and also at Mississippi State University and at the University of Wyoming. He was a Fulbright Scholar at the University of Massachusetts at Amherst in 1996–1997. Antecedent events to the Fulbright Scholar award were a visiting scientist at The Royal Veterinary & Agricultural University in Copenhagen, Denmark, the University of Reading U.K., and Harper Adams University College in U.K. He conducts agronomy research with impact in three general areas: agriculture, health, and the environment. The focus of Dr. Jeliazkov's research in general agriculture is on improving profitability of production systems with high value agricultural crops, products, and processes. He conducts research on plant chemicals and their biological activities, on developing approaches for obtaining essential oils with unique characteristics. He also works on multiple utilization potential of agronomic crops. He has authored 160 refereed journal publications and participated in numerous conferences.

Note: Valtcho is using the spelling Valtcho D. Zheljazkov in his research publications.

Dr. Charles Cantrell

Dr. Cantrell received a B.S. in physiology and zoology from Louisiana State University (LSU) in 1994. He obtained a Ph.D. in Chemistry from LSU in 1998 under the direction of Dr. Nikolaus H. Fischer. From 1998 to 2000 he conducted postdoctoral research in the Laboratory of Drug Discovery Research and Development, National Cancer Institute, in Frederick, Maryland. Research consisted of isolation and structure elucidation of cytotoxic constituents isolated from marine plants and animals. This research led to the discovery of the chondropsins, a new class of cytotoxic macrolides from the marine sponge *Chondropsis* sp. Following this postdoctoral position, he spent 2 years as a Research Chemist with the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) in Peoria, Illinois. Dr. Cantrell left ARS in 2001

to pursue a career in the private sector as Associate Director of Research and Development for Tanical Therapeutics, Inc. Research at Tanical focused on the identification of small molecule drug candidates from plants with a history of traditional usage against such diseases as cancer, rheumatoid arthritis, and benign prostate hyperplasia. Dr. Cantrell has also worked for both Hauser, Inc. and Sandoz Pharmaceuticals. Dr. Cantrell served as Treasurer of the Phytochemical Society of North America (PSNA) from 2002 to 2005, Editor of *The Cornucopia* from 2000 to 2004, Associate Editor of *Pest Management Science* from 2012 to present, and President of the PSNA in 2010. Dr. Cantrell is currently a Research Chemist for the USDA-ARS in Oxford, MS and an Associate Professor in the Department of Pharmacognosy at The University of Mississippi. As part of the Natural Products Utilization Research Unit, he is responsible for the discovery of natural product based pesticides. Dr. Cantrell has co-authored over 100 peer-reviewed publications, 5 book chapters, one U.S. patent and 2 international patents.

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