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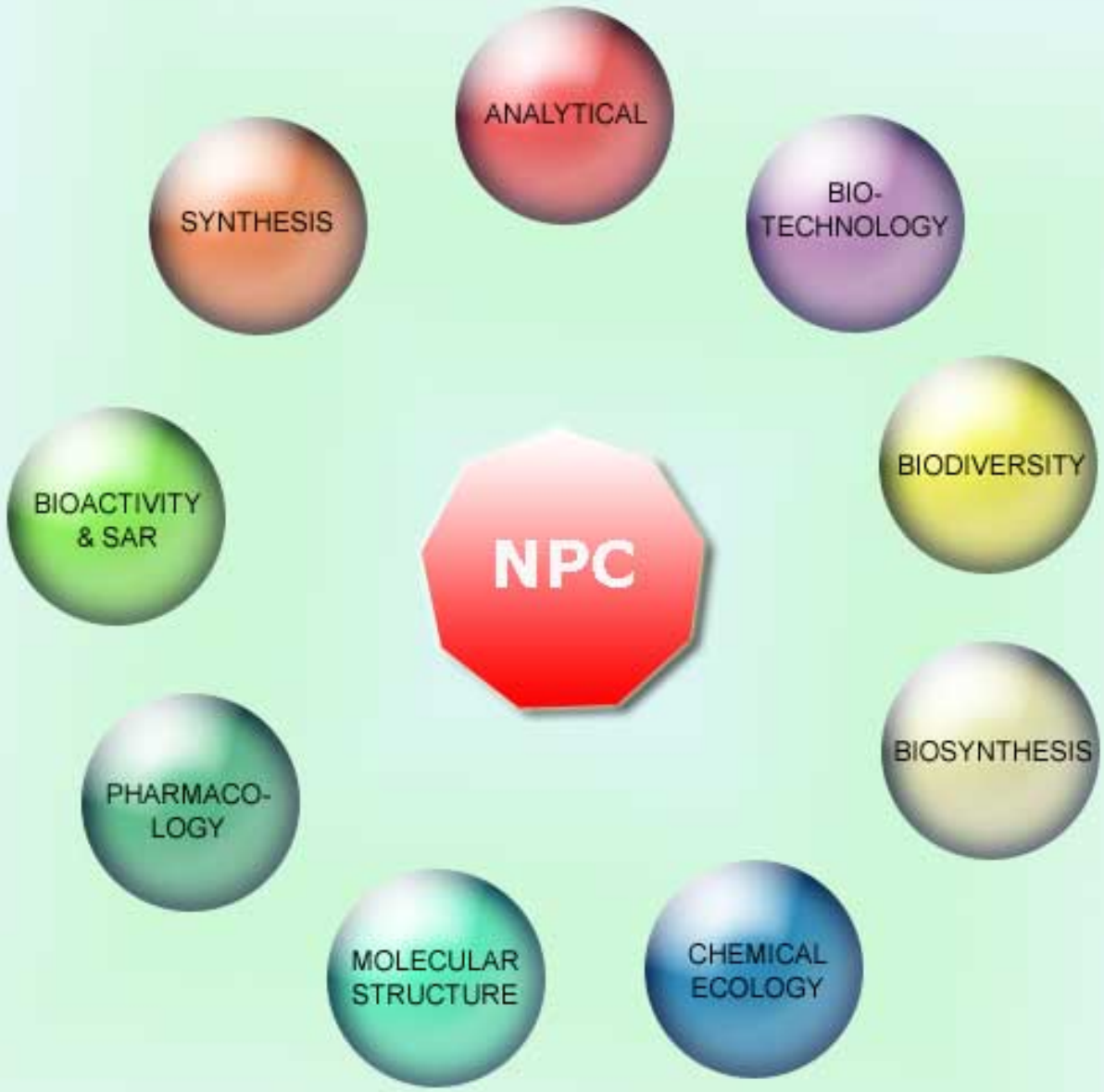

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Editorial

With the launch of *Natural Product Communications* in 2006, it was thought worthwhile to have thematic issues so that the community of natural product researchers could be aware of the views of some of the experts in that particular area. A Special Issue dedicated to Alkaloids was published in 2006 [*Natural Product Communications* **1** (10) 2006].

The present issue [*Natural Product Communications* **2** (12) 2007] is devoted to "Biologically Active Essential Oils" and includes original research papers as well as reviews on traditional uses, food preservation and potential applications of essential oils for medicinal purposes. I am, therefore, grateful to Professor William N. Setzer, The University of Alabama in Huntsville, Huntsville, AL, USA, who is a renowned researcher in this area, for accepting our invitation to act as Guest Editor. He was able to attract leading authors, and their contributions highlight the chemical and biological aspects of essential oils. The editors join me in thanking Professor Setzer, the authors and the reviewers for their efforts that have made this issue possible, and to the production department for putting this issue in print.

Pawan K. Agrawal
Editor-in-Chief

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Guest Editor's Foreword: Biologically Active Essential Oils

The use of volatile phytochemicals, essential oils, for food preservation, to alleviate pest infestation, and for ameliorating human illnesses has been around for hundreds of years. The essential oil of a plant is the concentrated, volatile, aromatic mixture of chemicals that is formed in various organs or tissues, including leaves, bark, flowers, fruits, and roots. Plants have evolved these volatile chemicals for a number of purposes. Essential oils help to protect plants from bacterial, fungal, and other microbial infections. Leaf volatiles serve to dissuade herbivory by marauding insects while floral volatiles attract pollinators. Humans have benefited from plant volatiles throughout history, not only as sources of pleasant fragrances and flavors, but also as therapeutic agents against disease and protection against pests.

This special issue of *Natural Product Communications* is devoted to the broad topic of biological activity of essential oils and includes original research papers as well as reviews on traditional uses, food preservation; potential applications of essential oils for medicinal purposes including antimicrobial, antiparasitic, and anticancer activities; the activities of essential oils against insects and other arthropod pests; as well as floral pollination.

This issue of *NPC* complements an excellent review on bioactivity of essential oils by Koroch and co-workers [1], and I am very grateful to the contributing authors for their outstanding support and cooperating in putting this special issue together.

[1] Koroch AR, Juliani HR, Zygadlo JA. (2007) Bioactivity of essential oils and their components. In *Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability*. Berger RG (Ed), Springer, Berlin. 87-115.

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Composition and Antinociceptive Activity of the Essential Oil from *Protium heptaphyllum* Resin

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The chemical composition of the essential oil from *Protium heptaphyllum* resin was analyzed by GC/MS and the oil examined for antinociceptive activity in chemical and thermal tests. Fourteen compounds were characterized, representing 95.8% of the total essential oil, with the monoterpenes α-phellandrene (10.4%), α-terpinene (13.7%) and 1,8-cineole (58.7%) as major components. Oral administration of the essential oil (50 and 100 mg/kg) significantly inhibited chemical nociception induced by capsaicin and formalin in mice. In rats, the oil also effectively enhanced the radiant heat-induced tail-flick latency response at a dose of 100 mg/kg. However, the essential oil, at either dose, was ineffective against thermal pain in the hot-plate test.

Keywords: Essential oil, antinociceptive activity, *Protium heptaphyllum*, chemical and thermal nociception.

The leafy parts of several species of Burseraceae, mainly of the genus *Protium*, are considered aromatic and medicinal [1,2]. Phytochemical investigation of the resin, fruits, leaves, and trunk of *P. heptaphyllum* led to the isolation of the monoterpene *p*-menth-3-ene-1,2,8-triol, α- and β-amyrin, quercetin, brein, quercetin-3-*O*-rhamnoside, (–)-catechin and scopoletin [3]. In folk medicine, gum and oleoresins from species of *Protium* have been popular for their anti-inflammatory, analgesic, expectorant and wound-healing effects [4]. Earlier studies in our laboratory have shown that the resin of *P. heptaphyllum* has gastroprotective and anti-inflammatory properties [5]. Furthermore, anti-inflammatory, antimicrobial and antioxidant effects of the essential oil from leaves and/or resin of *P. heptaphyllum* have been previously described [4-6]. Since many essential oils of plants and their volatile constituents are endowed with analgesic properties following their local or systemic applications [7], the present study was aimed at screening the essential oil extracted from the resin of *P. heptaphyllum* (EOPH) for a possible analgesic activity against chemical and thermal nociception.

Table 1: Chemical composition (%) of the essential oil of *Protium heptaphyllum.*

Compound	Kovat´s Indices	Percentage
α-Thujene	932	0.4
α-Pinene	937	0.9
Sabinene	977	1.1
β-Pinene	981	0.4
α-Phellandrene	1005	10.4
α-Terpinene	1017	13.7
1,8-Cineole	1031	58.7
Terpinolene	1091	0.7
Linalool	1099	1.0
cis-Limonene oxide	1144	0.2
Camphor	1148	0.2
α-Terpineol	1099	1.0
Piperitol	1196	0.6
γ-Terpineol	1201	7.7

Percentage of total oil identified 95.8%

The chemical composition of EOPH is presented in Table 1. Fourteen compounds were characterized, representing 95.8% of the oil. The major components present were the monoterpenes α-phellandrene (10.4%), α-terpinene (13.7%) and

1,8-cineole (58.7%). The effects of oral pretreatment with EOPH (50 and 100 mg/kg), in comparison with morphine (7.5 mg/kg; s.c.), on formalin-induced and capsaicin-induced nociception in mice are shown in Table 2. Vehicle-treated control mice showed extensive hind-paw licking in the formalin test at the first phase, as well as in the second phase. While morphine inhibited the licking response in both phases in a naloxone-sensitive manner, EOPH suppressed only the second phase response of formalin, which was resistant to naloxone. The two phases of mouse response to formalin have been attributed to different mechanisms, both peripheral and central [8,9], and since EOPH did not manifest antinociception in the first phase of the formalin test, we assume that its second phase analgesic effect is mainly due to the anti-inflammatory activity, which has been established by an earlier study [4]. In the capsaicin test, both EOPH (50 and 100 mg/kg) and morphine produced profound antinociception, as evidenced by suppression of the hind-paw licking response (Table 2). The extent of reduction in the respective groups of animals was in the order of 55 and 74% for the EOPH and 97% for morphine. Unlike that of morphine, the antinociceptive effect of EOPH in the capsaicin test was not reversed by pretreatment of mice with naloxone (2 mg/kg, s.c.), a μ-opioid receptor antagonist, suggesting the involvement of a non-opioid mechanism. The involvement of capsaicin-sensitive TRPV1 channel (transient receptor channel vanilloid 1 receptor) expressed in sensory neurons in nociception has been well documented [10]. It appears TRPV1 is up regulated in inflammatory disease conditions, such as inflammatory bowel disease and irritable bowel syndrome. Interestingly, EOPH attenuates the capsaicin-induced peripheral nociception, probably by desensitizing the primary sensory afferents.

Table 2: Effect of EOPH on formalin and capsaicin induced licking responses in mice.

Group	Dose mg/Kg)	Formalin test Paw licking (s)		Capsaicin test Paw licking (s)
		1st phase	**2nd phase**	
Control	-	82.75 ± 6.85	21.0 ± 8.45	80.33 ± 10.18
EOPH	50	64.25 ± 6.71	36.12 ± 9.51	36.00 ± 5.37[a]
	100	93.37 ± 8.11	33.50± 12.5	21.14 ± 8.41[a]
Morphine	7.5	33.14 ± 7.69[a]	1.14 ± 1.14[a]	2.12 ± 1.42[a]
Morphine + Naloxone	7.5 + 2.0	82.00 ± 10.0[b]	31.16 ± 9.38[b]	77.37 ± 8.17[b]
EOPH + Naloxone	100 + 2.0	68.71 ± 6.77	38.60 ± 12.31	46.73 ± 12.00

Each value is expressed as mean ± S.E.M. for six to eight animals in each. Statistical significance [a]$P<0.05$ vs control; [b]$P<0.05$ vs morphine.

Table 3: Effect of EOPH on tail-flick response latency in rats.

Group (mg/kg)	Response latency (s)				
	0´	**30´**	**60´**	**90´**	**120´**
Control	4.10 ± 0.82	4.56 ± 1.07	5.37 ± 0.95	5.82 ± 1.00	5.12 ± 0.75
EOPH 50	3.70 ± 0.76	6.42 ± 1.35	9.55 ± 1.45	8.63 ± 1.43	7.43 ± 1.02
EOPH 100	4.10 ± 0.79	11.57 ± 1.24**	11.57 ± 1.08**	10.92 ± 0.96**	7.27 ± 1.27

Each value is expressed as mean ± S.E.M. for six to eight animals in each. Statistical significance [a]$P<0.05$ vs control.

In the tail-flick test, EOPH (100 mg/kg) significantly prolonged the response latency (Table 3). It is well known that the hot plate test predominately measures supraspinally organized reflexes, while the tail flick test mostly measures spinal reflexes [11]. Since EOPH is effective only in the tail-flick test, we believe its antinociceptive action is likely at the spinal level. Mice treated with EOPH (50 and 100 mg/kg, i.p.) neither manifested any overt behavioral change in the open-field test nor demonstrated significant influence on pentobarbital sleeping time (data not shown), suggesting that it has neither central depressant nor sedative activity. These data suggest that the essential oil of *P. heptaphyllum* resin is an orally effective antinociceptive agent with peripheral and spinal levels of action.

Experimental

Plant material: The trunk wood resin of *Protium heptaphyllum* (Aubl.) March. was collected from the municipal areas of Timon, Maranhão State of Brazil, after its identification by botanist Roseli Farias de Melo Barros. A voucher sample (#18247) has been deposited at the Herbarium Graziela Barroso of the Federal University of Piauí, Teresina, Brazil.

Essential oil extraction and chemical composition: The essential oil from the resin was extracted by hydrodistillation and analyzed by GC/MS (Hewlett-Packard 5971 GC/MS) under the following conditions: column: dimethylpolysiloxane DB-1 fused silica capillary column (30 m x 0.25 mm, 0.1 μm film thickness); carrier gas: helium (1 mL/min); injector temperature: 250°C; detector temperature: 200°C; column temperature: 35°-180°C at 4°C/min, then 180-250°C at 10°C/min; mass spectra: electron impact, 70 eV. Individual components were identified by two computer library MS searches using retention indices as a preselection routine [12] and visual inspection of the mass spectra from literature for confirmation [13].

Animals: Male Swiss mice (20 – 25 g) and Wistar rats (150 – 180 g) maintained under standard environmental conditions were used. The animals had free access to a pellet diet (Purina chow) and tap water. The animals were fasted overnight for experimentation, but allowed free access to water. The Institutional Committee on the Care and Use of Animals for experimentation approved the experimental protocols in accordance with the guidelines of NIH, Bethesda. For experiments, animals were divided into groups of six to eight.

Formalin-induced nociception: Mice were pretreated with EOPH (50 and 100 mg/kg, p.o.), vehicle (3% Tween-80, 10 mL/kg in water), and morphine (7.5 mg/kg, s.c.) alone or in combination with naloxone (2 mg/kg, s.c.), 30 min prior to 20 μL of 1% formalin (in 0.9% saline, subplantar) and the total time (in seconds) that the animal spent licking the injected paw during the first 5 min (first phase) and then at 20 – 25 min (second phase) after formalin injection was quantified [8]. The pretreatment time period followed for the EOPH was 60 min and for morphine and naloxone, 30 min.

Capsaicin-induced paw licking: Mice pretreated as above with EOPH, vehicle or morphine alone or in combination with naloxone individually received subplantar injections of either capsaicin (1.6 μg, 20 μL) or a similar volume of vehicle into the right hind paw. The time in seconds that the animals spent licking the injected paw during the first 5 min after capsaicin injection was recorded [14].

Tail-flick test: A radiant heat tail-flick analgesiometer was used to measure response latencies in rats. The reaction time was recorded for animals pre-treated with EOPH (50 and 100 mg/kg, p.o.), vehicle or morphine (7.5 mg/kg, s.c.). Rats that showed tail-flick reaction of 5 seconds alone were included in the study [15].

Hot-plate test: In this test, mice were preselected on a hot-plate at 55 ± 0.5°C and only animals that showed a reaction time [time (s) required to start either licking of hind limb or jumping] within a 20 s period were included in the study. Animals were then treated with EOPH (50 and 100 mg/kg, s.c.), vehicle or morphine (7.5 mg/kg, s.c.) and the reaction time (s) was recorded for each mouse before and after the pretreatments, at intervals of 30 min, for a total period of 90 min. To avoid possible injury, a cut-off period of 45 s was followed while measuring the reaction time [16].

Locomotor activity (open-field test): Mice were observed for locomotion by placing them in an open-field arena and the locomotion frequency (number of floor units the animal entered) was counted for a period of 4 min, following 45 min of oral administration of either EOPH (50 and 100 mg/kg) or vehicle [17].

Pentobarbital-induced sleeping time: Sleeping times induced by pentobarbital (40 mg/kg, i.p.) were established in groups of mice, 45 min following oral treatment with either EOPH (50 and 100 mg/kg) or vehicle (10 mL/kg). The sleeping times were measured by observing the loss and the recovery of the righting reflex [18].

Statistical analysis: The data were expressed as mean ± S.E.M., and the statistical significance between groups was analyzed by means of analysis of variance (ANOVA), followed by Student-Newman-Keul´s test. *P*-values less than 0.05 were considered as indicative of statistical significance.

Acknowledgements - We acknowledge with gratitude the financial assistance of CNPq, and CAPES (PROCAD), Brazil.

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Cruzain Inhibitory Activity of Leaf Essential Oils of Neotropical Lauraceae and Essential Oil Components

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The leaf essential oils of twenty-three species of Lauraceae from Monteverde, Costa Rica, have been screened for inhibition of the cysteine protease cruzain. Of these, nine showed promising cruzain inhibitory activity (IC_{50} < 100 μg/mL), six showed marginal activity (IC_{50}, 100-500 μg/mL), and eight were inactive (IC_{50} > 500 μg/mL). The cruzain inhibitory activities of the essential oils can be attributed to active sesquiterpenoid components as well as synergistic effects between two or more components. The sesquiterpenes α-copaene, β-caryophyllene, α-humulene, and germacrene D are active (IC_{50} ~5-30 μg/mL) alone, but also show increased activity in combination with other essential oil components.

Keywords: *Beilschmiedia*, *Cinnamomum*, *Nectandra*, *Ocotea*, *Persea*, *Pleurothyrium*, Lauraceae, Monteverde, Costa Rica, leaf essential oil, composition, *Trypanosoma cruzi*, cruzain, synergy.

Parasitic protozoal infections such as trypanosomiasis continue to be a great cause of human morbidity and mortality, not only in developing nations where they are endemic, but also to people of industrialized countries due to world travel. An estimated 16-18 million people in tropical and subtropical America are infected by *Trypanosoma cruzi*, the protozoan responsible for Chagas disease [1]. Current chemotherapeutic treatments include nifurtimox and benznidazole, but these medicinal agents are accompanied by severe side effects and require prolonged use [2]. Vaccines for Chagas disease are currently unavailable [3]. Natural sources should not only provide new trypanocidal compounds with promise to combat these diseases, but also afford lead structures for synthetic modification and optimization of bioactivity [4]. Proteases play essential roles in the metabolism, replication, survival, and pathology of parasitic protozoa, and the cysteine protease cruzain has been identified as a potential target for *Trypanosoma cruzi* [5]. Plant pathogenic fungi [6], bacteria [7-9], plant viruses [10], pathogenic mites [11], and herbivorous insects [12, 13] utilize papain-family cysteine proteases in order to infect the host plant. It seems reasonable to presume that plants have developed cysteine protease inhibitors for protection from pathogenic pests and herbivory. Indeed, a number of proteins (cystatins) that inhibit cysteine proteases have been isolated and identified from plants [6,11,14-17]. We hypothesize that tropical rainforest plants have evolved small-molecule cysteine protease inhibitors in response to plant pathogens and herbivory, and that these compounds may be useful against human pathogens as well. In this work, we present the chemical compositions and the cruzain inhibitory activities of leaf essential oils from a number of species of the Lauraceae from Monteverde, Costa Rica.

The leaf essential oils of *Cinnamomum brenesii* (Standl.) Kosterm., *Cinnamomum costaricanum* (Mez & Pittier) Kosterm., *C. tonduzii* (Mez) Kosterm., *Persea americana* Mill., *P. caerulea* (Ruiz & Pav.) Mez, *Persea* new species "small leaf", and

Table 1: Leaf essential oils of Lauraceae from Monteverde, Costa Rica.

Plant	Voucher number	Collection Site (Date)	Mass of leaves	Yield of leaf oil
Cinnamomum brenesii	Haber 9945	Los Llanos Field Station (May 19, 2006)	53.2 g	69.5 mg (0.13%)
Cinnamomum costaricanum	Haber 9265	Los Llanos Field Station (May 19, 2006)	75.8 g	19.9 mg (0.026%)
Cinnamomum tonduzii	Haber 9120	Hotel El Bosque (May 24, 2003)	110.7 g	36.8 mg (0.033%)
Persea americana	Haber 9841	Monteverde Cloud Forest Preserve (May 23, 2006)	92.9 g	61.0 mg (0.066%)
Persea caerulea	Haber 9783	Upper Monteverde (May 23, 2005)	75.4 g	80.7 mg (0.11%)
Persea sp. "small leaf"	Haber 8503	Hotel El Bosque (May 18, 2006)	20.5 g	1.57 mg (0.0077%)
Pleurotheryrium palmanum	Haber 9526	Monteverde Cloud Forest Preserve (May 23, 2006)	81.2 g	19.5 mg (0.024%)

Pleurothyrium palmanum (Mez & Donn. Sm.) Rohwer were obtained as either colorless or pale yellow oils by hydrodistillation (Table 1). The chemical compositions of the leaf oils, as determined by GC-MS, of *Cinnamomum* spp, *Persea* spp, and *Pleurothyrium palmanum* are compiled in Table 2. The collection and GC-MS analyses of the five *Beilschmiedia* spp. [18], *Nectandra membranacea* [19], and the ten *Ocotea* species [20] have been previously reported. The essential oils were screened for cruzain inhibitory activity and the IC_{50} values determined. The cruzain inhibitory activities of *Beilschmiedia*, *Cinnamomum*, *Nectandra*, *Ocotea*, *Persea*, and *Pleurothyrium* leaf oils, along with some essential oil components, are summarized in Table 3.

Nine species showed pronounced cruzain inhibitory activity with IC_{50} values < 100 μg/mL. There is not an obvious correlation between cruzain inhibitory activity and the chemical compositions, however. The most active leaf oils in this study were those of *O. meziana*, *O. whitei*, *Ocotea* "los llanos", *Ocotea* "small leaf", *B. tilaranensis*, *Persea americana*, *B. brenesii*, *P. caerulea*, and *O. holdridgeana*. The leaf oils of all of these species are rich in sesquiterpene, with the exception of *Ocotea* "los llanos". Conversely, the inactive essential oils generally show diminished concentrations of sesquiterpenes.

The cruzain inhibitory activity can be attributed, in part, to the major sesquiterpenes present. Thus, α-copaene, β-caryophyllene, α-humulene, and germacrene D all show inhibitory activity. Other notably active compounds present in the leaf oils were the monoterpenes limonene and myrcene (IC_{50} = 42.1 and 46.5 μg/mL, respectively). α- and β-Pinene showed marginal inhibitory activities (IC_{50} = 111 and 132 μg/mL, respectively). While this may account for the activity of *O. tonduzii* leaf oil (~66% pinenes, IC_{50} ~150 μg/mL), *Pl. palmanum* oil (~60% pinenes) was inactive. The most active compound tested was α-copaene (IC_{50} = 5.20 μg/mL), but the leaf oil with the highest concentration of α-copaene (*N. membranacea* with 13%) was inactive.

It has been suggested that synergistic and/or antagonistic effects of essential oil components may account for observed biological activities in essential oils [21] including, for example, antimicrobial [22-24], insect antifeedant [25], insecticidal [26], acaricidal [27], antioxidant [28,29], cytotoxic [30,31], and enzyme inhibitory [32,33] activities. In order to test this, we have examined 1:1 binary mixtures of some commercially available essential oil components for potential synergistic and/or antagonistic effects in cruzain inhibition (Table 4).

Interestingly, while the sesquiterpenes α-copaene, β-caryophyllene, α-humulene, and germacrene D are active (IC_{50} = 5.2, 32.5, 28.2, and 22.1 μg/mL, respectively), combinations of these materials with other essential oil components generally show enhanced activity. In addition, caryophyllene oxide, which is inactive, significantly enhances the activity of inactive or marginally active components. The monoterpenes limonene and myrcene also show cruzain inhibitory activity (IC_{50} = 42.1 and 46.5 μg/mL, respectively), as well as enhanced activity with other components (for example, limonene + myrcene or myrcene + α-pinene).

The pronounced cruzain inhibitory activities (IC_{50} < 100 μg/mL) of *O. meziana*, *O. whitei*, *Ocotea* "small leaf", *B. tilaranensis*, *Persea americana*, *B. brenesii*, *P. caerulea*, and *O. holdridgeana* leaf oils may, therefore, be attributed to the high levels of sesquiterpenoids present in these species, especially

Table 2: Chemical compositions of leaf essential oils from *Cinnamomum* spp., *Persea* spp., and *Pleurothyrium palmanum* from Monteverde, Costa Rica.

RI	Compound	Percent Composition						
		Cinnamomum			*Persea*			*Pleurothyrium*
		brenesii	*costaricanum*	*paratriplinerve*	*americana*	*caerulea*	"small leaf"	*palmanum*
856	*cis*-3-Hexenol	---	---	---	trace	---	6.8	---
858	*trans*-3-Hexenol	---	---	0.4				---
859	*trans*-2-Hexenal	5.3	3.5	---	1.5	6.6	---	7.7
931	α-Thujene	---	---	---	trace	---	---	---
940	α-Pinene	14.7	8.7	---	5.6	1.6	3.3	39.7
957	Camphene	1.6	---	---	---	---	---	trace
968	Benzaldehyde	0.5	---	---	---	---	---	---
978	Sabinene	---	---	---	9.9	---	4.5	trace
980	β-Pinene	5.5	3.5	---	trace	trace	---	19.4
993	Myrcene	0.9	trace	---	0.7	trace	---	1.2
1006	α-Phellandrene	8.7	---	---	7.6	---	---	---
1015	Δ^3-Carene	0.4	trace	---	---	---	---	---
1019	α-Terpinene	0.8	---	---	4.3			---
1030	Limonene	3.6	1.4	---	---	trace	0.2	1.8
1033	1,8-Cineole	trace	---	0.6	7.0	trace	23.9	7.3
1043	*cis*-β-Ocimene	0.4	trace	---	trace	---	---	---
1048	Phenylacetaldehyde	0.1	---	---	---	---	---	---
1053	*trans*-β-Ocimene	0.3	---	---	0.1	---	---	---
1062	γ-Terpinene	0.4	trace	---	5.5	---	---	trace
1089	Terpinolene	1.3	0.5	---	1.6	---	---	trace
1094	2-Nonanone	---	---	---	trace	---	---	---
1101	Linalool	---	---	1.4	1.4	1.8	---	---
1112	*endo*-Fenchol	0.2	trace	3.0	---	---	---	---
1119	*cis*-*p*-Menth-2-en-1-ol	---	---	0.8	---	---	---	---
1125	α-Campholene aldehyde	---	---	0.4	---	---	---	---
1136	Nopinone	---	---	2.6	---	---	---	---
1146	Camphene hydrate	trace	---	1.8	---	---	---	---
1164	Borneol	0.2	---	4.2	---	---	---	---
1177	4-Terpineol	trace	---	1.4	8.9	---	---	---
1185	*p*-Cymen-8-ol	---	---	0.5	---	---	---	---
1189	*cis*-3-Hexenyl butyrate	---	---	---	---	0.6	---	---
1189	α-Terpineol	0.4	0.7	13.7	---	---	---	---
1196	Myrtenol	---	---	1.1	---	---	---	---
1207	Verbenone	---	---	0.4	---	---	---	---
1213	Unknown ($C_{10}H_{18}O$)	---	---	---	1.4	---	---	---
1267	*trans*-2-Decenal	---	---	---	trace	---	---	---
1288	Bornyl acetate	---	---	---	trace	---	---	---
1338	δ-Elemene	---	---	---	---	1.0	---	trace
1351	α-Cubebene	---	---	---	0.1	---	---	---
1360	Neryl acetate	---	---	---	0.2	---	---	---
1364	Eugenol	---	---	---	4.9	---	---	---
1372	α-Ylangene	0.2	---	---	---	0.3	---	---
1376	α-Copaene	1.0	trace	trace	---	1.5	1.0	trace
1386	β-Bourbonene	---	---	---	---	0.5	---	---
1390	β-Cubebene	---	---	---	---	---	2.0	---
1391	β-Elemene	0.2	8.3	---	---	3.1	trace	trace
1409	α-Gurjunene	0.2	---	trace	---	---	---	---
1410	Dodecanal	0.3	1.4	trace	---	0.5	---	---
1418	β-Caryophyllene	1.6	2.4	11.9	6.5	35.4	37.6	8.3
1434	γ-Elemene	---	---	---	---	0.3	---	---
1436	α-*trans*-Bergamotene	---	---	---	---	---	1.8	---
1438	Aromadendrene	---	---	---	trace	trace	---	trace
1439	α-Guaiene	---	0.3	trace	---	---	---	---

Table 2 (*Continued*)

1440	*cis*-β-Farnesene	0.6	---	---	---	---	---	---
1451	(*E*)-Isoeugenol	---	---	---	1.0	---	---	---
1454	α-Humulene	6.6	0.7	1.8	0.9	3.8	3.2	0.8
1461	Alloaromadendrene	1.2	trace	---	0.5	---	---	trace
1463	*trans*-β-Farnesene	---	---	---	---	5.6	---	---
1474	γ-Selinene	---	4.5	---	---	---	---	---
1476	γ-Muurolene	1.1	---	trace	---	---	---	---
1480	Germacrene-D	---	---	trace	3.1	15.6	4.8	1.8
1485	γ-Curcumene	0.6	---	---	---	---	---	---
1486	β-Selinene	1.1	14.7	---	---	1.3	---	---
1492	Valencene	---	---	---	---	0.3	---	---
1493	Ledene (= Viridiflorene)	3.9	---	0.6	---	---	---	---
1494	Bicyclogermacrene	---	---	---	---	9.0	---	5.2
1496	α-Selinene	trace	18.4	---	---	---	2.6	---
1499	α-Muurolene	1.5	---	---	---	trace	---	trace
1505	α-Bulnesene (= δ-Guaiene)	---	---	0.5	---	---	---	---
1505	Germacrene A	---	0.6	---	0.5	1.8	---	---
1510	Unknown ($C_{15}H_{24}$)	---	---	---	---	---	0.7	---
1511	(*E,E*)-α-Farnesene	---	0.5	---	---	---	---	---
1513	γ-Cadinene	0.6	0.7	0.4	0.1	0.5	5.5	trace
1519	*cis*-γ-Bisabolene	1.0	---	---	---	---	---	---
1519	7-*epi*-α-Selinene	---	0.4	---	---	---	---	---
1524	δ-Cadinene	1.5	1.2	1.9	0.5	1.2	2.3	1.5
1532	Cadina-1,4-diene	1.9	trace	---	1.8	0.1	---	---
1538	α-Cadinene	---	0.3	---	---	0.1	---	trace
1539	*trans*-γ-Bisabolene	7.2	---	---	---	---	---	---
1542	Selina-3,7(11)-diene	2.5	0.5	---	---	---	---	---
1549	Elemol	3.4	0.8	---	---	0.1	---	---
1556	Germacrene B	0.9	0.3	---	---	1.9	---	---
1564	*trans*-Nerolidol	0.5	---	2.4	---	1.4	---	---
1566	(*Z*)-Isoeugenol acetate	---	---	---	14.8	---	---	---
1569	α-Caryophyllene alcohol	0.4	trace	3.5	---	---	---	---
1574	Spathulenol	---	trace	1.7	---	---	---	trace
1579	Caryophylla-3,8(13)-dien-5β-ol	---	---	8.9	---	---	---	---
1581	Unknown ($C_{15}H_{26}O$)	trace	0.8	---	---	1.3	---	trace
1584	Globulol	0.7	---	---	---	---	---	---
1596	Guaiol	trace	0.5	---	---	---	---	---
1598	Unknown ($C_{15}H_{26}O$)	4.0	---	2.4	---	---	---	---
1605	Humulene epoxide II	---	---	0.9	---	---	---	---
1611	Unknown ($C_{15}H_{26}O$)	---	4.5	4.5	---	---	---	---
1615	Tetradecanal	0.9	---	---	---	---	---	---
1617	10-*epi*-γ-Eudesmol	---	---	---	---	0.2	---	---
1623	Unknown ($C_{15}H_{26}O$)	---	0.7	---	---	---	---	---
1627	1-*epi*-Cubenol	---	0.4	1.3	---	0.2	---	---
1630	γ-Eudesmol	trace	2.5	1.7	---	0.1	---	---
1634	Caryophylla-4(12),8(13)-dien-5β-ol	---	---	2.4	---	---	---	---
1636	Isospathulenol	---	---	0.8	---	---	---	---
1640	τ-Cadinol	1.4	1.5	4.3	4.2	0.4	---	1.0
1645	Torreyol	1.1	---	1.9	---	0.1	---	trace
1648	β-Eudesmol	---	---	0.9	---	0.4	---	---
1652	Kongol	---	13.1	---	---	---	---	---
1653	α-Eudesmol	1.7	---	---	---	1.2	---	2.2
1653	α-Cadinol	---	---	8.9	1.7	---	---	---
1655	Unknown ($C_{15}H_{24}O$)	---	---	2.8	---	---	---	---
1658	7-*epi*-α-Eudesmol	1.4	---	---	---	---	---	---
1669	Unknown ($C_{15}H_{24}O$)	---	---	1.4	---	---	---	---

Table 2 (*Continued*)

1669	Unknown ($C_{15}H_{26}O$)	0.8	---	---	---	---	---	---
1673	Unknown ($C_{15}H_{26}O$)	---	---	---	3.6	---	---	---
1673	β-Bisabolol	1.1	1.7	---	---	---	---	---
1686	α-Bisabolol	0.7	---	---	---	---	---	---
1688	Unknown ($C_{15}H_{26}O$)	---	---	---	---	---	---	2.0
1694	Juniper camphor	1.1	---	---	---	---	---	---
	Total identified	95.2	94.0	88.9	94.8	98.7	99.3	98.0
	Monoterpene hydrocarbons	38.5	14.1	0.0	35.2	1.6	8.0	62.1
	Oxygenated monoterpenoids	0.8	0.7	32.0	18.8	1.8	23.9	7.3
	Sesquiterpene hydrocarbons	35.3	53.8	17.0	14.1	83.3	61.3	17.7
	Oxygenated sesquiterpenoids	18.3	26.4	50.6	9.5	5.6	0.0	5.2
	Fatty-acid-derived compounds	6.5	4.9	0.4	1.5	7.7	6.8	7.7
	Aromatic compounds	0.6	0.0	0.0	20.8	0.0	0.0	0.0

Table 3: Cruzain inhibitory activity of leaf essential oils from Monteverde Lauraceae and some essential oil components (standard deviations are shown in parentheses).

Essential Oil	IC_{50} (μg/mL)	Compound	IC_{50} (μg/mL)
Beilschmiedia alloiophylla	160(7)	Borneol	>500
Beilschmiedia brenesii	61.9(8.1)	Bornyl acetate	>500
Beilschmiedia "chancho blanco"	>500	Camphene	117(36)
Beilschmiedia costaricensis	>500	β-Caryophyllene	32.5(6.4)
Beilschmiedia tilaranensis	23.6(2.4)	Caryophyllene oxide	>500
Cinnamomum brenesii	377(14)	1,8-Cineole	>500
Cinnamomum costaricanum	156(2)	α-Copaene	5.20(0.95)
Cinnamomum tonduzii	>500	*p*-Cymene	174(44)
Nectandra membranacea	>500	Eugenol	>500
Ocotea floribunda	323(11)	*endo*-Fenchol	>500
Ocotea holdridgeana	76.9(1.6)	Germacrene D	22.1(10.2)
Ocotea "los llanos"	17.1(0.3)	α-Humulene	28.2(6.3)
Ocotea meziana	14.9(0.9)	Limonene	42.1(6.4)
Ocotea sinuata	>500	Linalool	>500
Ocotea "small leaf"	19.2(0.1)	Myrcene	46.5(16.2)
Ocotea tonduzii	153(5)	Myrtenal	>500
Ocotea valeriana	177(4)	α-Pinene	111(9)
Ocotea veraguensis	>500	β-Pinene	132(22)
Ocotea whitei	15.8(0.2)	α-Terpineol	>500
Persea americana	>500	4-Terpineol	>500
Persea caerulea	62.5(7.1)		
Persea "small leaf"	50.6(1.0)		
Pleurothyrium palmanum	>500		

β-caryophyllene, α-humulene, or germacrene D, acting in synergy with other leaf oil components. Leaf oils with low sesquiterpenoid concentrations are generally inactive or marginally active. Interestingly, *Ocotea* "los llanos" leaf oil is very active (IC_{50} = 17.1 μg/mL), but contains only 9.8% sesquiterpene hydrocarbons [20]. It does, however, contain large amounts of both α- and β-pinenes, as well as limonene (4.5%) and myrcene (1.4%), which show pronounced synergy with one another other, along with 10.0% oxygenated sesquiterpenoids. In apparent contradiction, however, *O. floribunda* leaf oil also has large concentrations of both α- and β-pinenes, along with 15.7% total sesquiterpenoids, but was only marginally active.

O. sinuata leaf oil was rich in sesquiterpenoids, as well as pinenes [20], but the oil is inactive. Similarly, *Beilschmiedia* "chancho blanco" is also inactive, but the leaf oil contained 58.5% sesquiterpene hydrocarbons, along with 12.1% α-pinene and 7.7% β-pinene [18], so it is not obvious why these plant oils are inactive.

Table 4: Synergistic effects of essential oil components on cruzain inhibitory activity, IC_{50}, μg/mL (standard deviations are shown in parentheses).

	Borneol	Bornyl acetate	Camphene	β-Caryophyllene	Caryophyllene oxide	1,8-Cineole	α-Copaene	*p*-Cymene	Eugenol	*endo*-Fenchol
Borneol	> 500	> 500	421 (26)	90.9 (9.3)	15.1 (3.7)	> 500	6.43 (2.86)	> 500	> 500	> 500
Bornyl acetate	> 500	> 500	> 500	311 (45)	155 (17)	> 500	10.4 (4.8)	> 500	> 500	> 500
Camphene	421 (26)	>500	117 (36)	20.0 (2.7)	30.5 (9.8)	> 500	7.75 (5.16)	182 (43)	> 500	> 500
β-Caryophyllene	90.9 (9.3)	311 (45)	20.0 (2.7)	32.5 (6.4)	18.7 (8.0)	159 (31)	5.83 (2.45)	6.76 (2.70)	13.3 (6.1)	7.10 (4.51)
Caryophyllene oxide	15.1 (3.7)	155 (17)	30.5 (9.8)	18.7 (8.0)	> 500	98.5 (38.1)	25.3 (13.5)	5.73 (1.66)	35.3 (12.2)	43.1 (29.4)
1,8-Cineole	> 500	> 500	> 500	159 (31)	98.5 (38.1)	> 500	29.4 (9.4)	> 500	> 500	> 500
α-Copaene	6.43 (2.86)	10.4 (4.8)	7.75 (5.16)	5.83 (2.45)	25.3 (13.5)	29.4 (9.4)	5.20 (0.95)	6.60 (0.42)	7.23 (2.57)	4.82 (6.35)
para-Cymene	> 500	> 500	182 (43)	6.76 (2.70)	5.73 (1.66)	> 500	6.60 (0.42)	174 (44)	> 500	> 500
Eugenol	> 500	> 500	> 500	13.3 (6.1)	35.3 (12.2)	> 500	7.23 (2.57)	> 500	> 500	> 500
endo-Fenchol	> 500	> 500	> 500	7.10 (4.51)	43.1 (29.4)	> 500	4.82 (6.35)	> 500	> 500	> 500
Germacrene D	19.0 (3.1)	157 (45)	> 500	9.91 (3.48)	15.1 (8.6)	43.5 (24.8)	36.2 (31.0)	6.87 (1.50)	10.3 (2.9)	> 500
α-Humulene	> 500	> 500	13.4 (4.5)	296 (48)	159 (47)	> 500	8.45 (5.95)	7.41 (2.06)	130 (27)	>500
Limonene	> 500	> 500	427 (31)	7.22 (1.53)	24.2 (11.5)	> 500	5.13 (2.14)	> 500	> 500	> 500
Linalool	225 (44)	> 500	> 500	98.9 (28.6)	57.5 (14.2)	> 500	13.6 (9.7)	> 500	> 500	> 500
Myrcene	86.1 (45.7)	326 (32)	8.36 (2.95)	6.38 (2.67)	29.6 (3.9)	> 500	4.63 (1.27)	353 (17)	> 500	> 500
Myrtenal	> 500	> 500	> 500	12.2 (5.1)	16.4 (8.6)	> 500	7.48 (4.92)	> 500	> 500	> 500
α-Pinene	> 500	> 500	150 (27)	16.7 (4.8)	337 (40)	> 500	6.15 (2.60)	327 (49)	> 500	> 500
β-Pinene	> 500	> 500	23.2 (8.3)	11.0 (5.5)	372 (20)	>500	9.95 (7.57)	398 (29)	296 (35)	> 500
α-Terpineol	> 500	> 500	> 500	35.2 (16.5)	27.1 (3.9)	> 500	8.77 (5.38)	> 500	> 500	> 500
4-Terpineol	> 500	> 500	> 500	23.0 (6.6)	43.9 (12.7)	> 500	5.67 (3.84)	> 500	> 500	> 500

	Germacrene D	α-Humulene	Limonene	Linalool	Myrcene	Myrtenal	α-Pinene	β-Pinene	α-Terpineol	4-Terpineol
Borneol	19.0 (3.1)	> 500	> 500	225 (44)	86.1 (45.7)	> 500	> 500	> 500	> 500	> 500
Bornyl acetate	157 (45)	> 500	> 500	> 500	326 (32)	> 500	> 500	> 500	> 500	> 500
Camphene	> 500	13.4 (4.5)	427 (31)	> 500	8.36 (2.95)	> 500	150 (27)	23.2 (8.3)	> 500	> 500
β-Caryophyllene	9.91 (3.48)	296 (48)	7.22 (1.53)	98.9 (28.6)	6.38 (2.67)	12.2 (5.1)	16.7 (4.8)	11.0 (5.5)	35.2 (16.5)	23.0 (6.6)
Caryophyllene oxide	15.1 (8.6)	159 (47)	24.2 (11.5)	57.5 (14.2)	29.6 (3.9)	16.4 (8.6)	337 (40)	372 (20)	27.1 (3.9)	43.9 (12.7)
1,8-Cineole	43.5 (24.8)	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
α-Copaene	36.2 (31.0)	8.45 (5.95)	5.13 (2.14)	13.6 (9.7)	4.63 (1.27)	7.48 (4.92)	6.15 (2.60)	9.95 (7.57)	8.77 (5.38)	5.67 (3.84)
p-Cymene	6.87 (1.50)	7.41 (2.06)	> 500	> 500	353 (17)	> 500	327 (49)	398 (29)	> 500	> 500
Eugenol	10.3 (2.9)	130 (27)	> 500	> 500	> 500	> 500	> 500	296 (35)	> 500	> 500
endo-Fenchol	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
Germacrene D	22.1 (10.2)	19.7 (4.2)	7.55 (2.72)	19.8 (7.4)	11.9 (3.2)	5.96 (2.03)	19.3 (5.1)	> 500	45.9 (21.8)	11.9 (3.2)
α-Humulene	19.7 (4.2)	28.2 (6.3)	34.6 (20.2)	381 (27)	5.69 (1.47)	11.5 (3.5)	13.0 (2.8)	11.0 (4.8)	283 (26)	310 (37)
Limonene	7.55 (2.72)	34.6 (20.2)	42.1 (6.4)	> 500	13.5 (2.1)	> 500	61.5 (42.4)	79.3 (29.9)	>500	> 500
Linalool	19.8 (7.4)	381 (27)	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
Myrcene	11.9 (6.3)	5.69 (1.47)	13.5 (2.1)	> 500	46.5 (16.2)	> 500	11.8 (5.3)	27.6 (9.7)	> 500	> 500
Myrtenal	5.96 (2.03)	11.5 (3.5)	> 500	> 500	> 500	> 500	> 500	59.2 (21.6)	> 500	> 500
α-Pinene	19.3 (5.1)	13.0 (2.8)	61.5 (42.4)	> 500	11.8 (5.3)	> 500	111 (9)	17.9 (6.9)	> 500	> 500
β-Pinene	> 500	11.0 (4.8)	79.3 (29.9)	> 500	27.6 (9.7)	59.2 (21.6)	17.9 (6.9)	132 (22)	> 500	> 500
α-Terpineol	45.9 (21.8)	283 (26)	> 500	> 500	> 500	> 500	> 500	>500	> 500	> 500
4-Terpineol	11.9 (3.2)	310 (37)	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500

Experimental

Plant collection: The plants were collected from Monteverde, Costa Rica, and identified by William A. Haber. Voucher specimens have been deposited in the herbarium of the Missouri Botanical Garden and the National Herbarium of Costa Rica. Essential oils were obtained by hydrodistillation of freshly chopped leaves using a Likens-Nickerson apparatus with continuous extraction with chloroform [18-20]. Collection details and essential oil yields are compiled in Table 1.

Gas chromatographic-mass spectral analysis: The leaf oils of the plants were subjected to gas chromatographic-mass spectral analysis using an Agilent 6890 GC with Agilent 5973 mass selective detector, fused silica capillary column (HP-5ms, 30 m x 0.25 mm), helium carrier gas, 1.0 mL/min flow rate; inj temp 200°C, oven temp prog: 40°C initial temperature, hold for 10 min; increased at 3°/min to 200°C; increased 2°/min to 220°C, and interface temp 280°C; EIMS, electron energy, 70 eV. The samples were dissolved in $CHCl_3$ to give 1% w/v solutions; 1-Microliter injections, using a splitless injection technique, were used. Identification of oil components was achieved based on their retention indices (determined with reference to a homologous series of normal alkanes), and by comparison of their mass spectral fragmentation patterns with those reported in the literature [34] and stored on the MS

library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.08)]. The chemical compositions of the essential oils are summarized in Table 2.

Cruzain inhibition assay: The activity of essential oils and essential oil components against recombinant cruzain [35] was measured by a fluorescence assay using Z-Phe-Arg-AMC·HCl as the fluorescent enzyme substrate. The cruzain solution (4 nM) was prepared with 20 μL of cruzain per liter of 100 mM sodium acetate buffer with 5 mM DTT and a pH of 5.5. The substrate solution (40 μM) was prepared with 26 mg Z-Phe-Arg-AMC·HCl, first dissolved in DMSO, per liter of 100 mM sodium acetate buffer with 5 mM DTT and a pH of 5.5. The essential oils and components were prepared as 1% solutions in DMSO. For each well of a 96 well plate 475 μL of cruzain was mixed with 25 μL of the sample solution to be tested. Of this mixture, 100 μL was pipetted into each well. Each sample was tested in quadruplicate with DMSO negative controls and TLCK positive controls. After approximately 10 minutes incubation at room temperature, 100 μL of the substrate solution was pipetted into each well (the final sample concentration is 500 μg/mL). The plate was then immediately read using a SpectraMax M2 fluorescence plate reader. After an initial mixing period of 5 seconds the fluorescence was measured 9 times over a period of 5 minutes with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The slope given by the change in fluorescence was then exported into an Excel spreadsheet for the calculations of percent inhibition and standard deviation. Samples that showed >50% inhibition at 500 μg/mL were retested at 50 μg/mL and 5 μg/mL. IC_{50} values were determined using the Reed-Muench method [36]. The cruzain inhibitory activities of the leaf oils and components are presented in Table 3; the activities of 1:1 binary mixtures of essential oil components are summarized in Table 4.

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Cruzain Inhibitory Activity of the Leaf Essential Oil from an Undescribed Species of *Eugenia* from Monteverde, Costa Rica

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The leaf essential oil of *Eugenia* sp. nov. "San Bosco" inhibits cruzain, a cysteine protease from *Trypanosoma cruzi*, the parasitic protozoan responsible for Chagas disease, with an IC_{50} of 36.4 μg/mL. *Eugenia* "San Bosco" leaf oil is dominated by the sesquiterpene hydrocarbons zingiberene (24.7%) and germacrene D (11.1%), and these two compounds (IC_{50} = 8.6 and 21.2 μg/mL, respectively) are likely responsible for the cruzain inhibitory activity observed in the essential oil.

Keywords: *Eugenia*, essential oil, cruzain inhibition, zingiberene, germacrene D.

Chagas disease, caused by the parasitic protozoan *Trypanosoma cruzi*, is widely distributed in Latin America. There is currently no effective treatment for chronic Chagas disease, but the cysteine protease cruzain, which is essential for parasite replication within the host, has been identified as a potential biochemical target for drug discovery [1].

The Myrtaceae contains around 4620 species spread over 129 genera [2]. Members of the family are distributed in the Neotropics and Australia and are generally fragrant with oil glands in the leaves [3]. *Eugenia* is a large genus with more than 550 species found mainly in the Neotropics [2]. In this work, we describe the chemical composition and cruzain inhibitory activity of the leaf essential oil from an undescribed species of *Eugenia* from the Monteverde region of northwestern Costa Rica. To our knowledge, there have been no reports on the phytochemistry or the biological activity of this species.

Eugenia new species ("San Bosco") is a tree up to 20 m tall and 30 cm diameter at breast height. The twigs are smooth, without lenticels, slightly compressed, distinctly broader at nodes, light tan to purple-black, residual red-orange pubescence soon lost. The leaves are simple, opposite, petiole 10-17 mm, slender; blade to 3 x 7 cm, glabrous, elliptic, gradually acute, base cuneate, then minutely attenuate, weakly revolute, punctate above, smooth with visible glands below, midvein expressed above and below, lateral veins 9-11 per side, flat above and below, visibly lighter than blade above with light, darker and barely visible below, yellow against the light, marginal vein about 2 mm from edge, texture thick and leathery, not stiff, shiny dark green above, much paler below with residual red-orange hairs along midvein, odor strong. The inflorescences are axillary, almost sessile with 4-10 white flowers to 10 mm across. This species is rare, found on ridges along the Continental Divide at 1400-1500 m. Haber 12730

The chemical composition of the leaf essential oil of *Eugenia* sp. nov. "San Bosco" is presented in Table 1. The leaf oil of *Eugenia* "San Bosco" was dominated by sesquiterpene hydrocarbons (61.1%), oxygenated sesquiterpenoids (30.5%), and fatty acid derivatives (7.2%). The most abundant components

Table 1: Chemical composition of *Eugenia* sp. nov. "San Bosco" leaf essential oil.

RI[a]	Compound	Percent Composition
857	*trans*-2-Hexenal	7.2
1038	*cis*-Ocimene	0.2
1338	δ-Elemene	3.0
1370	α-Ylangene	trace
1376	α-Copaene	1.8
1384	β-Bourbonene	0.4
1390	β-Cubebene	0.1
1392	β-Elemene	0.3
1419	β-Caryophyllene	2.5
1429	β-Gurjunene	0.4
1434	γ-Elemene	0.2
1437	Aromadendrene	trace
1453	α-Humulene	2.0
1460	*trans*-β-Farnesene	0.5
1464	*epi*-Bicyclosesquiphellandrene	0.1
1483	Germacrene D	11.1
1486	β-Selinene	0.2
1491	*cis*-β-Guaiene	1.3
1497	Bicyclogermacrene	1.6
1498	Zingiberene	24.7
1509	Unidentified	1.1
1511	(*E,E*)-α-Farnesene	1.9
1527	δ-Cadinene	6.5
1533	Cadina-1,4-diene	0.6
1538	α-Cadinene	0.2
1542	α-Calacorene	0.1
1550	Elemol	0.9
1556	Germacrene B	1.5
1566	*trans*-Nerolidol	3.1
1585	Globulol	0.6
1591	Viridiflorol	1.3
1602	Guaiol	0.4
1612	1,10-di-*epi*-Cubenol	0.2
1616	10-*epi*-γ-Eudesmol	0.8
1627	1-*epi*-Cubenol	6.1
1632	γ-Eudesmol	1.3
1644	*epi*-α-Cadinol	5.7
1647	Torreyol	1.3
1650	α-Eudesmol	0.7
1653	Valerianol	1.4
1657	7-*epi*-α-Eudesmol	6.1
1667	Bulnesol	0.1
1685	α-Bisabolol	0.4

[a]Retention indices on HP-5ms fused silica capillary column.

of the essential oil of *Eugenia* "San Bosco" were zingiberene (24.7%), germacrene D (11.1%), *trans*-2-hexenal (7.2%), δ-cadinene (6.5%), 1-*epi*-cubenol (6.1%), 7-*epi*-α-eudesmol (6.1%), and *epi*-α-cadinol (5.7%). A notable characteristic of *Eugenia* "San Bosco" was the presence of its major component zingiberene, which has, to our knowledge, not been found in other *Eugenia* species [4].

The leaf essential oils of twelve species of Myrtaceae from Monteverde, Costa Rica, have been screened for inhibition of cruzain: *Calyptranthes pittieri* [5], *Eugenia austin-smittii*, *E. cartagensis*, *E. haberi*, *E. monteverdensis*, *Eugenia* "San Bosco", *E. zuchowskiae* [4], *Myrcia splendens* [6], *Myrcia* new species "fuzzy leaf" [7], *Myrcianthes fragrans* [8], *Myrcianthes* new species "black fruit" [9], and *Psidium guajava* [8]. Of these, only *Eugenia* "San Bosco" showed notable inhibitory activity (IC_{50} = 36.4 ± 0.9 μg/mL).

In order to determine if the cruzain inhibitory activity of *Eugenia* "San Bosco" leaf oil was due to the high concentrations of zingiberene or germacrene D, these materials were also tested for cruzain inhibitory activity. Both of these sesquiterpenoids showed activity: zingiberene (IC_{50} = 8.56 ± 3.38 μg/mL); germacrene D (IC_{50} = 21.2 ± 10.2 μg/mL) [10]. We conclude, then, that the cruzain inhibitory activity exhibited by *Eugenia* "San Bosco" leaf essential oil is due to the presence of high concentrations of zingiberene and germacrene D.

Both zingiberene and germacrene D have exhibited biological activity. Thus, for example, zingiberene has shown antirhinoviral [11], antiulcer [12], insect repellent [13], and insecticidal [14,15] activities; germacrene D has exhibited insect attractive [16,17], insect repellent [18], and cytotoxic [19] activities.

Experimental

Leaves of *Eugenia* "San Bosco" were collected on May 23, 2005, from a mature tree near Monteverde, Costa Rica (10.3442 N, 84.8317 W, 1420 m above sea level). The fresh leaves (94.9 g) were chopped and hydrodistilled employing a simultaneous distillation-extraction technique with a Likens-Nickerson apparatus [20] using $CHCl_3$ to continuously extract the distillate to give 65.5 mg essential oil. The GC-MS analysis of the leaf essential oil of *Eugenia* "San Bosco" was carried out as previously described [4]. The cruzain-inhibitory activity of *Eugenia* "San Bosco" leaf oil against recombinant cruzain was carried out as previously described [10]. Purified zingiberene was chromatographically isolated from commercial (100% Pure Essential Oils and Aromatherapy Products™) ginger (Zingiber officinalis) root oil (58% zingiberene) as described [11]; purified germacrene D was chromatographically isolated from commercial (Young Living Essential Oils™) goldenrod (Solidago canadensis) essential oil (34% germacrene D) as described [21].

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Biological Activities of Essential Oils from Monteverde, Costa Rica

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Essential oils from *Calyptranthes pittieri* (Lauraceae), *Cinnamomum tonduzii* (Lauraceae), *Croton niveus* and *C. monteverdensis* (Euphorbiaceae), *Dendropanax arboreus* (Araliaceae), *Eugenia austin-smithii* and *E. haberi* (Myrtaceae), *Myrcianthes fragrans* and *M. rhopaloides* (Myrtaceae), *Nectandra membranacea* (Lauraceae), *Ocotea floribunda* (Lauraceae), *Oreopanax xalapensis* (Araliaceae), *Piper umbellatum* (Piperaceae), *Psidium guajava* (Myrtaceae), *Stauranthus perforatus* (Rutaceae), *Zanthoxylum acuminatum*, *Z. melanostictum*, *Z. monophyllum*, and *Zanthoxylum* sp. nov. "brillante" (Rutaceae), have been screened for cytotoxic activity against a panel of human tumor cell lines, antibacterial activity against Gram-positive and Gram-negative bacteria, as well as brine shrimp (*Artemia salina*) lethality.

Keywords: essential oils, cytotoxicity, antibacterial, brine shrimp lethality, Monteverde, Costa Rica.

For thousands of years, plant products and their modified derivatives have been rich sources for clinically useful drugs. Even today, about 80% of the world's population relies predominantly on plants and plant extracts for health care. A recent study has shown that, of the top 150 proprietary drugs used in the United States, 57% contain at least one major active compound currently or once derived from (or patterned after) compounds derived from natural sources [1].

Tropical rainforests afford an abundance of plant species [2], but the phytopharmaceutical potential of these rainforests is still largely unexplored [3-6]. The potential medicinal value of tropical rainforests is due not only to the species richness of the tropical flora, but also to the diversity of pathogens, parasites, and herbivores against which the plants must defend themselves. The diversity of consumers has inevitably selected for a diversity of chemical defensive mechanisms (see, e.g., [7]). Many of these chemical defenses, because of their metabolic precision, can be used to treat human maladies.

The Monteverde region of the central Cordillera de Tilarán in northwestern Costa Rica is, like most tropical montane areas, physiographically and climatically diverse [8]. This environmental diversity results in an extraordinarily high between-site component of biodiversity; disjunct patches of tropical dry forest occupy edaphically dry narrow ridges on the upper Pacific slope only 4 km from true lower montane rain forests along the crest of the Cordillera [8,9]. Consequently the region is among the floristically most diverse in the world. The slopes of the Cordillera above 1200 m elevation contain ~1700 plant species – roughly the number in the La Selva Biological Station in the Caribbean lowlands of Costa Rica, or in the floodplains and upland terraces along the Rio Manú in the Amazonian lowlands of Peru – while the area above 700 m in the Cordillera de Tilarán contains ~3000 plant species.

In this work, we present the bioactivity screening of a variety of essential oils from 19 species of plants representing five families from the Monteverde region of Costa Rica. The leaf essential oils from *Calyptranthes pittieri* Standl., *Cinnamomum tonduzii* (Mez) Kosterm., *Nectandra membranacea* (Sw.)

Griseb., *Ocotea floribunda* (Sw.) Mez (Lauraceae), *Dendropanax arboreus* (L.) Decne. & Planch., *Oreopanax xalapensis* (Kunth) Decne. & Planch. (Araliaceae), *Eugenia austin-smithii* Standl., *E. haberi* Barrie, *Myrcianthes fragrans* (Sw.) McVaugh, *M. rhopaloides* (Kunth) McVaugh, *Psidium guajava* L. (Myrtaceae), *Piper umbellatum* L. (Piperaceae), *Stauranthus perforatus* Liebm., *Zanthoxylum acuminatum* (Sw.) Sw., *Z. melanostictum* Schltdl., *Z. monophyllum* (Lam.) P. Wilson, and *Zanthoxylum* sp. nov. "brillante" (Rutaceae), and the bark essential oils of *Croton niveus* Jacq. and *C. monteverdensis* Huft (Euphorbiaceae), have been screened for *in-vitro* cytotoxic activity against Hep G2 (hepatocellular carcinoma), MDA-MB-231 (estrogen-receptor negative mammary adenocarcinoma), MCF-7 (estrogen-receptor positive mammary adenocarcinoma), PC-3 (prostatic carcinoma), or SK-Mel-28 (malignant melanoma) human tumor cell lines; antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*; and lethality against brine shrimp (*Artemia salina*) (Table 1).

Nine of the nineteen essential oils (*C. pittieri*, *C. niveus*, *D. arboreus*, *E. austin-smithii*, *E. haberi*, *M. fragrans*, *M. rhopaloides*, *O. floribunda*, and *O. xalapensis*) showed notable cytotoxic activity (≥ 50% killing on at least one cell line). Four essential oils (*C. monteverdensis*, *N. membranacea*, *O. floribunda*, and *S. perforatus*) were active against brine shrimp (LC_{50} < 10 μg/mL). None of the essential oils was active against Gram-negative bacteria and only two (*C. niveus* and *O. floribunda*) showed notable activity against *S. aureus* (MIC = 78 μg/mL).

The essential oil with the broadest cytotoxicity was *Calyptranthes pittieri* (Myrtaceae) with ≥ 50% killing on three different cell lines. The most abundant components in *C. pittieri* leaf oil were linalool (54.6%), *trans*-2-hexenal (24.4%), α-terpineol (6.3%), and 4-terpineol (4.6%) [10]. Of these, both *trans*-2-hexenal [11,12] and 4-terpineol [13] are known to be cytotoxic, and likely account for the activity of the oil. *C. pittieri* leaf oil was not particularly toxic to brine shrimp and did not show appreciable antibacterial activity.

Croton monteverdensis (Euphorbiaceae) bark essential oil showed notable brine shrimp toxicity but was otherwise inactive. The abundant components in *C. monteverdensis*, α-pinene (17.1%) and β-pinene (10.5%) [14] may account, in part, for the observed brine shrimp lethality [15]. *C. niveus*, on the other hand, was slightly cytotoxic to MCF-7 cells and showed antibacterial activity on *S. aureus*. The major components of *C. niveus* bark oil, α-pinene (14%), 1,8-cineole (12%) and borneol (9%) [16] do not, by themselves, account for the observed activity.

Table 1: Bioactivity screening of Monteverde cloudforest essential oils[a].

Essential oil	Cytotoxic activity (% kill at 100 μg/mL, standard deviations in parentheses)					*Artemia salina* (LC_{50}, μg/mL)	Antibacterial activity (MIC, μg/mL)	
	Hep G2	MDA-MB-231	MCF-7	PC-3	SK-Mel-28		*B. cereus*	*S. aureus*
Calyptranthes pittieri (leaf)	**49.4(5.9)**	3.2(1.0)	28.4(4.3)	**46.6(3.8)**	**98.2(1.1)**	50.8	313	1250
Cinnamomum tonduzii (leaf)	NT[b]	2.9(0.6)	0	0	8.5(5.0)	37.9	625	1250
Croton monteverdensis (bark)	0	0	9.7(3.1)	11.3(4.0)	NT	**6.9**	625	156
Croton niveus (bark)	7.7(4.7)	0	**56.7(1.8)**	0	NT	18.2	625	**78**
Dendropanax arboreus (leaf)	NT	10.7(1.8)	7.1(1.6)	23.8(4.2)	**83.7(2.4)**	21.3	156	625
Eugenia austin-smithii (leaf)	NT	9.5(1.7)	39.2(2.2)	**49.4(9.0)**	**100**	38.2	625	1250
Eugenia haberi (leaf)	16.4(2.3)	0	10.4(9.9)	0	**52.3(5.9)**	31.6	313	1250
Myrcianthes fragrans (leaf)	**60.5(3.6)**	11.8(8.9)	0	11.9(0.4)	**71.0(3.8)**	43.3	625	1250
Myrcianthes rhopaloides (leaf)	NT	23.4(4.6)	13.4(4.8)	7.8(1.4)	**99.0(0.3)**	NT	313	1250
Nectandra membranacea (leaf)	5.3(4.5)	34.7(12.2)	18.1(6.9)	0	NT	**3.7**	1250	156
Ocotea floribunda (leaf)	**78.8(6.6)**	NT	25.5(8)	10.6(1.1)	NT	**3.7**	156	**78**
Oreopanax xalapensis (leaf)	16.1(6.9)	11.3(5.0)	0	0	**80.6(4.0)**	18.2	313	625
Piper umbellatum (leaf)	10.0(2.7)	NT	0	0	NT	29.1	1250	156
Psidium guajava (leaf)	NT	4.4(2.7)	16.2(6.6)	7.7(2.1)	0	29.5	625	1250
Stauranthus perforatus (leaf)	0	0	0	0	NT	**5.8**	625	313
Zanthoxylum acuminatum (leaf)	NT	0	0	0	0	29.7	1250	1250
Zanthoxylum melanostictum (leaf)	NT	0	0	0	0	28.1	625	1250
Zantoxylum monophyllum (leaf)	NT	2.8(1.0)	0	0	0	29.8	313	625
Zanthoxylum "brillante" (leaf)	NT	0	2.9(0.5)	0	0	31.6	1250	1250

[a]Notable bioactivity is indicated in **bold**.
[b]NT = not tested in this bioassay.

Table 2: Chemical compositions of *Dendropanax arboreus* and *Oreopanax xalapensis* leaf essential oils.

RI	Compound	% Composition	
		D. arboreus	*O. xalapensis*
856	*cis*-3-Hexenol	18.5	9.1
864	1-Hexanol	8.6	trace
1066	*cis*-Sabinene hydrate	---	0.5
1097	*trans*-Sabinene hydrate	---	0.3
1100	Linalool	trace	0.7
1176	4-Terpineol	trace	1.9
1337	δ-Elemene	0.4	0.5
1374	α-Copaene	0.4	0.4
1389	β-Cubebene	0.3	0.2
1392	β-Elemene	1.4	1.9
1417	β-Caryophyllene	2.9	3.2
1427	β-Gurjunene (= Calarene)	0.2	0.2
1438	Aromadendrene	trace	0.2
1442	6,9-Guaiadiene	0.8	1.2
1451	α-Humulene	1.0	1.1
1481	Germacrene-D	31.0	31.1
1495	Bicyclogermacrene	3.0	2.9
1503	Germacrene-A	0.7	0.9
1506	δ-Amorphene	3.8	5.0
1514	Cubebol	0.6	0.6
1518	Unidentified	trace	0.6
1522	δ-Cadinene	1.8	1.5
1548	Elemol	1.8	trace
1573	Germacrene D-4-ol	trace	4.1
1575	Spathulenol	5.8	trace
1590	Viridiflorol	trace	1.1
1619	1,10-di-*epi*-Cubenol	0.7	1.9
1627	Unidentified	trace	0.8
1636	Alloaromadendrene epoxide	1.4	0.6
1639	τ-Cadinol	1.6	1.6
1645	Torreyol (= α-Muurolol)	0.5	0.6
1652	α-Cadinol	2.6	3.3
1665	Intermediol	1.1	---
1684	Germacra-4(15),5,10(14)-trien-1α-ol	1.3	trace
1687	Shyobunol	7.6	22.0
	Total Identified	**100.0**	**98.5**

Both *Eugenia austin-smithii* and *E. haberi* (Myrtaceae) leaf oils were cytotoxic to SK-Mel-28 cells. The cytotoxicity observed is likely due to the relatively high concentrations of *trans*-2-hexenal in the two oils (33.6% and 22.1%, respectively) [16]. These leaf oils are also rich in α-terpineol (16.3% and 19.4%, respectively), but this compound has not been reported to be cytotoxic. 4-Terpineol has shown cytotoxic activity against melanoma cells [13], and this compound is present in *E. austin-smithii* and *E. haberi* leaf oils (5.7% and 4.7%, respectively) [16].

Myrcianthes fragrans (Myrtaceae), rich in *cis*-3-hexenol (10.0%), 1,3,5-trimethoxybenzene (15.7%), spathulenol (7.5%), caryophyllene oxide (7.8%), and α-cadinol (10.4%) [17], was cytotoxic to Hep G2 and SK-Mel-28 cells. Of these compounds, spathulenol [18], caryophyllene oxide [19], and α-cadinol [20] have been shown to be cytotoxic. *M. rhopaloides*, on the other hand, has abundant *trans*-2-hexenal (46.1%) in addition to 1,8-cineole (12.5%) and α-cadinol (6.7%) [17]. The high concentrations of *trans*-2-hexenal in addition to α-cadinol are likely responsible to the cytotoxicity of *M. rhopaloides* leaf oil on SK-Mel-28 cells.

Both *Nectandra membranacea* and *Ocotea floribunda* (Lauraceae) leaf oils showed remarkable toxicity toward brine shrimp (LC_{50} = 3.7 μg/mL). Both of these oils are rich in α- and β-pinenes (22.4% and 12.6%, respectively in *N. membranacea* [21], and 22.5% and 21.3% in *O. floribunda* [22]), and these compounds have shown brine shrimp lethality [15]. The pinenes, along with kaurene (34%) are probably responsible for the cytotoxicity of *O. floribunda* leaf oil toward Hep G2 cells [15].

Dendropanax arboreus and *Oreopanax xalapensis* (Araliaceae) leaf oils revealed cytotoxicity on the melanoma cell line, SK-Mel-28. Both of these oils are dominated by germacrene D (34% and 32%, respectively (Table 2), which has shown cytotoxicity [19]. In addition, both *D. arboreus* and *O. xalapensis* contain shyobunol (7.6% and 22.0%, respectively), a hydrate of δ-elemene, and *D. arboreus* contains the cytotoxic spathulenol (5.8%).

Stauranthus perforatus (Rutaceae) leaf oil, which was not active in any other screen, did show notable brine shrimp lethality. The oil had some α-pinene (8.4%) and some limonene (7.2%) with abundant germacrene D [23]. While α-pinene and limonene do show brine shrimp lethality [15], germacrene D is inactive [24].

Experimental

Collection and Analysis of Essential Oils: The collection, hydrodistillation, and GC-MS analyses of essential oils from *Calyptranthes pittieri* [10], *Cinnamomum tonduzii* [25], *Croton niveus* and *C. monteverdensis* [14], *Eugenia austin-smithii* and *E. haberi* [14], *Myrcianthes fragrans* and *M. rhopaloides* [17], *Nectandra membranacea* [21], *Ocotea floribunda* [22], *Piper umbellatum* [26], *Psidium guajava* [17], *Stauranthus perforatus* [23], *Zanthoxylum acuminatum*, *Z. melanostictum*,

Z. monophyllum, and *Zanthoxylum* sp. nov. "brillante" [27], have already been published.

Leaves of *Dendropanax arboreus* (127.3 g, Haber collection number 1637) and *Oreopanax xalapensis* (176.0 g, Haber collection number 4288) were collected from several individuals on June 11, 2003 from Monteverde, Costa Rica (10° 18.7′ N, 84° 48.6' W, 1350 m elevation). The fresh leaves were chopped and immediately hydrodistilled with continuous extraction with $CHCl_3$ using a Likens-Nickerson apparatus to give 24.2 mg *D. arboreus* leaf oil and 27.0 mg *O. xalapensis* leaf oil, respectively. The leaf essential oils were analyzed by GC-MS as previously described [28]. The chemical compositions of *D. arboreus* and *O. xalapensis* leaf oils are summarized in Table 2.

Cytotoxicity Screening: *In-vitro* cytotoxic activity against Hep G2 (ATCC No. HB-8065), MDA-MB-231 (ATCC No. HTB-26), MCF-7 (ATCC No. HTB-22), PC-3 (ATCC No. CRL-1435), and SK-Mel-28 (ATCC No. HTB-72) cells was carried out using the MTS method for cell viability as previously described [29].

Antibacterial Screening: Essential oils were screened for antibacterial susceptibility against Gram-positive bacteria, *Bacillus cereus* (ATCC No. 14579), *Staphylococcus aureus* (ATCC No. 29213); Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC No. 27853) and *Escherichia coli* (ATCC No. 25922), using the microbroth dilution technique as described previously [29].

Brine Shrimp Lethality Screening: Brine shrimp (*Artemia salina*) lethality tests were carried out using a modification of the procedure described by McLaughlin [30]. Solutions of crude extracts (1% w/w in DMSO) were added to brine shrimp suspensions to give final concentrations of 100, 10, 1, and 0.1 μg/mL (three replicates each plus DMSO controls). LC_{50} values (concentrations of extracts that are lethal to 50% of the organisms) were determined using the Reed-Muench method [31].

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Composition and Antibacterial Screening of the Essential Oils of Leaves and Roots of *Espeletiopsis angustifolia* Cuatrec

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Hydrodistillation of leaves and roots of *Espeletiopsis angustifolia* Cuatrec. (Asteraceae) yielded 0.18% and 0.15% essential oils, respectively. GC-MS analysis allowed identification of 24 components, which made up 92.9% of the total oil from the leaves, while only 16 compounds (67.2%) were identified in the roots. The most abundant compounds in the leaves were α-pinene (29.9%), β-caryophyllene (14.1%), α-gurjunene (9.9%), β-pinene (9.6%), and 19-oxo-*ent*-kaur-16-ene (5.3%). In the roots, the main ones were α-pinene (27.9%), β-pinene (10.9%), β-caryophyllene (10.2%), and bicyclogermacrene (8.6%). Antibacterial activity was tested against Gram-positive and Gram-negative bacteria using the agar diffusion method. Activity was observed only against Gram-positive bacteria. MIC values were determined for *Staphyloccocus aureus* ATCC 25923 (1000 μg/mL, both roots and leaves) and *Enteroccocus faecalis* ATCC 29212 (240 μg/mL, roots and 360 μg/ mL, leaves).

Keywords: *Espeletiopsis angustifolia*, Asteraceae, essential oil composition, antibacterial activity.

Espeletiopsis angustifolia Cuatrec. (Asteraceae) is one of 180 species of resinous plants that grow in the mountainous areas of northern South America above 2500 meters. These plants belong to the Espeletiinae subtribe [1] and have a characteristic rosette growth form. Sixteen *Espeletiopsis* species might be found in Colombia, while seven have been described for Venezuela [2-3].

A phytochemical study of five species of *Espeletiopsis* showed that these plants contained large amounts of kaurene derivatives, as well as monoterpenes and sesquiterpenes [4]. In the present study the composition of the essential oils isolated by hydrodistillation from the leaves and the roots of *E. angustifolia* is being reported.

Table 1 shows the percentage composition of the constituents of the essential oils from the leaves and roots of *E. angustifolia*. In the leaf oil, 24 compounds were identified, which made up 92.9% of the total oil. The most abundant constituents were α-pinene (29.9%), β-caryophyllene (14.1%), α-gurjunene (9.9%) and β-pinene (9.6%).

Table 1: Percentage composition of the essential oil from leaves and roots of *Espeletiopsis angustifolia*.

Peak	Constituents	leaves (%)	roots (%)	KI
1	α-Pinene	29.9	27.9	931
2	Sabinene	0.6	0.3	964
3	β-Pinene	9.6	10.9	968
4	β-Myrcene	0.6	0.6	979
5	α-Phellandrene	0.2	0.2	994
6	*p*-Cymene	0.5	0.8	1031
7	Limonene	0.6	0.6	1034
8	1,8-Cineole	-	0.4	1038
9	*trans*-Verbenol	0.4	-	1145
10	4-Terpineol	0.2	-	1177
11	α-Terpineol	0.2	-	1193
12	Myrtenol	0.3	-	1198
13	α-Gurjunene	9.9	-	1352
14	β-Caryophyllene	14.1	10.2	1422
15	α-*trans*-Bergamotene	0.9	-	1437
16	*trans*-β-Farnesene	0.3	-	1445
17	α-Humulene	0.5	1.2	1457
18	*ar*-Curcumene	2.4	-	1487
19	δ-Selinene	-	0.6	1494
20	α-Zingiberene	5.4	-	1500
21	Bicyclogermacrene	1.7	8.6	1501
22	δ-Cadinene	1.6	1.4	1526
23	Spathulenol	3.5	-	1580
24	Caryophyllene oxide	3.7	0.9	1586
25	Kaur-16-ene (Podocarpene A)	-	0.3	2040
26	19-oxo-*ent*-Kaur-16-ene	5.3	2.3	2255
27	19-hydroxi-*ent*-Kaur-16-ene	0.5	-	2348

KI: Kovats Indexes were determined by GC on a HP-5 column..

The oil was also relatively rich (5.3%) in 19-oxo-*ent*-kaur-16-ene, which was reported by Bohlmann *et al* [4] as one of the components from the flower stems of *Espeletiopsis guacharaca*. On the other hand, α-pinene has been found in all the essential oils of Espeletiinae studied so far [5-9]. On the contrary, only 16 compounds (67.2%) were identified in the oil from the roots, with α-pinene (27.9%), β-pinene (10.9%), β-caryophyllene (10.2%), and bicyclogermacrene (8.6%) as major components.

Results obtained in the antibacterial study of the essential oils are shown on Table 2. With the agar disc diffusion assay, growth inhibition was only observed with Gram-positive bacteria; both oils were found to be active against *Staphylococcus aureus* ATCC 25923 at a minimal inhibitory concentration (MIC) of 1000 μg/mL. Against *Enterococcus faecalis* ATCC 29212, the oil from the roots was found to be more active than the oil from the leaves; the oils showed MIC values of 240 μg/mL and 360 μg/mL, respectively.

Antimicrobial activities of essential oils are difficult to correlate to a specific compound due to their complexity and variability. In general, the antimicrobial activities have been mainly explained through C10 and C15 terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active sites of the target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters can contribute to the overall antimicrobial effect of essential oils [10].

On the other hand, enantiomers of α-pinene, β-pinene and limonene have a strong antibacterial activity [11-13]. These chemical components exert their toxic effects against these microorganisms through the disruption of bacterial and fungal membrane integrity [14-16]. It has been demonstrated that α-pinene and β-pinene are able to destroy cellular integrity, and thereby, inhibit respiration and ion transport processes.

Antimicrobial properties of caryophyllene and caryophyllene oxide have also been observed [17-18]. In addition, a great number of *ent*-kaurenes have displayed significant antibacterial activity against bacteria and yeasts [19-21]. Therefore, the antibacterial results observed in this investigation might be related to the presence of α-pinene, β-pinene and β-caryophyllene, although the synergistic effects of the diversity of major and minor constituents present in the essential oils should be taken into consideration to account for their biological activity. There are no previous reports on either the chemical composition or antimicrobial activity of the essential oils of this species.

Table 2: Antibacterial activity of the essential oils from leaves and roots of *Espeletiopsis angustifolia*.

Essential oil	Microorganisms				
	S. aureus *ATCC (25923)*	*E. faecalis* ATCC (29212)	*E. coli* ATCC (25992)	*K. pneumoniae* ATCC (23357)	*P. aeruginosa* ATCC (27853)
Disc diffusion assay					
Leaves	7*	10*	NA	NA	NA
Roots	7*	9*	NA	NA	NA
MIC (μg/mL):					
Leaves	1000	360	NT	NT	NT
Roots	1000	240	NT	NT	NT
Positive controls:					
Ampicillin-Sulbactam	29*	NT	NT	NT	NT
Vancomycin	NT	18*	NT	NT	NT
Streptomycin	NT	NT	15*	NT	NT
Aztreonam	NT	NT	NT	27*	NT
Cefoperazone	NT	NT	NT	NT	25*

*inhibition zone, diameter measured in mm, disc diameter 6 mm average of two consecutive trials
MIC: Minimal Inhibitory Concentration, concentration range: 10-1000 μg/mL
NA: Not active, NT: Not tested.
Ampicillin-Sulbactam® (10μg/10μg), Vancomycin® (30 μg), Streptomycin® (30 μg), Aztreonam® (30 μg), Cefoperazone® (75 μg).

Experimental

Plant material: Leaves and roots of *Espeletiopsis angustifolia* Cuatrec. were collected at San José páramo, Mérida State, in June 2006 at 2870 m above sea level (8º 21.002 N, 71º 18.447 W). The plant was identified by Prof. Juan Carmona. A voucher specimen (LBR 040) was deposited at the MERF Herbarium of the Faculty of Pharmacy and Bioanalysis, University of Los Andes.

Isolation of volatile compounds: Fresh leaves (900 g) and roots (800 g) of *E. angustifolia* were cut into small pieces and hydrodistilled in a Clevenger-type apparatus for 3 h. The oil samples were dried over anhydrous sodium sulfate and stored at 4°C in the dark.

Gas chromatography: GC analysis was performed on a Perkin-Elmer AutoSystem gas chromatograph equiped with a 5% phenyl methylpolysiloxane fused-silica capillary column (AT-5, Alltech Associates

Inc., Deerfield, IL, 60 m x 0.25 mm, film thickness 0.25 μm).

The initial oven temperature was 60°C. This was then increased to 260°C at 4°C/min, and the final temperature kept for 20 min. The column injector and detector temperatures were 200°C and 250°C, respectively, and the carrier gas was helium at 1.0 mL/min. A 1.0 μL sample was injected, using a split ratio of 1:100. Retention indices were calculated relative to C_8-C_{24} *n*-alkanes, and compared with values reported in the literature [22,23].

Gas chromatography - mass spectrometry: The GC-MS analysis was conducted on a Hewlett Packard GC-MS system, Model 5973, fitted with a 30 m long, cross-linked 5% phenyl methyl siloxane (HP-5MS, Hewlett Packard, USA) fused-silica column (0.25 mm, film thickness 0.25 μm). Source temperature 230°C; quadrupole temperature, 150°C; carrier gas helium adjusted to a linear velocity of 34 cm/s; ionization energy, 70 eV; scan range, 40-500 amu; 3.9 scans/s. The injected volume was 1.0 μL of 2% solutions of oil in *n*-heptane. A Hewlett-Packard ALS injector was used with a split ratio of 1:100. The identification of the oil components was based on a Wiley MS Data Library (6^{th} edn), followed by comparisons of MS data with published literature [23].

Antimicrobial Activity

The antimicrobial activity of the essential oils under study was evaluated by the agar disc diffusion method and the minimal inhibitory concentration (MIC) was determined.

Bacterial strains: The microorganisms used were *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25992), *Klebsiella pneumoniae* (ATCC 23357) and *Pseudomonas aeruginosa* (ATCC 27853).

Antimicrobial screening: The antimicrobial activity was determined according to the disc diffusion assay described by Rondón *et al*. [24]. The strains were maintained in agar at room temperature. Every bacterial inoculum (2.5 mL) was incubated in Mueller-Hinton agar at 37ºC for 18 h. The bacterial inoculum was diluted in sterile 0.85% saline to obtain a turbidity visually comparable to a McFarland Nº 0.5 standard (10^{6-8} CFU/mL).

Every inoculum was spread over plates containing Mueller-Hinton agar and a paper filter disc (6 mm) saturated with 10 μL of essential oil. The plates were left for 30 min at room temperature and then incubated at 37ºC for 24 h.

The inhibitory zone around the disc was measured and expressed in mm. A positive control was also assayed to check the sensitivity of the tested organisms using the following antibiotics: Ampicillin-Sulbactam ®, Vancomycin®, Streptomycin®, Cefoperazone® and Aztreonam®.

Determination of the minimal inhibitory concentration (MIC): The minimal inhibitory concentration (MIC) was determined only with microorganisms that displayed inhibitory zones. MIC was determined by dilution of the essential oils in dimethyl sulfoxide (DMSO) and pipetting 10 μL of each dilution into a filter paper disc. Dilutions of the oils within a concentration range of 10-1000 μg/mL were also carried out. MIC was defined as the lowest concentration that inhibited the visible bacterial growth [25].

A negative control was also included in the test using a filter paper disc saturated with DMSO to check possible activity of this solvent against the bacteria assayed. The experiments were repeated at least twice.

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GC-MS Analysis of the Leaf Essential Oil of *Ipomea pes-caprae*, a Traditional Herbal Medicine in Mauritius

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The chemical compositions of the essential oils of the fresh and dried leaves of *Ipomea pes-caprae* from Mauritius were studied for the first time by gas chromatography-mass spectrometry and 70 compounds were identified. The major components were found to be 8-cedren-13-ol (13.0%), (*E*)-nerolidol (7.0%), guaiol (6.2%), α-cadinol (6.2%) and limonene (6.1%) in fresh leaves and β-caryophyllene (36.6%), α-copaene (8.0%), germacrene D (7.3%), phytol (5.8%), δ-cadinene (5.7%), and α-humulene (5.4%) in the dried leaf samples. The relationship between the anti-hemorrhoidal activity of *Ipomea pes-caprae*, one of its traditional uses in Mauritius, and the chemical composition of the essential oil samples is also discussed.

Keywords: *Ipomea pes-caprae*, essential oil, monoterpenes, sesquiterpenes.

The genus *Ipomea* (Convolvulaceae) consists of more than 200 species widely distributed in tropical and subtropical countries. Some of them are frequently used in folk medicine for the treatment of several diseases [1]**.** *I. pes-caprae*, commonly known in Mauritius as "Liane batatran", has been traditionally used to cure stone fish stings and alleviate people suffering from hemorrhoids (personal communication with the fishermen of Mauritius). Pre-clinical and clinical investigations validated some of the ethnopharmacological properties of the plant. A light petroleum extract was shown to inhibit the contraction of the guinea-pig ileum stimulated by four different spasmogens in a dose-dependant manner [2]**.** β-Damascenone and (*E*)-phytol were later isolated and proved to be responsible for the antispasmodic activity exhibited by the plant [3]. Pongprayoon and colleagues [4] additionally isolated 2-hydroxy-4,4,7-trimethyl-1(4H)naphthalenone, (–)-mellein, eugenol, and 4-vinylguaiacol from the same fraction. These compounds were shown to exhibit anti-inflammatory properties *via* the inhibition of prostaglandin activity in a dose-dependant manner. The extract of *I. pes-caprae* also demonstrated ability to neutralize crude jellyfish venoms [5]. The extract of the leaves also exhibited antinociceptive activities [6]. In Mauritius, people suffering from hemorrhoids usually either take a bath with a decoction of the plant or sit on a recipient containing the hot decoction in order that the vapor reaches the hemorrhoids. It was hence deduced that the anti-hemorrhoid activity of the plant might reside, at least in part, in the constituents of the plant essential oil. The only available information regarding the essential oil of *I. pes-caprae* is its physical properties [7]. Therefore, in this paper, in an attempt to validate the use of *I. pes-caprae* in the treatment of hemorrhoids, we report for the first time the separation and identification of the components of its essential oils using GC-MS.

Separate hydro-distillation of fresh and dried aerial parts of *I. pes-caprae* yielded clear oils, the yields being 0.005 and 0.019 %, respectively. The oils were separately subjected to GC-MS analysis. The retention times, retention indices calculated according to [8], and percentages of the compounds identified in the essential oils from the fresh and dried leaves are detailed in Table 1. The components are listed in elution order on the DB-XLB column.

Table 1: Percentage composition of the essential oil from the fresh and dried leaves of *I. pes-caprae* (L.) R. Br.

Compounds	Rt	Retention Indices (FAME)	Fresh plant Area %	Dried plant Area %
Tricyclene	3.51	666.8	0.03	ND
α-Thujene	3.64	677.1	0.2	0.05
α-Pinene	3.70	681.9	3.2	0.88
Camphene	3.95	701.5	0.4	0.12
β-Pinene	4.40	731.9	1.4	0.38
β-Myrcene	4.67	750.1	0.8	0.25
δ-3-Carene	4.95	769.1	0.3	0.08
α-Terpinene	5.13	781.2	0.1	0.03
p-Cymene	5.30	792.7	4.6	1.61
Limonene	5.35	796.1	6.1	1.74
(*Z*)-β-Ocimene	5.42	800.8	0.1	0.05
(*E*)-β-Ocimene	5.58	811.4	ND	0.03
γ-Terpinene	5.80	826.0	0.6	0.24
(*Z*)-Linalool oxide (furanoid)	6.00	839.2	0.0	0.01
Terpinolene	6.19	851.8	0.05	0.07
Fenchone	6.31	859.7	0.05	0.09
Linalool	6.45	869.0	3.7	1.82
(*Z*)-Thujone (α-thujone)	6.68	884.2	0.1	0.24
Methyl octanoate	6.85	895.5	0.1	0.07
(*E*)-Pinocarveol	7.06	909.7	0.4	ND
(*E*)-Verbenol	7.19	918.5	0.4	0.03
Camphor	7.25	922.6	0.1	0.13
Menthone	7.54	942.3	0.3	0.08
Ethyl benzoate	7.64	949.1	0.07	0.06
Terpinen-4-ol	7.76	957.3	0.5	0.14
p-Cymen-8-ol	7.92	968.2	0.4	0.03
α-Terpineol	8.02	975.0	3.3	0.38
Estragol	8.08	979.0	ND	0.06
Safranal	8.14	983.1	0.64	0.17
Decanal	8.20	987.2	ND	0.05
Verbenone	8.31	994.7	1.01	0.05
(*E*)-Carveol	8.42	1002.3	0.2	0.4
(*Z*)-Carveol	8.46	1005.1	0.1	ND
Nerol	8.72	1023.7	0.5	0.36
Carvone	8.94	1039.4	0.2	0.02
2-(*E*)-Decenal	9.09	1050.1	0.5	0.29
Citral	9.18	1056.6	0.08	0.02
Thymol	9.24	1060.9	ND	1.28
Carvacrol	9.46	1076.6	0.2	ND
(*E,E*)-2,4-Decadienal	9.56	1083.7	0.1	0.12
δ-Elemene	9.80	1100.9	0.3	0.07
α-Cubebene	9.96	1112.9	ND	0.84
α-Terpinyl acetate	10.07	1121.2	0.2	0.12
Neryl acetate	10.20	1131.0	0.9	0.1
Eugenol	10.29	1137.7	2.7	0.05
α-Copaene	10.37	1143.8	0.2	7.97
Geranyl acetate	10.48	1152.0	1.7	4.21
(*E*)-β-Damascenone	10.51	1154.3	0.5	ND
β-Elemene	10.60	1161.1	0.4	0.41
β-Caryophyllene	10.95	1187.4	1.4	36.57
α-Humulene	11.45	1226.3	0.4	5.43
Geranyl acetone	11.52	1231.9	ND	0.03
γ-Muurolene	11.74	1249.4	0.4	0.13
Germacrene-D	11.83	1256.5	0.4	7.35
β-Ionone	11.91	1262.9	0.4	1.51
Cuparene	12.00	1270.0	ND	0.15
Tridecanal	12.06	1274.8	0.3	0.58
δ-Cadinene	12.26	1290.6	0.5	5.7
(*Z*)-Calamenene	12.38	1300.2	ND	0.09
(*E*)-Nerolidol	12.71	1327.7	7.0	0.12
Dodecanoic acid	12.79	1334.3	ND	0.22
Caryophyllene oxide	13.18	1366.8	2.0	3.94
Guaiol	13.36	1381.8	6.2	0.08
Cedrol	13.54	1396.8	0.2	0.41
α-Muurolol	13.97	1434.1	1.6	0.75
α-Cadinol	14.05	1441.0	6.2	0.3
α-Bisabolol	14.40	1471.5	2.2	0.02
8-Cedren-13-ol	15.12	1535.6	13.0	0.03
Hexahydrofarnesyl acetone	15.88	1605.0	0.2	3.02
Phytol	18.47	1864.8	0.3	5.84
Total			81.5	97.45

Rt, retention times on DB-XLB column
ND, not dedected.

It is to be noted that FAME (fatty acid methyl esters) have been used for indices calculation instead of *n*-alkanes since the DB-XLB is more polar than the ones normally used for Kovats and related indices calculation; so, indices based on FAME give higher specificity [8].

A total of 60 and 65 compounds, representing 81% and 97 % of the volatiles from the fresh and dried leaves respectively, were identified by means of their retention times and mass spectral fragmentation patterns. Unidentified components were present in such low amounts that either no mass spectrum could be recorded or the spectrum was too poor for interpretation. Some high boiling compounds were also identified from the essential oils due to the temperature gradient (up to 310°C) and the stationary phase (DB-XLB, extremely low bleeding) used.

From Table 1 it is evident that there are high quantitative differences in the compositions of both oils, albeit distilled from the same plant sample (fresh and dried). This stresses the importance of analysis of those oils and could explain differences in biological properties.

From Table 1 it is also clear that monoterpenoids and sesquiterpenoids constitute the main groups of compounds detected in both the fresh and dried leaves essential oils: they contain respectively 30.2% and 10.8% monoterpenoids and 42.5% and 70.4% sesquiterpenoids. This shows that drying of the leaves induces a loss of monoterpenoids, usually more volatile than sesquiterpenoids. Relative proportions are also very different, indicating that, during the drying process, not only evaporation but also transformations occur, which might be enzymatic or not. The major components (> 3%) of the fresh leaves essential oil were α-pinene (3.2%), *p*-cymene (4.6%), limonene (6.1%), linalool (3.7%), α-terpineol (3.3%), (*E*)-nerolidol (7.0%), guaiol (6.2%), α-cadinol (6.2%) and 8-cedren-13-ol (13.0%), while those of the dried leaves include α-copaene (8.0%), geranyl acetate (4.2%), β-caryophyllene (36.6%), α-humulene (5.4%), germacrene D (7.3%), δ-cadinene (5.7%), caryophyllene oxide (3.9%), hexahydrofarnesyl acetone (3.0%) and phytol (5.8%). The presence of some of these components can partially explain one of its traditional uses in Mauritius.

The three main signs and symptoms of hemorrhoids are severe pain, bleeding and inflammation [9]. The anti-inflammatory and antinociceptive activities of the oil could be imputed to the presence of the following compounds in quantitative amounts in the oil of the fresh leaves: α-pinene [10], limonene [11,12], linalool [13,14], α-terpineol [15], eugenol [2,16] and caryophyllene oxide [17]; compounds known to possess analgesic and/or anti-inflammatory properties on different models. For the essential oil from the dry leaves, β-caryophyllene [18], phytol [3,19] and caryophyllene oxide [18] are the main anti-inflammatory constituents.

Furthermore, the oil obtained from the fresh leaves of *I. pes-caprae* contains compounds which could help the permeation of the anti-inflammatory and antinociceptive agents through the skin. In fact limonene [20] is reported to promote percutaneous absorption of nonsteroidal anti-inflammatory drugs in rats while nerolidol has been shown to increase the skin permeation of naproxen® [21].

In light of the present study, the traditional usage of *I. pes-caprae* by Mauritian folks for its anti-hemorrhoidal activity is fully justified. As discussed above, oils obtained from both the dried and fresh leaves of the plant have been shown to possess several compounds that can synergistically reduce the symptoms of hemorrhoids and alleviate people suffering from the affliction. Additionally, this study emphasized that the use of fresh leaves of *I. pes-caprae* is expected to be more effective in the treatment of hemorrhoids than the dried leaves since the former retains most of its monoterpenes, which other studies have previously shown to possess biological activities relevant to the cure of hemorrhoids.

Experimental

Plant material: The leaves of *Ipomea pes-caprae* (L.) R. Br. (Convolvulaceae) were collected along the seashore of Grand Gaube, a small fishermen's village at the north-northeast part of the Island of Mauritius during January 2003 (summer). A voucher specimen of the plant, bearing No. MAU 23727, has been deposited at the National Herbarium at the Mauritius Sugar Industry Research Institute (MSIRI).

Preparation of extracts: Half of the collected leaf sample was immediately investigated and the other part was dried in shade at room temperature for two days and then analyzed (as dried plant material). The essential oils from the fresh and dried leaves of *I. pes-caprae* were obtained by hydro-distillation in a Clevenger-type apparatus according to the method recommended in the European Pharmacopoeia [22] with *n*-hexane. The essential oil was collected in *n*-hexane and stored at 4°C in the dark. Essential oil yields from fresh and air dried plant material were 0.005 and 0.019% respectively (based on fresh and dried mass of samples).

Gas chromatography-mass spectrometry: GC-MS analyses were carried out on a Thermo Quest Trace GC 2000 coupled to a Trace MS mass spectrometer, equipped with PTV split-splitless injector, fused silica capillary column (DB-XLB, 15m x 0.25 mm) and electron impact detector. Samples were injected (1 µL of the 10% solution of essential oils in *n*-hexane) in split mode (1:40). Injector temperature was 220°C. Column temperature was programmed as follows: isothermal at 40°C for 1 min, then increased to 250°C, at a rate of 10°C min^{-1}, and subsequently at a rate of 15°C min^{-1} to 310°C. This temperature was held isothermally for 15 min. Helium was used as carrier gas (flow rate: 1 mL/min). Mass spectra were recorded in the scan mode at 70 eV (40-415 U). The ion source temperature was 230°C.

Qualitative and quantitative determination: Triplicate analyses of each oil sample were performed and quantitative results are presented as a mean of data derived from GC–MS analyses. Identification of individual constituents was made by comparing their mass spectra with the NIST library of mass spectra and literature [23], as well as by comparison of their retention indices to those of authentic samples, when available. Quantitative analysis (in % of the total peak areas) was performed by peak area measurement (TIC).

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Chemical Composition, Insecticidal Effect and Repellent Activity of Essential Oils of Three Aromatic Plants, Alone and in Combination, towards *Sitophilus oryzae* L. (Coleoptera: Curculionidae)

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Essential oils of aromatic plants with insecticidal properties are nowadays considered as alternative insecticides to protect stored products from attack by insect pests. A combination of some of these plants in the granaries is a current practice in certain localities of northern Cameroon. The aim of the present work was to analyze the impact of the combinations of the essential oils of *Vepris heterophylla* (Rutaceae), *Ocimum canum*, and *Hyptis spicigera* (both Lamiaceae), the three most used local aromatic plants because of their insecticidal activity and their repellent effect on *Sitophilus oryzae*. The present work revealed that these plants are rich in monoterpenoids. The GC/MS analyses have shown that monoterpenoids represented 65.5% for *H. spicigera*, 92.1% for *O. canum* and 47.0% for *V. heterophylla*. The crude essential oil of *O. canum* was the most insecticidal with a LD_{50} of 42.9 ppm. The most repellent effect was obtained by a combination of the essential oils of *H. spicigera* and *O. canum*, with a repellent percentage at 77.5%. These results suggest a suitable strategy for pest management of stored products.

Key words: Aromatic plants, combination, essential oils, repellent effect, stored products.

In northern Cameroon, the most important insect grain pests are *Sitophilus zeamais* and *S. oryzae* (Coleoptera: Curculionidae), *Callosobruchus maculatus* (Coleoptera: Bruchidae) and *Tribolium castaneum* (Coleoptera: Tenebrionidae) [1]. Smallholders lose up to 80% of their stock each year because of insects [2]. To prevent the losses, producers usually rely on a relish of chemical insecticides. These tools, used frequently and abusively, consequently result in pollution of the environment and intoxication of consumers. There is, therefore, an urgent need to develop user-friendly storage methods with minimal adverse effects on the environment and on consumers. Essential oils of aromatic plant that have insecticidal properties could be considered as alternative insecticides [3,4]. These oils are volatile with high insecticidal efficiency and very low persistence. Most of the active compounds of the essential oil are specific to particular insect groups and not to mammals [5], and, therefore, should be considered in pest management strategies. One of the most important qualities of aromatic plants is their odors, which confer them their repellent effects. To maximize the effects of these

Table 1: Yields of essential oil from 3 aromatic plants of Northern Cameroon.

Aromatic plant	Part collected	Yield (%)
Vepris heterophylla	leaves	5.8 ± 1.2a
Ocimum canum	Leaves and flowers	3.3 ± 0.9a
Hyptis spicigera	flowers	1.7 ± 0.2a
Chi square		2.4 (df=2)

The yields followed by the same letter do not differ significantly ($p<0.01$)

Table 2: Major components of the three essential oils.

Compounds	*Vepris heteropylla*	*Ocimum canum*	*Hyptis spicigera*
α-Thujene		0.2	0.5
α-Pinene	0.2	2.1	9.1
β-Pinene		8.8	5.7
Sabinene	17.3		
Myrcene	1.9	1.6	
Cymene (*p*/*o*)	0.2		
Limonene	4.0	49.2	
(*E*)-β-ocimene	10.2		
γ-Terpinene	0.7		
1,8-Cineol			24.5
Terpinolene	1.4		
Linalool	0.9		8.4
Sabinol			1.1
Terpinen-4-ol	1.5		4.7
α-Terpineol	1.2		8.3
Safrole	3.0		
(*E*)-Caryophyllene	2.3	8.6	22.2
Carvacrol			1.9
Germacrene D	1.6		
γ-Amorphene	0.4		
δ-Cadinene	3.2		
Elemene		3.2	1.2
Elemol	19.4		
Guaiol	15.2		
Humulene epoxide II	1.6		
α-Eudesmol + Valerianol	1.1		

plants, farmers in the past utilized many of them in the same granary. This present work investigates the insecticidal and repellent efficiency of three local aromatic plants, *Vepris heterophylla* (Engl.) Letouzey (Rutaceae), *Ocimum canum* Sims (Lamiaceae), and *Hyptis spicigera* Lam. (Lamiaceae), frequently used alone and in combination.

The essential oil yields obtained ranged from 1.7 to 5.8% (Table 1). Flowers of *H. spicigera* produced less essential oil than the leaves of *V. heterophylla* and *O. canum.*

The GC/MS analyses of each of the three essential oils showed that they contain abundant monoterpenes (Table 2): 65.5% for *H. spicigera*; 92.1% for *O. canum* and 47.0% for *V. heterophylla*. The amount of sesquiterpenes observed was also different between the essential oils. That of *V. heterophylla* had the highest percentage, 51%, and that of *O. camum* the lowest, 7%. The most abundant active compounds in these essential oils differed from one oil to another. Thus, 49.2% of *O. canum* was composed of limonene, 8.8% of α-pinene and 3.2% of elemene. The essential oil of *H. spicigera* had two main components, 1,8-cineol (24.0%) and (*E*)-caryophyllene (22.2%). Other active compounds found in this essential oil were α-pinene (9.1%), β-pinene (5.7%), α-terpineol (8.3%) and linalool (8.4%). The essential oil of *V. heterophylla* contained elemol (19.4%), sabinene (17.3%), (*E*)-β-ocimene (10.6%), guiaol (15.3%), limonene (4.0%), (*E*)-caryophyllene (2.3%) and additional compounds such as myrcene and terpinolene.

Table 3: Chemical composition of combinations of the essential oils.

Compounds	*Vh + Oc*	*Oc + Hs*	*Vh + Hs*
α-Pinene		4.3	4.5
β-Pinene		6.1	2.6
Sabinene	8.6		
Myrcene	2.1		
Limonene	27.6	26.2	
(*E*)-β-Ocimene	5.2		5.7
1,8-Cineol		14.4	11.8
Linalool		54.1	3.0
Terpinen-4-ol		2.9	2.1
α-Terpineol		4.1	
Safrole	1.6		
(*E*)-Caryophyllene	5.4	17.8	
Elemene		1.3	
Elemol	9.5		10.2
Guaiol	7.8		9.8

The chemical composition of combinations of essential oils (Table 3), as expected, represent averages of the percentages of each of the components in the individual oils. The LD_{50} values obtained for each of the essential oils, as well as their combinations, are presented in Table 4. The most active essential oil, with the lowest LD_{50} value, was that of *O. canum* oil.

The insecticidal activity of an essential oil depends on its chemical composition and the sensitivity of the target pest to the active compounds [6]. The essential oil of *O. canum*, which is the most toxic, contains 49% limonene, according to the GC/MS analysis. It has been shown that limonene is highly toxic to Coleopterans [7]. All the essential oils tested showed remarkable insecticidal activity, the least active of which was *Vepris heterophylla* with an LD_{50} of 349.8 ppm. *H. spicigera* oil showed a high concentration of 1,8 cineol (24.5%) and (*E*)-caryophyllene (22.2%).

These compounds, along with α-phellandrene, terpinolene, and (+)-limonene have shown high toxicity towards *S. oryzae* [8]. The insecticidal efficiency observed is due to both major and minor components of each active oil [4,7-9]. These synergistic effects could explain the differences between observed LD_{50} values and what would be expected based on average activities of the individual

Table 4: Insecticidal activity (LD_{50}) of the three essential oils and their combinations towards *Sitophilus oryzae*.

	LD_{50} (ppm)		
Plant species	Observed	Expected	CHI^2
Hyptis spicigera	112.0		
Ocimum canum	42.9		
Vepris heterophylla	349.8		
Hyptis + Ocimum	75.8	77.5	0.017 ns
Hyptis + Vepris	182.1	230.9	5.76*
Ocimum + Vepris	103.8	196.0	28.3***

Table 5: Duration of insecticidal potency of the essential oils tested alone and in combination towards *Sitophilus oryzae*.

	LD_{50}		
Plant species	Observed	Expected	CHI^2
Hyptis spicigera	6h 2 min		
Ocimum canum	5h 4 min		
Vepris heterophylla	14h 5 min		
Hyptis +Ocimum	4h 2 min	5h 5 min	16.8***
Hyptis + Vepris	13h 4 min	10h 5 min	18.7***
Ocimum + Vepris	7h 4 min	10h 2 min	23.5***

Table 6: Insect repellent activity of the essential oils tested alone and in combination towards *Sitophilus oryzae*.

	Repellent rate (McDonald class)		
Plant species	Observed	Expected	CHI^2
Hyptis spicigera	62.5 (IV)		
Ocimum canum	33.7 (II)		
Vepris heterophylla	42.5 (III)		
Hyptis +Ocimum	77.5 (IV)	48.1 (III)	6.9***
Hyptis + Vepris	41.2 (III)	52.5 (III)	1.3 ns
Ocimum + Vepris	62.5 (IV)	38.1 (II)	9.5***

essential oils (Table 4). This synergistic effect has already been demonstrated between essential oils of five aromatic plants used in north Cameroon [10].

The activity of the essential oils decreased with time due to their high volatility, although the decrease was not the same for the three oils tested (Table 5). Those oils with a high proportion of hydrocarbon components lost their activity more rapidly than those composed mainly of oxygenated compounds [4,11].

The essential oil that exhibited the most repellent activity was *H. spicigera*, with a repellent percentage (RP) of 62.5% (Table 6). The least repellent oil, however, was *O. canum*, which had an RP of 33.7%. For the essential oil combinations, *Hyptis* + *Ocimum* was the most repellent (RP >77%), whereas the combination was expected to have an RP of 48%. The synergy between *O. canum* and *H. spicigera* has increased their repellent effects. Comparable results were observed for *O. canum* + *V. heterophylla*. The repellent effect of *V. hetrophylla* has previously been shown on *S. oryzae*. [8]. Leaves of *V. heterophylla, H. spicigera* and *O. canum* are used in traditional medicine against diseases and as purgatives. Their use in combinations in granaries could prove to be beneficial to prevent attack of post harvest insect pests.

Experimental

Plant collection: Leaves of *V. heterophylla* and flowers of *O. canum* were collected at Maroua, far north of Cameroon (10° 39.214′ N, 14° 24.145′ E, 375 m elevation). Flowers of *H. spicigera* were collected near the campus of the University of Ngaoundéré (7° 25.609′ N, 13° 33.549′ E, 1100 m elevation). These data were recorded with a GPS Garmin Geko 301. The collection of all plant materials was made in December 2005. After collection, the plant material was dried in the shade under laboratory conditions for 24 h, cut in pieces, weighed, and hydrodistilled for 4 h using a Clevenger-type apparatus. The essential oils obtained were stored at 4°C until their use for the bioassays.

GC/MS chemical analysis: GC/MS analysis utilized an HP-5MS column (5% phenyl methyl siloxane), 30 m long and 250 μm in diameter. The carrier gas was helium; the temperature program applied was from 40°C to 230°C at a rate of 5°C/min and then maintained at 230°C for 5 min. The pressure of the carrier gas was 49.9 KPa with a flux of 74.1mL/min. The ion-source temperature was 230°C and the ion scan range was 50-350 amu. The mass spectrum of each compound was compared with those of the Wiley 275 L library [12,13].

Insects: Insects used for the test were reared in the *in vivo* collection at the Storeprotect laboratory at the University of Ngaoundéré in Cameroon. They were derived from a strain collected in November 2003 from a granary in Beka hosséré (Ngaoundéré, Cameroon).

Insecticidal activity: In preliminary tests, several doses were chosen between those having no killing effect on the experimental population to the minimal one killing 100% of this population, in order to establish the LD_{50} of each essential oil. With a micropipette (Rainin Magnetic-assist), the precise volume of essential oil was added to acetone and diluted to 5 mL. From this, 0.5 mL of solution was uniformly applied to a 9 cm disk of filter paper (Whatman N°1) and placed in a Petri dish. Twenty adult insects, less than one month old, were introduced into the dish 5 min later and the dish was covered. A control with acetone alone, was made. For each preparation, 5 replications were made. The number of dead insects was determined 24 h after the application.

Insect repellent activity: Repellent effects of essential oils and their combinations were evaluated at doses of 0.031, 0.062, 0.125, and 0.251 µL/cm². The test was conducted in a 9-cm diameter Petri dish in which two half circles of filter paper were introduced. One half was treated with either essential oil or a combination of essential oils, while the second half was treated with acetone. Twenty insects were placed in the middle of the Petri dish and, after two h, the distribution of insects on each part of the paper was noted. The repellent percentage of the different oils, their combinations and the class were calculated according to the McDonald formula [14,15].

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Chemical Composition and Larvicidal Activity against *Aedes aegypti* of Essential Oils from *Croton zehntneri*

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The chemical composition of the essential oils from leaves, stalks and inflorescences of *Croton zehntneri* obtained by hydrodistillation were analyzed by GC-MS and CG-FID. *E*-Anethole was the main component of the essential oils of all plant parts. Essential oils of leaves, stalks, inflorescences and *E*-anethole were tested at different concentrations against instar III larvae of *Aedes aegypti* and showed LC_{50} values of 56.2 ± 0.3, 51.3 ± 0.3, 57.5 ± 0.1 and 69.2 ± 0.5 μg/mL, respectively.

Keywords: *Croton zethntneri*, essential oil, *E*-anethole, *Aedes aegypti*, larvicidal activity.

Croton zehntneri Pax et Hoff is an aromatic plant native to northeastern Brazil, and popularly known as "canela de cunhã". The species is used in traditional medicine as a sedative, appetite stimulator, antianorexigen, and for the relief of gastrointestinal disturbances [1]. The essential oil also acts as an intestinal muscle relaxant [2,3], central nervous system depressant [4], and antinociceptive agent [5]. *Aedes aegypti* is one of the mosquito species responsible for the transmission of both dengue fever and dengue haemorrhagic fever. In recent years, essential oils have received much attention as potent bioactive compounds against *A. aegypti.* [6-10].

Furthermore, because *C. zehntneri* is characterized by a strong and pleasant odor reminiscent of anise and clove, extracts of its barks and leaves are used in perfumes and as sweeteners in foods and in beverages [11]. The literature reports the chemical composition and larvicidal activity of the essential oil of leaves from *C. zehntneri* [12-14]. *E*-anethole is an important substance used as flavoring in the manufacture of candy, ice cream, chewing gum and alcoholic beverages [15]. As far as we know, there are no reports of either the chemical composition of the essential oils from stalks and inflorescences of *C. zehntneri* or of their larvicidal activity.

As part of our program to evaluate essential oils from northeastern Brazilian flora, this work reports the composition and larvicidal activity of the essential oils from the leaves, stalks and inflorescences of *C. zehntneri*, as well as of their major component, *E*-anethole, against *A. aegypti*.

The essential oils extracted from leaves, stalks and inflorescences of *C. zehntneri* were analyzed by CG/MS and the constituents identified and quantified (Table 1). A total of 30 compounds were identified in the three sample oils and they are arranged in Table 1 in the order of elution from a DB-5 column. The oils were characterized by high amounts of phenylpropanoids.

Table 1: Chemical composition of essential oil from leaves, stalk and inflorescences of *C. zehntneri*.

Compounds	RI[a]	Leaves (%)	Stalks (%)	Inflorescences (%)
Sabinene	975	0.1		
Myrcene	991	2.5		
1,8-Cineole	1031	4.3	0.9	
E-*β*-ocimene	1050	1.3		
Camphor	1146	0.3	2.5	
Borneol	1169	0.4	1.8	
α-terpineol	1189	0.8		
Estragole	1196	4.9	5.7	2.0
p-Anisaldehyde	1250		16.5	
E-anethole	1285	74.5	35.8	90.5
Anisyl formate	1332		9.1	
Eugenol	1359		3.4	
Isoledene	1376		0.5	
β-elemene	1391	0.1	4.9	
Methyl eugenol	1404		2.9	
Anisyl acetate	1413		7.0	
E-caryophyllene	1419	2.0	0.3	1.6
E-α-bergamotene	1435	0.09	0.6	
γ-elemene	1437	0.1		
α-humulene	1455	0.2		
Acetovanillone	1483		1.0	
Germacrene D	1485	1.3		1.2
α-selinene	1498		0.5	
Bicyclogermacrene	1500	3.9		1.7
E-*β*-guaiene	1503		0.4	
δ-cadinene	1523	0.1		
Spathulenol	1578	1.2	0.7	
Caryophyllene oxide	1583	0.5	0.5	
Globulol	1585			0.6
Viridiflorol	1593		1.4	
Total		98.8	96.5	97.6

[a] Retention indices

Twenty constituents (98.8%) were identified in the oil from leaves, representing eight monoterpenes. A comparison of our results with those previously reported for leaves of *C. zehntneri* reveal significant differences. In the earlier report, estragole and eugenol were identified as the main constituents [12]. The essential oils from leaves, stalks and inflorescences and their major constituent, *E*-anethole, were evaluated against instar larvae of *A. aegypti* in order to determine their potential as larvicidal agents, and the results are presented in Table 2. Temephos® (*O,O'*-(thiodi-4,1-phenylene)bis(*O,O*-dimethyl phosphorothioate) was used as a control positive.

Results of larvicidal evaluation showed that the essential oils and *E*-anethole were very active agents against larvae of *A. aegypti*, with LC values for the leaf oil of 56.2 ± 0.3 μg/mL, of the stalk oil 51.3 ± 0.3 μg/mL, of the inflorescence oil 57.5 ± 0.1 μg/mL, and of *E*-anethole 69.2 ± 0.5 μg/mL. The oils were slightly more active than the major compound. This effect may be due to the presence of terpenoid constituents. These substances can serve to increase the transmembrane absorption of lipophilic drugs [16].

Table 2: LC_{50} values for larval mortality caused by the essential oils and *E*-anethole.

Essential oil	LC_{50} (μg/mL)
Leaves	56.2 ± 0.3
Stalks	51.3 ± 0.3
Inflorescences	57.5 ± 0.1
E-Anethole	69.2 ± 0.5
Temephos®	1.4 ± 0.2

Therefore, it is possible that other constituents of the essential oils work synergistically with *E*-anethole. GC-MS and CG-FID analysis showed that the major constituent in the essential oils is *E*-anethole and these results suggest that these essential oils can be used as flavoring and as a potent natural larvicide.

Experimental

Plant material: Leaves, stalks and inflorescences of *C. zehntner*i were collected in August 2004 in Tianguá County, State of Ceará, northeast Brazil. A voucher specimem (#EAC33546) is deposited at the Herbário Prisco Bezerra, Departamento de Biologia, Universidade Federal do Ceará, Brazil.

Extraction of the essential oils: The fresh leaves (930 g), stalks (720 g) and inflorescences (100 g) of *C. zehntneri* were subjected to hydrodistillation in a Clevenger-type apparatus for 2 h to afford 1.04%, 0.46% and 0.30% of pale yellow oils, respectively. The yields (w/w) were calculated based on the fresh weight of the plant materials. The isolated oils, after drying over anhydrous sodium sulfate and filtration, were stored in sealed glass vials and maintained under refrigeration before analysis.

Gas chromatography: GC-FI for the quantitative analysis was carried out on a Shimadzu GC-17A gas chromatograph using a dimethylpolysiloxane DB-5 fused silica capillary column (30 m x 0.25 mm, film thickness 0.25 μm). H_2 was used as the carrier gas at a flow rate of 1 mL/ min and 30 psi inlet pressure; split, 1:30; temperature program: 35-180°C at 4°C/ min, then heated at a rate of 17°C/ min to 280°C and held isothermal for 10 min; injector temperature, 250°C; detector used FID, detector temperature, 250°C.

Gas chromatography-mass spectrometry: GC-MS for the analysis of the volatile constituents was carried out on a Hewlett-Packard Model 5971 GC/MS using a non-polar DB-5 fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness); carrier gas helium, flow rate 1 mL/min and with split mode. The injector and detector

temperatures were 250ºC and 200ºC, respectively. The column temperature was programmed from 35°C to 180ºC at 4ºC/min and then 180°C to 250ºC at 10ºC/min. Mass spectra were recorded from 30 – 450 *m/z*. Individual components were identified by matching their 70 eV mass spectra with those of the spectrometer data base using the Wiley L-built library and two other computer libraries using retention indices as a preselection routine [17], as well as by visual comparison of the fragmentation pattern with those reported in the literature [18].

Larvicidal bioassay: Aliquots of the essential oils tested (12.5 to 500 μg/mL) were placed in a beaker (50 mL) and dissolved in DMSO/H_2O 1.5% (20 mL). Instar III larvae of *Aedes aegypti* (50) were delivered to each beaker. After 24 h, at room temperature, the number of dead larvae was counted and the lethal percentage calculated. A control using DMSO/H_2O 1.5% was carried out in parallel. For each sample, three independent experiments were run [19].

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Composition and Larvicidal Activity of Essential Oil from *Stemodia maritima* L.

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The leaves and stems of *Stemodia maritima*, collected in the state of Ceara, Brazil, were subjected to hydrodistillation and their essential oils were analyzed by combined GC and GC/MS. The major components found in the leaf oil were β-caryophyllene and 14-hydroxy-9-*epi*-β-caryophyllene, while in the stem oil β-caryophyllene and caryophyllene oxide were the most abundant constituents. Furthermore, the oils were examined with respect to their larvicidal properties against the larvae of *Aedes aegypti* and showed LC_{50} values of 55.4 ± 1.03 and 22.9 ± 0.85 ppm for the leaves and stems, respectively.

Keywords: *Stemodia maritima* Linn., Scrophulariaceae, essential oil, *Aedes aegypti*, larvicidal activity.

Stemodia maritima Linn. (Scrophulariaceae) is a very common shrub that grows wild in northeastern Brazil near the sea, where it is known as "melosa". It is used to treat stomach ache, dropsy, and swelling by the local population, although some toxic effects in cattle have been reported [1]. Diterpenes possessing antiviral and cytotoxic properties have been isolated from *S. maritima* [2], but there is no previous report on its essential oil.

Aedes aegypti is responsible for the transmission of yellow fever in Central and South America and in west Africa, and it is also a vector of dengue hemorrhagic fever, which is endemic to South East Asia, the Pacific Islands area, Africa and the Americas [3]. As the control of the mosquito population in the larval stage is much easier than in the adult stage, new strategies are needed for controlling the proliferation of the larvae of *A. aegypti*. Several studies have focused on natural products as insecticides for controlling *A. aegypti* larvae. Compounds and essential oils from herbal plants have demonstrated larvicidal activity [4-8], which motivated our group to search for new insecticides from Brazilian plants. The results of the analysis of the volatile components from leaves and stems of *S. maritima* are listed in Table 1, in order of elution from the DB-5 column. *S. maritima* gave sesquiterpenic oils, devoid of monoterpenes and there are similarities and dissimilarities between these oils. The major component detected for both oils was β-caryophyllene, being 31.5% for leaves and 42.0% for the stems. However, the content of caryophyllene oxide was higher in the stems (37.7%) than in the leaves, which showed only 7.4% of this compound. The percentages of oxygenated sesquiterpenes were approximately the same in the leaves (48.7%) and in the stems (48.2%); the

Table 1: Chemical composition (%) of the essential oil from leaves and stems of *Stemodia maritima*.

Constituents[a]	RI[b]	Leaf oil	Stem Oil
β-Caryophyllene	1419	31.5	42.0
α-Humulene	1455	2.1	3.1
(*Z*)-Nerolidol	1533	4.0	-
(*E*)-Nerolidol	1563	2.0	2.7
Caryophyllene oxide	1583	7.4	37.7
cis-Isolongifolanone	1613	4.7	-
Caryophylla-4(14),8(15)-dien-5α-ol	1641	8.6	7.4
14-Hydroxy-9-*epi*-β-caryophyllene	1670	14.4	-
(*Z*)-Santalol	1633	3.4	-
(*E*)-Santalol acetate	1869	4.2	0.5
Total		**82.3**	**93.4**

[a]Constituents listed in order of elution from DB-5 column. [b]Retention indices.

sesquiterpenoids, caryophylla-4(14),8(15)-dien-α-ol and caryophylla-4(14),8(15)-dien-α-ol were found in significant amounts in the leaf and stem oils (8.6 and 7.4%, respectively). 14-Hydroxy-9-*epi*-β-caryophyllene (14.4%) was detected only in the leaf oil.

The essential oils from the leaves and the stems were examined with respect to their larvicidal properties against the larvae of the mosquito, *Aedes aegypti*, and gave LC_{50} values of 55.4 ± 1.03 and 22.9 ± 0.85 ppm, respectively**.** The larvicidal proprieties of terpenes, such as β-caryophyllene, have been reported previously [9]. In an effort to evaluate the contribution of the caryophyllene oxide, the pure sesquiterpene was tested under identical conditions to the oil and gave an LC_{50} value of 50.4 ± 1.20. This suggested that the greater larvicidal activity found for the *S. maritima* stem essential oil could be attributed to the larger content of this compound in its composition.

Experimental

Stemodia maritima L. was collected in January 2006, during its flowering stage, in Freixeiras-Ceara State (northeast Brazil). A voucher specimen, #38483, has been deposited at the Herbarium Prisco Bezerra (EAC) of the Universidade Federal do Ceará, Brazil.

Fresh leaves were subjected to hydrodistillation in a Clevenger-type apparatus for 4 h, to afford 0.02 % of a pale yellow oil, which was dried over sodium sulfate and stored in a sealed glass vial at low temperature before analysis. The same procedure was applied to the fresh stems to yield 0.08% of a pale yellow oil. The yields (w/w) were calculated based on the fresh weight of the plant materials. The essential oils were analyzed using GC-FID and GC-MS. GC-FID analysis was performed on a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector using a non-polar DB-5 fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness). Hydrogen was used as carrier gas at a flow rate of 1 mL/ min^{-1} and 30 psi inlet pressure; split ratio 1:30. The column temperature was programmed from 35ºC to 180ºC at a rate of 4ºC min^{-1}, then heated at a rate of 17ºC min^{-1} to 280ºC and held isothermal for 10 min; both injector temperature and detector temperature were 250ºC.

The GC-MS analysis was carried out on a Hewlett-Packard Model 5971 GC/MS using a non-polar DB-5 fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness); carrier gas helium, flow rate 1 mL min^{-1} and with split mode. The injector temperature and detector temperature were 250ºC and 200ºC, respectively. The column temperature was programmed from 35ºC to 180ºC at 4ºC min^{-1} and then 180ºC to 250ºC at 10ºC min^{-1}. MS were recorded from 30 – 450 *m/z*. Individual mass spectra were compared with those of the MS data base of the Wiley L-built library and two other MS computer library searches using retention indices as a pre-selection routine [10,11], as well as by visual comparison of the fragmentation pattern with those reported in the literature [12,13]. The chemical components identified in the essential oil of *S. maritima* are presented in Table 1.

The larvicidal activity assays were developed using known methodology [14]. Aliquots of oil were placed in beakers (50 mL) and dissolved in H_2O/DMSO 1.5% (v/v) at concentrations of 1-500 ppm, followed by the addition of 50 larvae at the third stage. Temephos®, a synthetic larvicide, (3.22 ppm) and distilled water containing 1.5% DMSO served as positive and negative control, respectively. Mortality was recorded after 24 h of exposure, and no nutritional supplement was added. The experiment was carried out at 28±2°C and performed in triplicate. Data were evaluated through regression analysis. From regression line, the LC_{50} values were read representing the lethal concentration for 50% larval mortality of *A. aegypti*. The bioassays were performed at the Laboratorio de Entomologia, Núcleo de Endemias, Secretaria de Saúde do Estado do Ceará, Brazil.

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Cytotoxic Leaf Essential Oils from Neotropical Lauraceae: Synergistic Effects of Essential Oil Components

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The leaf essential oils of *Beilschmiedia* sp. nov. "chancho blanco", *Cinnamomum costaricanum*, *Ocotea meziana*, *Ocotea* sp. nov. "los llanos" and *Ocotea* sp. nov. "small leaf" showed notable *in-vitro* cytotoxic activity on MCF-7 cells. In order to examine possible synergistic effects of essential oil components, cytotoxic activities of 1:1 binary mixtures of a number of volatile compounds were determined. Notable synergistic cytotoxic enhancement was observed for mixtures of various compounds with citral, citronellal, and artemisia ketone. The cytotoxic activity of α-humulene, on the other hand, was antagonized by pinenes, thujene, and camphene. Likewise, camphene and terpinen-4-ol reduced the activity of β-caryophyllene.

Keywords: cytotoxicity, MCF-7, synergism, Monteverde, Costa Rica, *Beilschmiedia*, *Cinnamomum*, *Ocotea*.

Synergism, in contrast to simple dose addition or additive responses, represents the interaction or dynamic interplay of two or more components to produce an enhancement (potentiation) or inhibition (antagonism) [1]. Synergistic activity has been observed with the components of essential oils [2a]. Thus, for example, thymol and carvacrol, in combination with other essential oil components, exhibited enhanced antibacterial activity [2b]. Conversely, γ-terpinene and *p*-cymene have been found to reduce the antibacterial activity of terpinen-4-ol [2c]. Synergistic effects of essential oil components have also been observed for insecticidal and insect antifeedant activity [3a,3b], and enzyme inhibitory activity [4]. In this work, we present the cytotoxic activities of leaf essential oils from members of the Lauraceae from Monteverde, Costa Rica, as well as activities of essential oil components, both individually and in combination.

The leaf essential oils of *Beilschmiedia* sp. nov. near *brenesii* ("chancho blanco") [5a], *Cinnamomum costaricanum* [4b], *Ocotea meziana*, *Ocotea* sp. nov. "los llanos", and *Ocotea* sp. nov. "small leaf" [5b], exhibited *in-vitro* cytotoxic activity on MCF-7 human mammary adenocarcinoma cells (100% killing at 100 μg/mL). *Beilschmiedia* "chancho blanco" essential oil was dominated by the sesquiterpene hydrocarbons β-caryophyllene (16.6%), bicyclogermacrene (14.1%), germacrene D (6.6%), δ-cadinene (6.1%), and α-humulene (5.6%), in addition to large concentrations of the monoterpene hydrocarbons α-pinene (12.1%), *cis*- and *trans*-β-ocimene (5.1% and 4.1%, respectively) [5a]. The leaf oil of *C. costaricanum* was composed largely of the sesquiterpenoids α-selinene (18.4%), β-selinene (14.7%), kongol (13.1%), and β-elemene (8.3%), as well as the monoterpene α-pinene (8.7%) [4b].

Both *Ocotea meziana* and *Ocotea* "small leaf" leaf essential oils were rich in germacrene D (50.6% and 60.4%, respectively), while *O. meziana* also had large amounts of β-caryophyllene (13.2%) and δ-cadinene (8.0%) [5b]. The leaf oil of *Ocotea* "los llanos", on the other hand, was dominated by the monoterpene hydrocarbons α- and β-pinene (27.5% and 17.2%, respectively), and *trans*-β-ocimene (24.1%) [5b].

Table 1: *In-vitro* cytotoxic activities on MCF-7 cells for essential oil components (% kill at the concentrations given, standard deviations in parentheses).

Compound	100µg/mL	50 µg/mL
Artemisia ketone	0	0
Borneol	3.6 (5.1)	0
Bornyl acetate	19.3 (5.8)	0
Camphene	41.8 (7.3)	0
Camphor	13.4 (13.0)	0
β-Caryophyllene	100	28.5 (12.0)
Caryophyllene oxide	79.0 (3.9)	0
1,8-Cineole	36.0 (6.8)	0
Citral	92.3 (7.7)	29.6 (8.9)
Citronellal	35.4 (6.8)	0
Citronellol	31.2 (5.1)	0
α-Copaene	100	7.5 (7.1)
Eugenol	33.3 (5.5)	12.6 (6.0)
Fenchone	0	0
Geraniol	22.1 (1.9)	9.2 (1.8)
Hexanal	14.2 (1.5)	0
α-Humulene	100	86.5 (12.4)
Limonene	0	0
Linalool	0	0
Myrtenal	19.5 (17.0)	0
trans-Pinocarveol	22.3 (3.9)	0
α-Pinene	35.8 (5.9)	22.4 (15.5)
β-Pinene	98.8 (1.2)	0
Terpinen-4-ol	32.3 (8.2)	0
α-Terpineol	19.9 (4.3)	0
α-Thujene	14.7 (7.3)	0
α/β-Thujone	20.3 (11.3)	0
1,3,5-Trimethoxybenzene	32.8 (14.3)	0

Cytotoxic activity against the MCF-7 cell line has been observed for α-pinene, β-pinene, β-caryophyllene, α-humulene, and germacrene D [5c]. δ-Cadinene [5d] and β-elemene [5e,5f] have shown cytotoxic activity on a number of tumor cell lines. While the high concentrations of these cytotoxic components may explain, in part, the observed cytotoxicities of the Lauraceous essential oils, synergistic effects are also likely to enhance the cytotoxicities. The cytotoxic activities of a number of essential oil components, as well as 1:1 binary mixtures, have been determined and are summarized in Tables 1 and 2, respectively. At 100 µg/mL, β-caryophyllene, citral, α-copaene, α-humulene, and β-pinene showed greater than 80% kill ratios against MCF-7 cancer cells. The percentage kill ratios are much lower for 50 µg/mL. Thus, for example at 100 µg/mL, β-pinene killed 99% of the cells and at 50 µg/mL, killed none. Similarly, β-caryophyllene killed 100% of the cells at 100 µg/mL, but only killed 29% of the cells at 50 µg/mL.

To test the hypothesis that synergistic effects may be occurring with the components of essential oils, 1:1 binary mixtures of a number of components found in essential oils have been prepared and tested for cytotoxic activity (Table 2). In most cases, there is an enhancement of activity. That is, the cytotoxic activity of the mixture is greater that what should be expected if the activities of the two materials are additive. For example, it was found that β-caryophyllene, when mixed in equal quantities with either citronellal or hexanal showed pronounced synergistic enhancement. Similarly, artemisia ketone, in combination with bornyl acetate, caryophyllene oxide, fenchone, and thujone, showed notable enhancement. Conversely, the cytotoxic activity of α-humulene was antagonized upon mixture with monoterpene hydrocarbons such as pinenes, thujene, and camphene. Likewise, camphene and terpinen-4-ol reduced the activity of β-caryophyllene.

Beilschmiedia "chancho blanco" essential oil contained 12% α-pinene and 17% β-caryophyllene [5a]. This study, however, has revealed that α-pinene and β-caryophyllene are antagonistic, so these compounds together cannot account for the cytotoxic activity of *Beilschmiedia* "chancho blanco". Likewise, *Ocotea* "los llanos" was rich in pinenes (28% and 17% α- and β-pinene, respectively) [5b], but the relatively weak synergistic effects of these two compounds cannot account for the cytotoxicity of *Ocotea* "los llanos" leaf oil.

Essential oils are generally complex mixtures of compounds, and potential synergistic and antagonistic effects should be taken into account when evaluating the biological activities of essential oils. Although this present study begins to reveal potential synergistic effects of essential oil components, much additional research is needed to look at ternary and higher order mixtures of these compounds.

Experimental

Plant material: Plants were collected, identified, and the leaf essential oils obtained as previously described [4b,5a,5b].

Cell culture: MCF-7 cells (American Type Culture Collection (ATCC) # HTB-22; Manassas, VA) are a cancer cell line derived as a pleural effusion from human mammary gland adenocarcinoma from a Caucasian female. The MCF-7 cells are estrogen receptor positive (ER+) and Fibroblast Growth Factor 1Receptor positive (FGFR+). The MCF-7 cells were grown in 25 cm^2 tissue culture flasks (Corning; Corning, NY) with feeding media consisting of Institute in Buffalo, New York and purchased from Mediatech Cellgro; Herndon, VA), containing phenol, supplemented with 10% fetal bovine serum

Table 2: *In-vitro* cytotoxic activities of 1:1 binary mixtures of various essential oil components on MCF-7 cells (% kill at 50 μg/mL of each component; diagonal elements, shaded, are 50 μg/mL of single component. Notable cytotoxicities (> 80% kill) are shown in **bold**.

	Artemisia ketone	Borneol	Bornyl acetate	Camphene	Camphor	β-Caryophyllene	Caryophyllene oxide	1,8-Cineole	Citral	Citronellal
Artemisia ketone	0	13	**99**	3	14	13	**90**	29	**89**	36
Borneol	13	0	18	8	0	32	21	23	**85**	8
Bornyl acetate	**99**	18	0	12	0	21	4	33	**81**	77
Camphene	3	8	12	0	0	0	28	0	25	0
Camphor	14	0	0	0	0	48	70	14	76	36
β-Caryophyllene	13	32	21	0	48	29	54	13	60	**100**
Caryophyllene oxide	**90**	21	4	28	70	54	0	0	45	76
1,8-Cineole	29	23	33	0	14	13	0	0	72	0
Citral	**89**	**85**	**81**	25	76	60	45	72	30	**85**
Citronellal	36	8	77	0	36	**100**	76	0	**85**	0
Citronellol	11	8	0	23	28	72	20	11	59	32
α-Copaene	0	6	10	8	47	71	28	14	57	21
Eugenol	20	14	18	12	27	35	16	31	**90**	34
Fenchone	**84**	25	16	22	6	53	45	38	**84**	0
Geraniol	27	18	26	35	30	37	34	18	52	39
Hexanal	**80**	0	28	27	21	**100**	**89**	46	**86**	43
α-Humulene	**100**	62	69	36	**100**	65	58	27	**87**	**100**
Limonene	6	9	0	10	14	52	9	7	**87**	24
Linalool	8	4	10	18	0	63	19	8	**88**	22
Myrtenal	20	38	47	19	21	20	10	18	78	28
trans-Pinocarveol	10	19	0	13	12	21	21	12	20	0
α-Pinene	39	0	6	6	19	2	26	22	49	79
β-Pinene	6	7	0	14	18	41	30	0	13	**93**
Terpinen-4-ol	6	10	7	15	0	0	5	17	35	0
α-Terpineol	0	0	3	11	15	9	24	0	28	0
α-Thujene	0	0	17	0	4	21	8	21	36	0
α/β-Thujone	**81**	5	20	12	40	69	**92**	57	**97**	0
1,3,5-Trimethoxybenzene	62	9	21	6	0	15	0	21	49	0

Table 2: cont.

	Citronellol	α-Copaene	Eugenol	Fenchone	Geraniol	Hexanal	α-Humulene	Limonene	Linalool
Artemisia ketone	11	0	20	**84**	27	**80**	**100**	6	8
Borneol	8	6	14	25	18	0	62	9	4
Bornyl acetate	0	10	18	16	26	28	69	0	10
Camphene	23	8	12	22	35	27	36	10	18
Camphor	28	47	27	6	30	21	**100**	14	0
β-Caryophyllene	72	71	35	53	37	**100**	65	52	63
Caryophyllene oxide	20	28	16	45	34	**89**	58	9	19
1,8-Cineole	11	14	31	38	18	46	27	7	8
Citral	59	57	**90**	**84**	52	**86**	**87**	**87**	**88**
Citronellal	32	21	34	0	39	43	**100**	24	22
Citronellol	0	38	22	28	52	30	**95**	15	12
α-Copaene	38	7	3	11	30	26	77	17	0
Eugenol	22	3	13	22	27	38	56	21	34
Fenchone	28	11	22	0	40	16	**100**	2	3
Geraniol	52	30	27	40	9	26	39	7	0
Hexanal	30	26	38	16	26	0	**100**	6	16
α-Humulene	**95**	77	56	**100**	39	**100**	**87**	69	30
Limonene	15	17	21	2	7	6	69	0	22
Linalool	12	0	34	3	0	16	30	22	0
Myrtenal	17	18	22	16	14	19	23	33	33
trans-Pinocarveol	23	48	22	0	21	22	27	3	23
α-Pinene	6	23	15	54	37	54	9	0	6
β-Pinene	12	24	2	42	14	**86**	2	0	5
Terpinen-4-ol	21	17	29	0	22	0	10	7	21
α-Terpineol	23	13	12	0	12	0	53	18	18
α-Thujene	18	0	11	0	0	9	21	0	9
α/β-Thujone	31	8	13	0	36	26	**95**	11	0
1,3,5 -Trimethoxybenzene	54	11	21	0	48	16	**100**	11	8

(Atlanta Biologicals; Lawerenceville, GA), 30 mM HEPES, 100 U/mL penicillin with 0.1 mg/mL streptomycin (Sigma; St. Louis, MO) at 37°C in a 5% CO_2 incubator. Media was replaced every 2 days to ensure optimum growth conditions.

Cytotoxicity screening*: In-vitro* cytotoxic activity of the essential oils, pure compounds, and binary mixtures on MCF-7 cells was carried out using the MTT assay as previously described [5g]. Cytotoxicities were determined at 100 μg/mL for the essential oils, 100 and 50 μg/mL for essential oil components and 50 + 50 μg/mL for binary mixtures of compounds.

Table 2: cont.

	Myrtenal	*trans*-Pinocarveol	α-Pinene	β-Pinene	Terpinen-4-ol	α-Terpineol	α-Thujene	α/β-Thujone	1,3,5 - Trimethoxybenzene
Artemisia ketone	20	10	39	6	6	0	0	**81**	62
Borneol	38	19	0	7	10	0	0	5	9
Bornyl acetate	47	0	6	0	7	3	17	20	21
Camphene	19	13	6	14	15	11	0	12	6
Camphor	21	12	19	18	0	15	4	40	0
β-Caryophyllene	20	21	2	41	0	9	21	69	15
Caryophyllene oxide	10	21	26	30	5	24	8	**92**	49
1,8-Cineole	18	12	22	0	17	0	21	57	21
Citral	78	20	49	13	35	28	36	**97**	49
Citronellal	28	0	79	**93**	0	0	0	0	0
Citronellol	17	23	6	12	21	23	18	31	54
α-Copaene	18	48	23	24	17	13	0	8	11
Eugenol	22	22	15	2	29	12	11	13	21
Fenchone	16	0	54	42	0	0	0	0	0
Geraniol	14	21	37	14	22	12	0	36	48
Hexanal	19	22	54	**86**	0	0	9	26	16
α-Humulene	23	27	9	2	10	53	21	**95**	**100**
Limonene	33	3	0	0	7	18	0	11	11
Linalool	33	23	6	5	21	18	9	0	8
Myrtenal	0	0	13	0	10	19	2	21	20
trans-Pinocarveol	0	0	0	4	13	0	10	2	17
α-Pinene	0	0	22	8	22	0	7	50	35
β-Pinene	4	4	8	0	0	0	3	21	0
Terpinen-4-ol	10	13	22	0	0	8	15	0	0
α-Terpineol	19	0	0	0	8	0	0	0	0
α-Thujene	2	10	7	3	15	0	0	0	18
α/β-Thujone	21	2	50	21	0	0	0	0	0
1,3,5 - Trimethoxybenzene	20	17	35	0	0	0	18	0	0

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Chemical Composition and Antibacterial Activity of the Essential Oil of *Baccharis latifolia* Pers. and *B. prunifolia* H. B. &K. (Asteraceae)

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The essential oils from leaves of *Baccharis latifolia* and *B. prunifolia* collected in January 2006 were analyzed by GC/MS. The yields of oils extracted by hydrodistillation were 0.27 and 0.29% for *B. latifolia* and *B. prunifolia*, respectively. Sixteen (*B. latifolia*) and twenty nine (*B. prunifolia*) components were identified by comparison of their mass spectra with the Wiley GC-MS Library data and by their retention indices (RI). The identified products may be divided into four different groups: monoterpenes (9.0% *B. latifolia*; 43.9% *B. prunifolia*), oxygenated monoterpenes (0.8% *B. latifolia*; 5.4% *B. prunifolia*), sesquiterpenes (20.4% *B. latifolia*; 45.9% *B. prunifolia*) and oxygenated sesquiterpenes (69.8% *B. latifolia*; 1.9% *B. prunifolia*). The oils showed antibacterial activity only against Gram positive bacteria, with MIC values for *Staphylococcus aureus* (ATCC 25923) of 80 μg/mL (*B. latifolia*) and *Enterococcus faecalis* (ATCC 29212) of 90 μg/mL and 260 μg/mL (*B latifolia* and *B. prunifolia*, respectively).

Keywords: *Baccharis latifolia*, *B. prunifolia*, Asteraceae, essential oil, antibacterial activity.

The family Asteraceae is comprised of about 1,500 genera and 25,000 species, distributed worldwide. In Venezuela, around 210 genera and 760 species are known [1a]. From the Andes area of the country, 15 species of *Baccharis* have been reported [1b]. *B. latifolia* Pers. is located in Mérida State in San Rafael de Mucuchies, between Santo Domingo and Chachopo paramo, La Mucuy, Timotes, Apartaderos paramo and on the way to Torondoy. *B. prunifolia* H. B. & K. is located in Laguna Negra, La Mucuy, Chachopito (near San Rafael), Piedras Blancas paramo, Santo Domingo paramo, Mucubají lake, El Molino paramo, El Águila paramo, Sai-Sai mini waterfalls and La Sal paramo [1b].

Species of this genus have been used in traditional medicine as a febrifuge, for their antirheumatic, antispasmodic, diuretic, antifungal, antiviral, antileukemic, analgesic, antioxidant and anti-inflammatory properties, and to treat hepatobiliary disorders, diabetes and skin ulcerations [2]. Previous investigations of the essential oil of different species of *Baccharis* have reported a variety of compounds, such as sabinene, limonene, α-pinene, β-pinene, (*E*)-nerolidol, α-muurolol, isocaryophyllene, β-caryophyllene, caryophyllene oxide, β-selinene, terpinen-4-ol, α-tuyene, spathulenol, cubenol, germacrene-D and carvacrol [3]. In the present study, the compositions of the essential oils of *B. latifolia* and *B. prunifolia* collected from La Culata, Mérida State are reported, as well as their antibacterial activity.

Leaves of *B. latifolia* and *B. prunifolia* collected from the same location in January 2006 yielded 0.27% and 0.29% essential oil, respectively. GC/MS analyses

performed on the two oils showed the presence of 16 and 29 components, respectively. A list of identified components, along with their percentages of the total oil, is given in Table 1. The identified products may be divided into four different groups: monoterpenes (9.0% *B. latifolia*; 43.9% *B. prunifolia*), oxygenated monoterpenes (0.8% *B. latifolia*; 5.4% *B. prunifolia*), sesquiterpenes (20.4% *B. latifolia*; 45.9% *B. prunifolia*) and oxygenated sesquiterpenes (69.8% *B. latifolia*; 1.9% *B. prunifolia*). Three compounds in the essential oil of *B. latifolia* could not be identified. One peak gave a mass spectrum [*m/z* (rel. int.): M^+ 216 (75), 201 (100), 185 (30)] that is very similar to that of andro encecalinol [*m/z* (rel. int.): M^+ 216 (35), 201 (100), 185 (32)] [4a]. The MS produced by another peak [*m/z* (rel. int.): M^+ 218 (100), 203 (78), 161 (80), 133 (60)] is similar to that of aristolone [*m/z* (rel. int.): M^+ 218 (50), 161 (45), 203 (100), 133 (48)] [4a]. An important component was observed with the mass spectral features, *m/z* (rel. int.): M^+ 232 (100), 161 (100), 147 (40), but, unfortunately, we were unable to find a mass spectrum corresponding to this compound during analysis on both the polar (HP-5MS) and nonpolar (AT-WAX) columns, and it was not comparable with any of the compounds listed in either the library data base or the literature consulted [4a,4b]. Unfortunately, the lack of pure reference samples made it difficult to have complete identification of these compounds.

According to the references consulted, there have been no studies on the composition of the essential oil of *B. prunifolia*. However, a previous investigation of the essential oil of *B. latifolia* collected from Cochabamba, Bolivia, reported germacrone (41.3%), limonene (23.6%), α-tuyene (10.9%), α-pinene (6.3%), γ-elemene (4.3%) and verboccidentafurane (5.6%) as the major components [4c]. There are a number of differences between the compositions of the essential oils of *B. latifolia* and *B. prunifolia* collected from the same location in Venezuela and that of *B. latifolia* collected in Bolivia.

In the present investigation, antibacterial activity was observed only against Gram positive bacteria with MIC values for *Staphylococcus aureus* (ATCC 25923) of 80 μg/mL (*B. latifolia*) and *Enterococcus faecalis* (ATCC 29212) of 90 μg/mL and 260 μg/mL for *B latifolia* and *B. prunifolia,* respectively (Table 2). Antibacterial activity against *S. aureus* (ATCC 25923) has been reported previously for *Baccharis nitida* using different extracts (ethanol, acetone and water) of the aerial parts of the plant [5], while

Table 1: Composition of the essential oil of *B. prunifolia* and *B. latifolia*.

Components	KI	*B. latifolia* (%)	*B. prunifolia* (%)
α-Thujene	923	-	0.4
α-Pinene	930	0.6	2.3
Sabinene	964	0.3	1.3
β-Pinene	968	0.3	2.3
Myrcene	981	0.2	19.2
α-Phellandrene	994	-	1.2
δ-3-Carene	1000	-	4.9
α-Terpinene	1006	-	0.6
Limonene	1019	7.6	5.4
trans-β-Ocimene	1039	-	5.2
γ-Terpinene	1052	-	1.1
Linalool	1099	-	2.3
trans-Verbenol	1138	0.1	-
4-Terpineol	1178	-	1.8
1-α-Terpineol	1191	-	0.9
Myrtenol	1197	0.7	-
Geraniol	1259	-	0.4
α-Copaene	1378	-	0.3
α-Gurjunene	1411	-	5.4
β-Caryophyllene	1422	-	25.3
α-Humelene	1456	-	1.8
trans-β-Farnesene	1458	2.2	-
γ-Gurjunene	1475	-	3.9
γ-Curcumene	1483	12.2	-
Germacrene-D	1484	-	1.8
β-Selinene	1489	-	1.9
Bicyclogermacrene	1500	1.5	1.8
α-Muurolene	1503	-	0.6
β-Bisabolene	1512	2.8	-
δ-Cadinene	1527	1.7	2.7
trans-Nerolidol	1566	-	0.3
Caryophyllene oxide	1586	-	0.6
1,10-di-epi-Cubenol	1619	7.9	-
t-Cadinol	1629	-	0.7
m/z : 216 (75), 201 (100), 185 (30)	1638	30.5	-
m/z: 218 (100), 203 (78), 161 (80)	1652	4.5	-
Cubenol	1654	-	0.3
m/z : 232 (100), 161 (100), 147 (40)	1678	26.9	-

*The composition of the essential oil was determined by comparison of the mass spectrum of each component with Wiley GC/MS library data and also from its retention index (RI).

B. grisebachii from Argentina was active against both methicillin sensitive and resistant strains of *S. aureus* with a MIC of 125 μg/mL [6a,6b]. *S. aureus* and *E. faecalis* are well known for causing several human infections [6c] and for showing resistance to antibacterial treatment using commercial patented medicines [6d,6e]. Antimicrobial activity of essential oils is difficult to correlate to a specific compound due to their complexity and variability. It has been mainly explained through C10 and C15 terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active site of target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters can contribute to the overall antimicrobial effect of essential oils [7a]. However, *β*-caryophyllene, *γ*-curcumene, *trans*-*β*-ocimene and limonene, observed at important concentrations in the essential oil of the species analyzed in the present investigation, are well known to possess antibacterial activity [7b,7c]. Previous investigations have reported activity of these

compounds against *Escherichia coli*, *Staphylococcus aureus, Pseudomonas aeruginosa*, *Enterococcus hirae*, *Salmonella typhi*, *Bacillus subtilis*, *Acinetobacter calcoaceticus*, *Clostridium sporogenes*, and *Yersinia enterocolitica* [7d,7e]. Thus, the antibacterial results observed in this investigation might be related to the presence of these compounds.

Table 2*:* Antibacterial activity of the essential oil of *B. latifolia* and *B. prunifolia*.

Essential oil	Microorganisms				
	S. aureus *ATCC (25923)*	*E. faecalis* ATCC (29212)	*E. coli* ATCC (25992)	*K. pneumoniae* ATCC (23357)	*P. aeruginosa* ATCC (27853)
B. latifolia	8*	9*	NA	NA	NA
MIC (µg/mL): [a]	80	90	NT	NT	NT
B. prunifolia	NA	9*	NA	NA	NA
MIC (µg/mL): [b]	NT	260	NT	NT	NT
Antibiotics:					
Sulbactam - Ampicilli	29*	NT	NT	NT	NT
Vancomycin®	NT	19*	NT	NT	NT
Streptomycin®	NT	NT	15*	NT	NT
Aztreonam®	NT	NT	NT	27*	NT
Cefoperazone®	NT	NT	NT	NT	25*

Sulbactam -Ampicillin® (10µg/10 µg), Vancomycin® (30 µg), Streptomycin® (10 µg), Aztreonam® (30µg), Cefoperazone® (75 µg), NA: not active, NT: not tested.*inhibition zone, diameter measured in mm, disc diameter 6 mm, average of two consecutive assays. MIC: Minimal inhibitory concentration, concentration range: [a] 10-160 µg/mL, [b] 10-340 µg/mL.

Experimental

Plant material and isolation of essential oils: Aerial parts of *B. latifolia* and *B. prunifolia* were collected from La Culata, Mérida State, Venezuela at 2900 m above sea level. Voucher specimens LBR 034 (*B. latifolia*) and LBR 035 (*B. prunifolia*) were deposited in the Faculty of Pharmacy and Bioanalysis MERF herbarium, University of Los Andes, Venezuela. Leaves [*B. latifolia* (800 g) and *B. prunifolia* (850 g)] were cut into small pieces and subjected to hydrodistillation for 3 h, using a Clevenger-type apparatus. The oil was dried over anhydrous sodium sulfate and stored at 4°C.

Gas chromatography: GC analyses were performed on a Perkin-Elmer AutoSystem gas chromatograph equipped with flame ionization detectors. Two capillary columns of different polarities were used: a 5% phenylmethyl polysiloxane fused-silica column (HP-5MS, Hewlett Packard, USA) 60 m x 0.25 mm, film thickness 0.25 µm, and a polyethylene glycol fused-silica column (AT-WAX, Alltech Associates Inc., Deerfield, IL) of the same dimensions. The initial oven temperature was 60°C; it was then heated to 260°C at 4°C/min, and the final temperature was maintained for 20 min. The injector and detector temperatures were 200°C and 250°C, respectively. The carrier gas was helium at 1.0 mL/min. The sample was injected using a split ratio of 1:100. Retention indices were calculated relative to C_8-C_{24} n-alkanes, and compared with values reported in the literature [4a,4b].

Gas chromatography-mass spectrometry: The GC-MS analyses were carried out on a Hewlett Packard GC-MS system, Model 5973, fitted with a 30 m long, cross-linked 5% phenylmethyl siloxane (HP-5MS, Hewlett Packard, USA) fused-silica column (0.25 mm, film thickness 0.25 µm). The source temperature was 230°C, the quadrupole temperature 150°C, the carrier gas helium, adjusted to a linear velocity of 34 m/s, the ionization energy 70 eV, and the scan range 40-500 amu at 3.9 scans/s. The injected volume was 1.0 µL of a 2% dilution of oil in n-heptane. A Hewlett-Packard ALS injector was used with split ratio 1:100. The identification of the oil components was based on a Wiley MS Data Library (6^{th} edn), followed by comparisons of MS data with published literature [4a].

Microbiological analysis

Bacterial strains and Antimicrobial method: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25992), *Pseudomonas aeruginosa* (ATCC 27853) and Klebsiella pneumoniae (ATCC 23357) were used in this study. The antimicrobial activity test was carried out according to the disc diffusion assay described by Rondón et al. [7f]. The strains were maintained in agar conservation at room temperature. Every bacterial inoculum (2.5 mL) was incubated in Mueller-Hinton broth at 37ºC for 18 h. The bacterial inoculum was diluted in sterile 0.85% saline to obtain turbidity visually comparable to a McFarland Nº 0.5 standard (10^{6-8} CFU/mL). Every inoculum was spread over plates containing Mueller-Hinton agar and a paper filter disc (6 mm) saturated with 10 µL of essential oil. The plates were left for 30 min at room temperature and then incubated at 37ºC for 24 h. The inhibitory zone around the disc was measured and expressed in mm. A positive control was also assayed to check the sensitivity of the tested organisms using the following antibiotics: Sulbactam-Ampicilin® (10µg/10 µg), Vancomycin® (30 µg), Streptomycin® (10 µg), Cefoperazone® (75 µg) and Aztreonam® (30 µg) (Table 2). The minimal inhibitory

concentration (MIC) was determined only with microorganisms that displayed inhibitory zones. MIC was determined by dilution of the essential oil in dimethylsulfoxide (DMSO) and pipetting 10 µL of each dilution onto a filter paper disc. Dilutions of the oils within a concentration range of 10-160 µg/mL (*B. latifolia*) and 10-340 µg/mL (*B. prunifolia*) were also carried out. MIC was defined as the lowest concentration that inhibited the visible bacterial growth [7g]. A negative control was also included in the test using a filter paper disc saturated with DMSO to check possible activity of this solvent against the bacteria assayed. The experiments were repeated at least twice.

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Biological Activity and Composition of the Essential Oil of *Tetrataenium nephrophyllum* (Apiaceae) from Iran

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The aerial parts of *Tetrataenium nephrophyllum* were collected at the flowering stage, hydrodistilled, and the essential oil was analyzed by GC and GC-MS. Forty components accounting for 97.9% of the total oil were identified. Germacrene D (38.5%), 2-ethylhexyl acetate (11.2%), *n*-octyl 2-methylbutanoate (9.2%) and geranyl isovalerate (8.3%) were the major constituents. Sesquiterpene hydrocarbons (51.3%) and aliphatic esters (40.4%) were found to be the main group of compounds. The antimicrobial activity of the essential oil of *T. nephrophyllum* was determined against seven Gram-positive and Gram-negative bacteria (*Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*), as well as three fungi (*Candida albicans*, *Saccharomyces cerevisiae* and *Aspergillus niger*). The bioassay showed that the oil exhibited moderate to high antimicrobial activity.

Keywords: *Tetrataenium nephrophyllum*, essential oil, germacrene D, antimicrobial activity.

The genus *Tetrataenium* Manden. belongs to the family Apiaceae and consists of two perennial species distributed in Iran of which *T. nephrophyllum* (Leute) Mandenova is endemic to the country [1,2]. In Iran leaves, flower buds and fruits of *Tetrataenium* species are used ethnobotanically as flavoring agents and as a spice for foods.

Volatile constituents and antimicrobial activity of the essential oil of *T. lasiopetalum* have previously been investigated, and germacrene D was found as the main component. The antimicrobial activity of the essential oil was reported as moderate to high [3]. Therefore, in continuation of our research on the composition and biological activities of the essential oils of Iranian aromatic and medicinal plants [4-9], the objectives of this study were aimed to assess the chemical composition and *in vitro* antimicrobial activity of the essential oil of *T. nephrophyllum* from Iran, which has not been previously investigated.

Hydrodistillation of *T. nephrophyllum* yielded a yellow oil in 0.1% (w/w) yield, based on the dry weight of plant. The identified constituents are presented in Table 1, where all compounds are listed in order of their elution from the DB-1 column. Forty components were characterized, representing 97.9% of the total oil. Sesquiterpene hydrocarbons (51.2%) and aliphatic esters (40.5%) were found as the major groups of compounds.

Among the sesquiterpene hydrocarbons, germacrene D (38.5%), β-bourbonene (5.3%) and bicyclogermacrene (4.7%) were the principal compounds. From the aliphatic ester group, 2-ethylhexyl acetate (11.2%), *n*-octyl 2-methyl-butanoate (9.2%) and geranyl isovalerate (8.3%) were the major components. Compounds not in either of these two groups accounted for 6.2% of the total oil.

The essential oil of *T. nephrophyllum* was tested against four Gram-positive and three Gram-negative bacteria, as well as three fungi. The results, presented in Table 2, show that the oil exhibited moderate to high biological activity against all tested fungi and bacteria except for two resistant Gram-negative bacteria, *Pseudomonas aeruginosa* and *Klebsiella*

Table 1: Essential oil composition of *T. nephrophyllum*.

Component	RI (DB1)	%
Nonane	898	0.3
2-Nonene	909	0.2
α-Pinene	934	0.2
β-Pinene	976	0.2
1-Octanol	1053	0.8
Isopentyl 2-methylbutanoate	1084	0.2
2-Methylbutyl 2-methylbutanoate	1088	0.3
n-Hexyl isobutanoate	1130	0.1
2-Ethylhexyl acetate	1196	11.2
Hexyl 2-methylbutanoate	1221	2.1
Nerol	1234	0.1
n-Octyl propionate	1282	0.1
n-Octyl isobutanoate	1328	1.4
Citronellyl acetate	1332	0.3
Bicycloelemene	1338	0.8
Neryl acetate	1358	0.1
Octyl butanoate	1370	0.6
α-Copaene	1380	0.3
β-Bourbonene	1391	5.3
7-Decen-1-yl acetate	1403	2.1
n-Octyl 2-methylbutanoate	1421	9.2
9-Decen-1-yl acetate	1431	2.1
Aromadendrene	1449	0.2
allo-Aromadendrene	1459	0.4
(*E*)-β-Farnesene	1476	0.1
Germacrene D	1494	38.5
Bicyclogermacrene	1505	4.7
Germacrene A	1508	0.1
Geranyl isobutanoate	1514	0.1
β-Sesquiphellandrene	1519	0.8
Citronellyl butanoate	1536	0.4
Geranyl butanoate	1556	0.5
9-Decen-1-yl butanoate	1563	0.4
Spathulenol	1574	0.7
Geranyl isovalerate	1585	8.3
9-Decen-1-yl pentanoate	1629	0.8
Aromadendrene oxide	1676	0.6
Geranyl hexanoate	1726	0.2
Neophytadiene	1828	2.3
(*Z*)-Falcarinol	1994	0.8
Monoterpene hydrocarbons		0.4
Oxygenated monoterpenes		0.1
Sesquiterpene hydrocarbons		51.2
Oxygenated sesquiterpenes		2.1
Aliphatic esters		40.5
Others		3.6
Total identified		**97.9**

RI, retention indices relative to C_6 – C_{24} *n*-alkanes on the DB-1 column.

pneumoniae, as well as a Gram-positive bacterium, *Enterococcus faecalis:* The most sensitive microorganisms were *Bacillus subtilis* and *Escherichia coli*, with inhibition zones of 21 and 18 mm and MIC values of 3.75 and 7.5 mg/mL, respectively. Other microorganisms were found to be less sensitive to the oil with inhibition zones ranged from 8 to 14 mm and MIC values from 5 to 15 mg/mL.

Comparing the composition of *T. nephrophyllum* oil with that of another species, *i.e.*, *T. lasiopetalum* [3] revealed some differences and similarities especially in the first two major components. In the case of other constituents of the essential oils profile either qualitative or quantitative differences were also observed.

With respect to sensitivity screening, the bioactivity of the essential oil of *T. nephrophyllum* was very similar to that of *T. lasiopetalum.* The result was expected owing to the similarity of the compositions of the oils. It is conceivable that the antimicrobial property of the essential oil from *T. nephrophyllum* might be ascribed to its high content of alkyl esters and sesquiterpenoids, which constitute the major percentage of the total oil and which have been shown previously to be antimicrobial [10,11].

Experimental

Plant material: The aerial parts of *T. nephrophyllum* were collected on July 2004, at the flowering stage, from West Azarbaijan province, Takab, Belgheis Mountain, Iran at an altitude of 2500 m. A voucher specimen (MP-908) has been deposited at the Medicinal Plants and Drugs Research Institute Herbarium of Shahid Beheshti University, Tehran, Iran.

Isolation of the essential oil: The air-dried and ground aerial parts of the plant (100 g) were subjected for 4 h to hydrodistillation using a Clevenger-type apparatus. The obtained oil was dried over anhydrous sodium sulfate and stored at 4°C until tested and analyzed.

Essential oil analysis: GC analysis was performed by using a Thermoquest gas chromatograph equipped with a flame ionization detector (FID). The analysis was carried out on a fused silica capillary column (DB-1, 60 m × 0.25 mm i.d., film thickness 0.25 μm). The operating conditions were as follows: injector and detector temperatures, 250˚C and 300˚C, respectively; carrier gas, nitrogen at a flow rate of 1 mL/min; oven temperature program, 60˚C – 250˚C at a rate of 5˚C/min, and finally held isothermally for 10 min.

GC-MS analysis was accomplished by using a Thermoquest-Finnigan gas chromatograph coupled with a TRACE mass spectrometer. Helium was used as carrier gas at a flow rate of 1.1 mL/min. Ion source and interface temperatures were kept at 200˚C and 250˚C, respectively. The quadrupole mass spectrometer was scanned from 43-456 mass units with an ionization voltage of 70 eV. Gas chromatographic conditions were the same as those given above for GC.

Oil components identification: Retention indices (RI) for all constituents were calculated according to the Van den Dool approach, using *n*-alkanes (C_6 – C_{24}) as standards and the essential oil on a DB-1 column under the same chromatographic conditions. The identification of each component was made based on comparison of its mass spectrum with those of the internal computer reference mass spectra libraries (Wiley 7 and NIST), as well as by comparison of its retention index with published data [12], and in some cases by co-injection with authentic compounds.

Antimicrobial activity: The *in vitro* antibacterial and antifungal activities of the oil were evaluated by the disc diffusion method using Mueller-Hinton agar for bacteria and Sabouraud Dextrose agar for fungi [13]. Discs containing 15 µL and 30 µL of the oil were used and growth inhibition zones were measured after 24 h and 48 h of incubation at 37˚C and 24˚C for bacteria and fungi, respectively. Gentamicin and tetracycline for bacteria, and nystatin for fungi were used as positive controls.

The microorganisms used were: *Bacillus subtilis* ATCC 9372, *Enterococcus faecalis* ATCC 15753, *Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *Eschericia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27852, *Klebsiella pneumoniae* ATCC 3583, *Candida albicans* ATCC 5027, *Saccharomyces cerevisiae* ATCC 9763 and *Aspergillus niger* ATCC 16404. Minimum inhibitory concentration (MIC) values were measured by the microdilution broth susceptibility assay recommended by NCCLS [14].

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Table 2: Antimicrobial activity of the essential oil of *T. nephrophyllum*.

Microorganism	Essential oil		Antibiotics		
	IZ[a]	MIC[b]	Tetracycline (30 µg/disk)	Gentamicine (10 µg/disk)	Nystatine (30 µg/disk)
Bacillus subtilis	21 ± 0.8	3.75	21 ± 0.4	-	nt
Staphylococcus epidermidis	13 ± 0.4	15	34 ± 0.8	-	nt
Enterococcus faecalis	-	nt	9 ± 0.4	-	nt
Staphylococcus aureus	10 ± 0.4	15	20 ± 0.8	-	nt
Klebsiella pneumoniae	-	nt	-	20 ± 0.8	nt
Pseudomonas aeruginosa	-	nt	-	12 ± 0.4	nt
Escherichia coli	18 ± 0.8	7.5	-	23 ± 0.8	nt
Aspergillus niger	8 ± 0.4	10	nt	nt	16 ± 0.8
Candida albicans	14 ± 0.8	5	nt	nt	18 ± 0.4
Saccharomyces cerevisiae	12 ± 0.4	5	nt	nt	18 ± 0.4

[a]Inhibition zone diameter (mm), including diameter of sterile disk 6 mm; values are given as mean ± SD.
[b]Minimum inhibitory concentration values as mg/mL.
Essential oil tested at 15 µL/disc for bacteria and 30 µL/disc for fungi.
(-), Inactive; (7-14), moderately active; (>14), highly active; nt, not tested.

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Volatile Constituents of *Calamintha origanifolia* Boiss. Growing Wild in Lebanon

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The essential oil of aerial parts of *Calamintha origanifolia* Boiss. (Lamiaceae), growing wild in Lebanon, was obtained by hydrodistillation and was analysed by GC and GC-MS. 49 compounds, representing 92.2% of the oil, were identified. The major components, belonging to the class of oxygenated monoterpenes, were pulegone (22.5%), isomenthone (12.2%) and piperitenone (9.6%). The oil showed a slight antimicrobial activity against three bacterial strains.

Keywords: *Calamintha origanifolia*, essential oil, GC-MS, oxygenated monoterpenes, pulegone, isomenthone, piperitenone.

Calamintha (syn. *Cyclotrichium*) is a genus of about thirty species that belongs to the tribe Mentheae, subfamily Nepetoideae, family Lamiaceae. It is native to the northern temperate regions of Europe and Asia. According to Marin *et al.* [1], the genus *Calamintha* Miller is closely related to *Micromeria* Benth, *Satureja* L., *Clinopodium* L. and *Acinos* Miller, and for this reason the use of chemotaxonomic markers is essential to better differentiate these genera.

Many *Calamintha* species are used as spices in various culinary recipes because of their pleasant mint-like smell. Besides, they are known for different medicinal uses. Common calamint is used as diaphoretic, in syrups for coughs and colds and as an expectorant. The tea is used to help with gas and colic [2]. Externally, it is useful in poultices for bruises and as a strengthener and nerve soother. The essential oil shows different activities. The oil of *C. sylvatica* subsp. *ascendens* exerts significant sedating and antipyretic activities in the rat, due to the presence of the monoterpenes pulegone, menthone and eucalyptol [3]. Monoterpenes, particularly pulegone and isopulegone, are also reported to be the responsible of the strong antibacterial and antifungal activities showed by essential oils from different *Calamintha* species [4]. Due to its good antimicrobial activity, *C. officinalis* essential oil has been proved to be effective as preservative in two current formulations (cream and shampoo) [5].

Calamintha origanifolia Boiss. (syn. *Cyclotrichium origanifolium* (Labill.) Manden & Scheng.) is a strongly aromatic, suffruticose, much branched species wild growing in the Horsh Ehden reserve that is located on the northern part of the Lebanese western mountain range, just below Cornet As Sawda, the highest mountain peak in Lebanon. The Reserve represents a mountainous ecosystem on the elevated slopes of the northern Mt. Lebanon chain. In this paper, as a continuation of our studies on the essential oils from Lamiaceae growing wild in Lebanon [6], we report on the chemical composition of the essential oil of *Calamintha origanifolia* collected in the Lebanese Horsh Ehden reserve.

Table 1: Essential oil composition of *Calamintha origanifolia* Boiss.

I[a]	I[b]	Component	Method[c]	%[d]
798		Hexanal	*I*,MS	0.1
930		α-Thujene	*I*,MS	t
963	1543	Benzaldehyde	*I*,MS,Co-GC	0.3
973	1132	Sabinene	*I*,MS	0.1
980	1118	β-Pinene	*I*,MS,Co-GC	0.3
1025	1280	*p*-Cymene	*I*,MS,Co-GC	0.2
1030	1203	Limonene	*I*,MS,Co-GC	0.6
1034	1213	1,8-Cineole	*I*,MS,Co-GC	0.6
1111		*p*-Mentha-1,3,8-triene	*I*,MS	0.7
1117	1152	*trans*-*p*-Menth-2-en-1-ol	*I*,MS	0.9
1125	1540	Chrysanthenone	*I*,MS	0.9
1138	1475	Menthone	*I*,MS,Co-GC	7.7
1145	1663	*cis*-Verbenol	*I*,MS	1.9
1163	1502	Isomenthone	*I*,MS	12.2
1175	1582	Isopulegone[#]	*I*,MS	5.8
1177	1755	Dihydrocarveol	*I*,MS	0.1
1182	1652	Menthol	*I*,MS,Co-GC	0.7
1233	1662	Pulegone	*I*,MS,Co-GC	22.5
1244	1750	Carvone	*I*,MS	1.5
1293	2198	Thymol	*I*,MS,Co-GC	0.8
1299	2239	Carvacrol	*I*,MS,Co-GC	1.1
1329	1949	Piperitenone	*I*,MS	9.6
1343	1748	Piperitone	*I*,MS	6.9
1353	2186	Eugenol	*I*,MS,Co-GC	0.2
1363		Piperitenone oxide	*I*,MS	0.7
1372	1493	α-Ylangene	*I*,MS	0.2
1377	1497	α-Copaene	*I*,MS	0.1
1382		β Cubebene	*I*,MS	t
1385	1535	β-Bourbonene	*I*,MS	0.1
1387	1600	β-Elemene	*I*,MS	0.2
1415	1612	Caryophyllene	*I*,MS,Co-GC	1.9
1451	1868	Geranyl acetone	*I*,MS	0.3
1452	1673	(*E*)-β-Farnesene	*I*,MS	t
1455	1689	α-Humulene	*I*,MS	0.1
1477	1726	Germacrene D	*I*,MS	0.1
1515	1776	γ-Cadinene	*I*,MS	t
1520	1839	*cis*-Calamenene	*I*,MS	0.2
1526		δ-Cadinene	*I*,MS	t
1640	2187	τ-Cadinol	*I*,MS	4.0
1642	2209	τ-Muurolol	*I*,MS	0.7
1649	2255	α-Cadinol	*I*,MS	1.2
1835	2131	Hexahydrofarnesylacetone	*I*,MS	1.6
1957	2931	Hexadecanoic acid	*I*,MS,Co-GC	1.4
2500	2500	Pentacosane	*I*,MS	0.3
2600	2600	Hexacosane	*I*,MS	0.2
2700	2700	Heptacosane	*I*,MS	1.0
2800	2800	Octacosane	*I*,MS	0.1
2900	2900	Nonacosane	*I*,MS	1.3
3100	3100	Hentriacontane	*I*,MS	0.8
		Total identified		**92.2**

[a]: HP-5 MS column; [b]: HP Innowax; [c]: *I* is the retention index, MS = mass spectrum, Co-GC = co-injection with authentic compound; [d]: t = trace, less than 0.05%; [#]: correct isomer not identified.

Great variations occur in the volatile compounds from *Calamintha* genus, but the major components in the oils generally belong to the C-3 oxygenated *p*-menthanes such as pulegone, isomenthone, menthone, piperitone and piperitenone with their oxides [4a,4c-4f,5,7-9]. According to Baldovini *et al.* [8], three types of oils can be distinguished: in the first pulegone is the major component, associated with different compounds such as menthone and/or isomenthone, menthol and its isomers, piperitenone, piperitone and piperitenone oxides. The second type is characterized by the predominance of piperitone oxide and/or piperitenone oxide. Last type is distinguished by the presence of carvone and 1,8-cineole as main components [8 and references cited therein].

Table 2: Antimicronial activity of *Calamintha origanifolia* oil (**C**).

Strain	MIC (MBC)µg/mL	
	C	**Ch**
Bacillus subtilis ATCC 6633	50 (100)	12.5
Staphylococcus aureus ATCC 25923	100 (>100)	25
Staphylococcus epidermidis ATCC 12228	25 (50)	3.12
Streptococcus faecalis ATCC 29212	100	25
Escherichia coli ATCC 25922	50 (100)	12.5
Klebsiella pneumoniae ATCC 10031	100	50
Proteus vulgaris ATCC 13315	100 (>100)	25
Pseudomonas aeuriginosa ATCC 27853	>100	100

Ch: Chloramphenicol

The essential oil of *C. origanifolia* belongs to the first type, as pulegone (22.5%) is the most abundant component. In total, forty-nine constituents have been identified; representing 92.2% of the total oil; their retention indices and percentage composition are given in Table 1, where the components are listed in order of elution from a HP 5MS column. As reported in the literature for other *Calamintha* species [9], the oxygenated monoterpenes were the most abundant components of the oil, particularly those with *p*-menthane skeleton, and their content represented 59.7% of the oil. The most abundant compounds of this fraction were pulegone (22.5%), isomenthone (12.2%) and piperitenone (9.6%). The high content of isomenthone can be considered a characteristic of the present oil because this compound is reported in lower amounts in other *Calamintha* oils. Isomenthone was detected in a quite similar extent only in the oils of *C. grandiflora* (15.2%) [9b] and *C. sylvatica* ssp. *sylvatica* in the pre-blossom phase (13.4%) [9e]. The greatest amount of isomenthone was detected in the oil of *C. sylvatica* ssp. *ascendens* (36.8-43.3%) [9f]. Other ketones identified in the oil were chrysanthenone (0.9%), geranyl acetone (0.3%) and hexahydrofarnesyl acetone (1.6%). Also a few monoterpene hydrocarbons were present but they represented only 1.9% of the oil, ranging between 0.7% (*p*-mentha-1,3,8-triene) and traces (α-thujene). Twelve sesquiterpene hydrocarbons were detected. Caryophyllene represented the 1.9% of the oil whereas the other sesquiterpene hydrocarbons were present in low content, from traces to 0.2%. Three

oxygen-containing sesquiterpenes were present and τ-cadinol (4.0%) was the major component of this fraction. In the oil were also identified three phenols that amounted to the 2.1%. Carvacrol (1.1%) and thymol (0.8%) were the most abundant while eugenol represented the 0.2% of the oil. Data obtained allow us to ascribe the oil of *Calamintha origanifolia* Boiss. growing wild in Lebanon to a type pulegone/isomenthone oil.

The MIC and MBC values of the essential oil against eight selected micro-organisms are reported in Table 2. The oil showed action mainly against *B. subtilis*, *S. epidermidis* and *E. coli*.

Experimental

Plant material: Aerial parts of *C. origanifolia* Boiss were collected at the full flowering stage from plants growing wild on rocky soil at Oyoun Ouvghanch, 2200 m a.s.l., in June 2005. The required authorizations for the plant collection were given by the Lebanese authorities to Apostolides Arnold. A voucher specimen (leg. & det. N. Arnold s. n., confirm. Th. Raus) was deposited in the Herbarium of the Botanischer Garten, Berlin Universität.

Essential oil isolation: The oil from air-dried and ground aerial parts of plants was isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method recommended in the *European Pharmacopoeia* [10]

The oil was dried over anhydrous sodium sulphate and stored under N_2 at +4°C in the dark until tested and analysed. The sample yielded 0.13% of yellow oil (w/w), with a pleasant smell of mint.

GC analysis: Analytical gas chromatography was carried out on a Perkin-Elmer Sigma 115 gas chromatograph fitted with a HP-5 MS capillary column (30 m x 0.25 mm i.d.; 0.25 μm film thickness). Helium was the carrier gas (1 mL min^{-1}). Column temperature was initially kept at 40°C for 5 min, then gradually increased to 250°C at 2°C min^{-1}, held for 15 min and finally raised to 270°C at 10°C min^{-1}. Diluted samples (1/100 v/v, in *n*-hexane) of 1 μL were injected manually at 250°C, and in the splitless mode. Flame ionization detection (FID) was performed at 280°C. Analysis was also run by using a fused silica HP Innowax polyethylenglycol capillary column (50 m x 0.20 mm i.d.; 0.20 μm film thickness).

GC-MS analysis: GC-MS analysis was performed on an Agilent 6850 Ser. II apparatus, fitted with a fused silica HP-1 capillary column (30 m x 0.25 mm i.d.; 0.33 μm film thickness), coupled to an Agilent Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V. Gas chromatographic conditions were as reported above; transfer line temperature, 295°C.

Qualitative and quantitative analyses: Most constituents were identified by gas chromatography by comparison of their retention indices (*I*) with either those of the literature [11,12] or with those of authentic compounds available in our laboratories. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8-C_{24}) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with either those stored in NIST 02 and Wiley 275 libraries or with mass spectra from the literature [11,13] and our home made library. Component relative concentrations were calculated based on GC peak areas without using correction factors.

Antimicrobial activity: The antibacterial activity was evaluated by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) using the broth dilution method as previously described [6e]. Eight bacteria species, selected as representative of the class of Gram positive and Gram negative, were tested: *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus faecalis* (ATTC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), *Proteus vulgaris* (ATCC 13315) and *Pseudomonas aeruginosa* (ATCC 27853).

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Essential Oil from *Chenopodium ambrosioides* as a Promising Antileishmanial Agent

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Chenopodium ambrosioides has been used traditionally against parasitic diseases. The essential oil of the plant is a complex mixture of compounds with a rich structural diversity. This review focuses on recent evaluation of the essential oil from *C. ambrosioides* as a promising antileishmanial agent. The tested product showed activity against promastigotes and amastigotes of *Leishmania amazonensis* and *L. donovani*. An optimal dose of 30 mg/Kg was effective by intraperitoneal and oral routes in experimental cutaneous leishmaniasis. The chenopodium oil had a moderate toxicity against peritoneal macrophages of BALB/c mice and no side effects were detected in animals treated by the oral route. Isolates of *L. amazonensis* from treated mice were susceptible to the essential oil. Synergic effects were observed when the essential oil was incubated in conjunction with pentamidine on *L. amazonensis* promastigote cultures. Future studies focusing on formulation, toxicity and mechanism of action may help in the development of chenopodium oil as a new antileishmanial drug.

Keywords: *Chenopodium ambrosioides*, essential oil, antileishmanial agent, BALB/c mice, leishmaniasis.

Leishmaniasis is an infection caused by various species of *Leishmania* protozoa, which are usually transmitted by phlebotomine female sandflies [1]. The disease is endemic in 88 countries throughout Latin America, Africa, Asia and southern Europe. Approximately 350 million people are thought to be at risk with a worldwide prevalence of 12 million and annual incidence of 2 million new cases [2]. Moreover, multiple factors such as the AIDS epidemic, increased international travel, a lack of effective vaccines, difficulties in controlling vectors, international conflicts and the development of resistance to chemotherapy could increase the cases of leishmaniasis [3].

The epidemiology and clinical features of leishmaniasis are highly variable due to the interplay of numerous factors in the parasites, vectors, host, and environments involved. Three principal clinical manifestations are recognized in leishmaniasis: cutaneous, mucocutaneous and visceral [4]. Primary prevention relies on managed control of the maintenance host and sandfly bite prevention measures. Secondary and tertiary prevention are dependent on medical assistance using the clinical guidelines [5].

Currently, there is no immunoprotection available, although prospects for a vaccine remain high [6]. The main drugs to treat this disease are derivatives of pentavalent antimonial compounds (sodium stibogluconate and meglumine antimoniate), amphotericin B, and pentamidine [7,8]. However, theses agents are far from ideal. Problems associated with the most commonly used drugs are: toxicity, parenteral administration, drug resistance and high cost [9]. Miltefosine is the only oral antileishmanial drug available, but pregnant women can not be given this compound due to its teratogenic effects [10]. For all the reasons previously mentioned, leishmaniasis continues to take an enormous toll on human health, particularly in endemic areas.

Many people in rural areas depend largely on popular treatments to alleviate the symptoms [11]. In traditional medicine, the most common treatment consists of the use of plants, which are potential sources of wide chemistry with a remarkable

diversity, and are readily accessible in nature. Recently, the Tropical Diseases Program of the World Health Organization (TDR/WHO) with the Drug Discovery Research Program has considered the pharmacological investigation of plants to be a priority [12].

Our laboratory has initiated and developed original investigations on alternative compounds to control the growth of *Leishmania*, with the objective to validate traditional medicine, as well as search for plant-derived drugs that could lead to new strategies for treatment of leishmaniasis. We began from the selection of plants with ethnomedical uses. Several studies have been addressed to recover the traditional expertise. Franca et al, in 1996, reported some plants used in the treatment of leishmanial ulcers [13]. We centered our attention on *Chenopodium ambrosioides* for three reasons: (i) It is an aromatic herb with a large history of use in the population; (ii) in the course of screening for leishmanicidal compounds, we found promising pharmacological results with the essential oil; and (iii) it is easily cultivated. A review of the experimental results with this product on the *Leishmania* parasite is presented in this article.

Chemical properties and composition of the essential oil from *C. ambrosioides:* The essential oil was obtained by distillation, under laboratory conditions, of the aerial parts of the plant. The efficiency was approximately 1% and the density of the essential oil was 0.8893 g/mL. The composition of the essential oil was determined by high resolution gas chromatography-mass spectrometry (HRGC-MS). The chromatogram showed 68 peaks and the nine major components were identified as carvacrol (62.4%), ascaridole (22.5%), caryophyllene oxide (5.6%), apiole (2.0%), isoascaridole (1.9%), hexyl tiglate (1.0%), *p*-cymene (0.8%), Δ^4-carene (0.8%) and neomenthyl acetate (0.6%) [14].

Chenopodium ambrosioides: *C. ambrosioides*, popularly know as "apazote" in Cuba, is an aromatic plant, with its branched stem often prostrated. It is an annual or biannual herb, between 80 and 100 cm in height, with centuplicated leaves, which are oblong-lanceolate and serrated, with small green flowers in dense terminal panicles of glomerules, each with five sepals. The plant is sylvan and grows in all geographic area of Cuba. A voucher specimen (No. 4639) is kept at the Experimental Station of Medicinal Plants "Dr. Juan Tomás Roig", Cuba [14].

Antileishmanial *in vitro* studies: *In-vitro* activities of chenopodium oil against *Leishmania amazonensis*, the causal agent of cutaneous leishmaniasis, were determined. The growth of promastigotes and intracellular amastigotes forms of the parasite was inhibited by 100% at concentrations of 28 and 16 µg/mL, respectively. The 50% inhibitory concentration (IC_{50}) was determined to be 3.7 µg/mL against promastigotes and 4.6 µg/mL against amastigotes [14]. Surprisingly, the IC_{50} values of the essential oil were similar against both forms of *L. donovani* (IC_{50} promastigotes = 4.5 µg/mL and IC_{50} amastigotes = 5.1 µg/mL), the causal agent of visceral leishmaniasis [15].

Antileishmanial *in vivo* studies: An initial experiment was carried out in order to evaluate the activity of chenopodium oil against an experimental model of cutaneous leishmaniasis, caused by *L. amazonensis* in BALB/c mice [14]. In this case, the objective was to validate the *in-vitro* activity previously obtained. Animals were infected and treatment began 15 days after inoculation. A significant reduction ($P < 0.05$) in the size of the lesions was observed in animals treated with 30 mg/Kg of the essential oil, in comparison with placebo groups of animals (Figure 1).

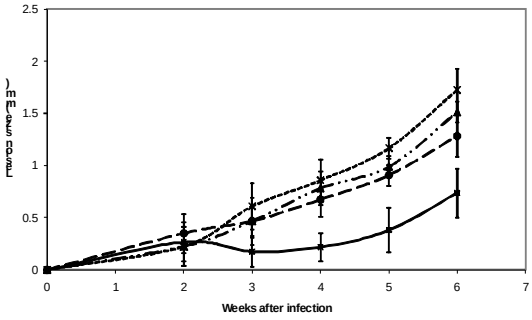

Figure 1: Effects of treatment with the essential oil of *C. ambrosioides* (30 mg/Kg), Miglyol (0.1 mL) and Amphotericin B (1 mg/Kg) administered daily for 15 days by intraperitoneal routes, during the course of infection of BALB/c mice with *L. amazonensis*. Each point represents the mean ± the standard deviation of the mean difference in lesion size between infected and uninfected footpads of twelve mice. Stars = Untreated mice; squares = Essential oil; circles = Amphotericin B; triangles = Miglyol (vehicle).

A second experiment was performed to compare the activity of chenopodium oil after either intraperitoneal or oral administration [16]. The treatment started 30 days after inoculation of parasites and animals received two cycles of treatment for 15 days. The mice treated with the essential oil by the oral route developed significantly similar lesions ($P > 0.05$) to those in mice treated by

Table 1: Evaluation of the toxicity of essential oil after injection by the intraperitoneal route in BALB/c mice, during 15 days.

Mice treated with the essential oil	Mortality (%)	Mice Gain or Loss of Weight (g)						
		Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
15 mg/Kg	0	0.0	0.4	0.7	0.9	1.1	1.2	1.9
30 mg/Kg	0	-0.4	-0.2	0.2	0.3	0.9	1.0	0.8
60 mg/Kg	100	-2.3	-3.6	-2.1	-2.5	D[a]	D	D
0.1 ml Miglyol	0	-0.9	-0.1	-0.2	0.1	0.0	0.3	0.2
Untreated	0	0.2	0.7	0.6	1.5	1.5	1.8	1.5

[a]: D; animal death before the treatment finished

the intraperitoneal route (Figure 2). Nevertheless, from day 75 post-infection, an increase of the lesion size in animals treated by the oral route was observed.

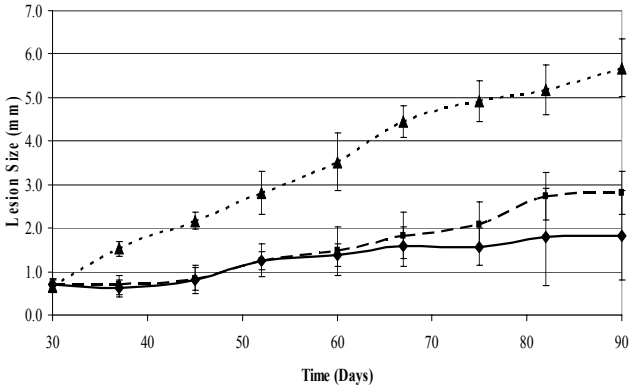

Figure 2: Effect of essential oil from *C. ambrosioides* on lesion growth using different routes of administration, during the course of infection of BALB/c mice with *L. amazonensis*. Animals treated with the essential oil: 30 mg/Kg/day by intraperitoneal route (♦); 30 mg/Kg/day by oral route (●); untreated mice (▲). Lesion size was measured at the indicated times (mean ± standard deviation).

Toxicological evidence: Preliminary experiments were carried out to examine the toxicity of chenopodium oil *in-vitro* and in animal models. The essential oil showed an IC_{50} of 58.8 µg/mL against peritoneal macrophages from BALB/c mice [14].

The 50% lethal dose (LD_{50}) was 100 mg/Kg of the essential oil after one administration by the intraperitoneal route in BALB/c mice. Then, we determined the maximum tolerated dose (MTD), which is the dose that does not cause either death or weight loss in more than 10% of the mice treated during 15 days by the intraperitoneal route. The treatment of animals with a dose of 60 mg/Kg caused 100% mortality before the end of the treatment. The group of mice treated with 15 and 30 mg/kg did not show death and the loss of weight was small (Table 1). The MTD selected was 30 mg/Kg/day by the intraperitoneal route [14].

In order to compare the intraperitoneal with the oral route, gross-pathological changes in the thoracic and abdominal cavity was verified after 15 administrations of 30 mg/Kg/day of the essential oil. Intraperitoneal administration caused some perforations in the peritoneal cavity. Oral treatment did not show signs of toxicity [16].

Resistance level of the parasite after treatment: Isolates of *Leishmania* parasites from BALB/c mice were treated with two cycles of 30 mg/Kg/day of chenopodium oil during 15 days by intraperitoneal and oral routes [16]. Promastigotes showed similar susceptibility compared with the wild type promastigotes of reference strains (Table 2).

Table 2: Influence of treatment with the essential oil on sensitivity of *L. amazonensis* promastigote strains.

Leishmania strains	*MIC*[a] (ug/mL)	IC_{50}[b] (ug/mL)	Resistance Index[c]
Wild Type	27.8	3.7	-
After IP [d]	30.1	6.7	1.8
After O [e]	27.8	5.5	1.5

[a]: *MIC*; concentration of the essential oil that caused 100 % mortality.
[b]: IC_{50}; concentration of the essential oil that caused 50 % mortality.
[c]: Resistance Index; IC_{50} of the isolated line/IC_{50} of wild type line.
[d]: *Leishmania* strain after intraperitoneal treatment with the essential oil.
[e]: *Leishmania* strain after oral treatment with the essential oil.

Synergistic effect: The incubation of the essential oil from *C. ambrosioides* in conjunction with pentamidine shows a synergic activity against promastigotes of *L. amazonensis* (Table 3). This result was demonstrated throughout isobologram analyses. However, an indifferent effect has been found for combinations of either meglunine antimoniate or amphotericin B and the essential oil [17].

General considerations: Essential oils are aromatic oily liquids obtained from plant material, which were used at first as fragrances in perfume, but they are perceived to be alternative medicines due to their protective roles. Different pharmacological properties have been explored related to the function of the compounds in the plant [18]. However, there are few reports about the antileishmanial effects of essential oils. Our recent studies provide evidence that the essential oil from *C. ambrosioides* can constitute a promising alternative to the development of a new therapy against leishmaniasis.

Table 3: Activity of each compound studied against promastigotes of *L. amazonensis* and the results of the combinations of the studied compounds expressed as FIC index.

Compound	*MIC*[a] ± SD[b] (μg/mL)	IC_{50}[c] ± SD (μg/mL)	FIC Index[d]	Interaction[e]
Meglumine antimoniate	-	-	1.790	Indifference
Amphotericin B	0.160 ± 0.002	0.030 ± 0.003	1.622	Indifference
Pentamidine	3.100 ± 0.010	0.370 ± 0.010	0.453	Synergism

[a]: *MIC*; lowest concentration that caused 100 % inhibition of the parasite growth; [b]: SD; standard deviation; [c]: IC_{50}; concentration that caused 50 % inhibition of the parasite growth; [d]: FIC Index; [A]/IC_{50A} + [B]/ IC_{50B}, where IC_{50A} and IC_{50B} are the IC_{50} of each compound alone and [A] and [B] are the IC_{50} of the essential oil and the other compounds when used in combination.; [e]: Interaction; terminology for describing results of combination testing

Previous reports have described ascaridole as the major constituent of the essential oil from Brazil and Canada [19, 20]. This endoperoxide is responsible for the anthelmintic effect, which was demonstrated by Smillie and Pessoa in 1924 [21]. However, the chemical analysis carried out in our study did not identify ascaridole as the main component. One reason could be the known variation in the chemical composition of plants, according to the geographic area. Fester et al found between 16 to 20% of ascaridole in the essential oil from plants collected in Cordoba, Argentina [22].

Surprisingly, the IC_{50} value for the promastigotes and amastigotes of *L. amazonensis* were similar ($P > 0.05$) to that found for *L. donovani*. Taking this result into account, we should consider the possibility that this essential oil acts either on a molecule or inhibits a metabolic pathway conserved in the *Leishmania* genus, which might be equally important for the viability of both morphophysiological forms. Another possible explanation is that the activity of the essential oil on both parasitic forms is the result of action of several compounds present in the oil, which could act on different molecules or metabolic pathways of *Leishmania*.

The mechanism of action by which the essential oil kills *Leishmania* is still unknown. However, some authors have shown that ascaridole generates free radicals, which act on parasitic DNA. This property is due to cleavage of the O-O bond in the endoperoxide [23a-23c]. Other experiments are necessary to search for the mechanism of action of the essential oil.

The intraperitoneal route was the most effective in controlling the disease after its establishment. Oral administration of the essential oil produced the same effect as treatment of the mice by the intraperitoneal route, except for a slight transient recrudescence in lesion size between 8 and 12 week post-infection. The effectiveness of the oral route results in a good absorption of the essential oil through the gastrointestinal tract. For that reason, it must also be assumed that the principal active agent was metabolized at low levels and is transported via the systemic circulation from the intestinal mucosa to the infected tissue. However, a partial loss of the drug may occur due to exchange interactions through different compartments such as the blood, the liver and others.

Preliminary experiments were carried out to examine the potential toxicity of the essential oil *in-vitro* and *in-vivo*. The essential oil showed a moderate toxicity against mouse peritoneal macrophages, approximately 15-fold more selective against *Leishmania* parasite compared to mammalian cells. This result suggests that the product may be safe for host cells. Oral administration of essential oil in BALB/c mice did not exhibit any observable signs of toxicity in these animals, which could facilitate long term treatment, in order to produce a consistent protection against cutaneous leishmaniasis.

Complete cure of the animals treated with the essential oil did not occur. However, while untreated animals develop the inexorable disease, mice treated with the essential oil by the intraperitoneal and oral routes had small lesions and low parasite burden. The model of cutaneous leishmaniasis due to *L. amazonensis* is not a perfect model, because it is a highly virulent strain and causes a disseminating, "noncure" and fatal diseases in BALB/c mice [23d].

Leishmania parasites are evolutionarily successful organisms, and they must develop highly sophisticated actions to combat the host's killing mechanisms [24], that include the immune response of the host and chemotherapy.

The development of drug resistance in the parasites is another major impediment in the successful treatment with conventional drugs [25]. In our work, the resistance index was less than twice compared with the wild type strains. We can thus assume that the drug pressure received by strains was very low to develop the expression of other phenotypes like drug resistance. In others studies, a high resistance level was found in *L. infantum* isolates from dogs that received two treatment cycle of meglumine

antimoniate (20.4 mg Sb^V/Kg/12 h/10 days) [26]. As part of a series of studies on the antileishmanial activity of some compounds (miltefosine, atovaquone), promastigote resistant lines have been selected by stepwise increases in drug pressure *in-vitro*. In this study, selection of resistant lines *in vitro* had shown a high level of resistance, but this induction had been found after five or more treatments [27,28].

After increased unresponsiveness to most of the monotherapeutic regimens, combination therapy has found new scope in the treatment of both cutaneous and visceral leishmaniasis [29]. Additionally, the combination of antileishmanial drugs could reduce the potential toxic side effects and prevent drug resistance. For these reasons, it is important to critically evaluate the role of combination therapy as new data. Several works have shown that some drugs increased their antileishmanial effect in conjunction with new antileishmanial agents.

Synergism among antileishmanial agents might occur in one of several ways. The inhibition of different stages of the same biochemical pathway represents one type of mechanism of synergism [30]. It is possible that this mechanism could explain the synergistic effect found between pentamidine and the essential oil. Multiple mechanisms of action had been proposed for pentamidine in kinetoplastid parasites, including DNA binding [31,32]. On the other hand, the exact mechanism of action of the essential oil is not known, but some authors hypothesized that ascaridole (endoperoxide), an active molecule, generates free radicals that can act on parasitic DNA [23a,23b]. Investigations are in progress in our laboratory to identify the mechanism involved in the antileishmanial activity. We observed electronic perturbations in the essential oil after increased amounts of *Leishmania* DNA, due to a hypochromism effect (data not shown). These perturbations could suggest an interaction of the DNA with the compounds present in the essential oil. Although we are thinking in terms of a correlation between DNA binding affinity and synergism observed, this may not be the only factor contributing to increase of antileishmanial activity.

Table 5: Summary of main results on antileishmanial activity of essential oil from *Chenopodium ambrosioides* according standard criteria.

Standard Criteria (Pink 2005)	Result of the essential oil from *C. ambrosioides* against *Leishmania* parasites
1. Active *in-vitro* with *IC50* ≤ 1 μg/mL	1. *IC50* between 3.7 and 5.1 μg/mL
2. Selective (at least tenfold more active against parasite than against a mammalian cell)	2. 15-fold approximately more active against parasite than against a mammalian cell
3. Active *in-vivo* at a dose ≤ 100 mg/Kg	3. Active at 30 mg/Kg by intraperitoneal route
4. Active *in-vivo* by oral route at a dose ≤ 100 mg/Kg	4. Similar activity at 30 mg/Kg by oral route
5. Not toxic in animals at efficacious dose	5. At 30 mg/Kg/day during 15 days by oral route: - No death - No weight loss of more than 10 % - No gross-pathological damages in the thoracic and abdominal cavity

Conclusions: Plant essential oils can be used as alternatives to current antiparasitic therapies [33]. Our results demonstrated that chenopodium oil is effective *in-vitro* and *in-vivo* against *Leishmania*, in addition to having acceptable biological properties (easily extracted oil, bioavailability by oral route, toxicity or tolerability of the animals, the small resistance induced, and the synergistic effect in conjunction with pentamidine). These results are in concordance to standard criteria (Table 5), shown by Pink et al, concerning the drug discovery process [9]. The promising results and the relative cost of the product are important considerations that suggest continuation of the study of the essential oil from *C. ambrosioides* as an antileishmanial drug for people in developing countries. Future studies should be performed in order to develop a formulation with the desired pharmacokinetic and toxicological properties, accessible to endemic populations.

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Selective Cytotoxic Activities of Leaf Essential Oils from Monteverde, Costa Rica

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The leaf essential oils of *Eugenia cartagensis*, *Myrcia* sp. nov. "fuzzy leaf", *Ocotea veraguensis*, *O. whitei*, and *Persea americana*, have been obtained by hydrodistillation and the essential oil compositions determined by GC-MS. The essential oils have been screened for *in-vitro* cytotoxic activity against a panel of human tumor cell lines, and each of the species shows selective cytotoxic activity. *E. cartagensis* was active against HCT-15 and SW 620 human colorectal carcinoma cells, *O. veraguensis* and *Myrcia* "fuzzy leaf" were cytotoxic to MDA-MB-231 and MDA-MB-468 mammary adenocarcinoma cells, and *O. whitei* and *Persea americana* were toxic to M-14 melanoma cells.

Keywords: *Eugenia*, *Myrcia*, *Ocotea*, *Persea*, essential oils, chemical composition, cytotoxicity.

The American Cancer Society estimates that about 1,444,920 new cancer cases will be diagnosed in the United States in 2007 [1]. Cancer is the second most common cause of death in the U.S. (after cardiovascular disease), and about 559,650 people in the U.S. are expected to die of cancer this year. The deadliest forms of cancer in the U.S. include lung (both men and women), prostate (men), breast (women), and colorectal (both men and women) cancers.

In earlier times, all drugs and medicinal agents were derived from natural substances, and most of these remedies were obtained from higher plants. Even today, about 80% of the world's population relies predominantly on plants and plant extracts for health care. Not only do higher plants continue to serve as important sources of new drugs, but phytochemicals derived from them are also extremely useful as lead structures for synthetic modification and optimization of bioactivity. As part of our program investigating the phytopharmaceutical potential of the Monteverde region of Costa Rica [2], we have examined a number of essential oils from rainforest plants for potential medicinal utility. In this work, we describe the cytotoxic activity of the leaf essential oils of *Eugenia cartagensis*, *Ocotea veraguensis* [3], *O. whitei* [3], *Persea americana*, and an undescribed species, *Myrcia* new species "fuzzy leaf". To our knowledge, the cytotoxic activities of these essential oils have not been previously examined.

Eugenia cartagensis O. Berg (Myrtaceae) is a tree, 4-10 m in height, endemic to Costa Rica. It is common on the Pacific slope at 1200-1500 m elevation.

Myrcia new species "fuzzy leaf" (Myrtaceae) is a sub-canopy tree of the secondary forest and edge, 8-15 m in height. The twigs are round, pubescent when young, with smooth, gray bark when older; leaves are simple, opposite, entire, petiole to 3 mm, blade to 4 x 10 cm, lanceolate, apex acuminate, base rounded to obtuse, mid-vein expressed above and remaining pubescent, blade and veins remaining densely soft pubescent below with erect rusty hairs,

lateral veins 12-14 per side, a distinct marginal vein 1.5 mm from edge. The inflorescences are axillary and terminal, 3-6 cm long; flowers white with pedicel 0-3 mm, flowers 3 mm long x 4 mm diameter at anthesis, 5 calyx lobes to 1 mm long, 5 round white petals 3 mm long; fruit to 12 mm, globose, white to pink to purple black when mature. This tree is uncommon on the Pacific slope of the Monteverde region at 1300-1450 m elevation.

Ocotea veraguensis (Meissn.) Mez is a subcanopy tree, 5-15 m tall and *O. whitei* Woodson is a canopy tree, 10-30 m in height.

Persea americana Mill. is a canopy tree, up to 30 m tall and 90 cm diameter at breast height. In Monteverde, this tree is typically found in primary forest and pastures at 1400-1600 m elevation on the Pacific slope and from the lowlands to about 1300 m on the Atlantic slope.

The leaf essential oils were screened for cytotoxic activity against a panel of human tumor cell lines (Table 1). The leaf oil of *E. cartagensis* was especially cytotoxic against HCT-15 and SW 620 (colorectal carcinoma) cells, but was less active against MCF7 and MDA-MB-468 (mammary adenocarcinoma), M-14 and SK-Mel-28 (malignant melanoma), and was inactive on Malme-3M and UACC-257 (malignant melanoma), MDA-MB-231 (mammary adenocarcinoma), MDA-MB-435 (mammary ductal carcinoma), and OVCAR-5 (ovarian adenocarcinoma).

Myrcia "fuzzy leaf" essential oil showed notable *in-vitro* cytotoxicity to the mammary adenocarcinoma cell lines, MDA-MB-231 and MDA-MB-468, but was less active against Malme-3M, MDA-MB-435, EKVX (non-small-cell lung carcinoma), SK-Mel-28, MCF7, and UACC-257, and was inactive against M-14 and OVCAR-5. *Ocotea veraguensis* leaf oil was also selective against MDA-MB-131 and MDA-MB-468 cells, but either less active or inactive against the other cell lines tested.

Myrcia "fuzzy leaf" essential oil showed notable *in-vitro* cytotoxicity on the mammary adenocarcinoma cell lines, MDA-MB-231 and MDA-MB-468, but was less active against Malme-3M, MDA-MB-435, EKVX (non-small-cell lung carcinoma), SK-Mel-28, MCF7, and UACC-257, and was inactive against M-14 and OVCAR-5. *Ocotea veraguensis* leaf oil was also selective against MDA-MB-131 and MDA-MB-468 cells, but less active or inactive against the other cell lines tested.

Both *Ocotea whitei* and *Persea americana* showed activity against M-14 melanoma cells, and the *Persea* was also active against MDA-MB-231. None of the leaf essential oils showed any cytotoxic activity against the ovarian tumor line, OVCAR-5.

Notable in the pattern of cytotoxicity on the breast cancer lines is the difference between the estrogen receptor (ER) positive cell line, MCF-7, and the two estrogen receptor negative cell lines MDA-MB 231 and MDA-MB-468. Specifically, *Myrcia* "fuzzy leaf" and *Ocotea veraguensis* essential oils were both very active against the ER negative lines, but were not cytotoxic to the ER positive line. Both of the ER negative lines express the epidermal growth factor receptor (EGFR) suggesting a possible mechanism of action involving the EGFR. Interestingly, *Persea americana* leaf oil was active on only one of the ER negative, EGFR positive cell lines, suggesting a more specific mechanism of action than just working through the EGFR. It should be noted that although the MDA-MB-435 cell line is listed as a breast

Table 1: Cytotoxic activity of leaf essential oils.

Cell line	% kill at 100 µg/mL[a]				
	Eugenia cartagensis	*Myrcia* "fuzzy leaf"	*Ocotea veraguensis*	*Ocotea whitei*	*Persea americana*
HCT-15	100	NT[b]	NT	NT	NT
SW 620	84.1(8.1)	NT	NT	NT	NT
MCF7	73.5(12.8)	32.7(8.1)	45.0(16.8)	65.0(8.3)	37.2(4.9)
MDA-MB-231	0	100	93.0(6.3)	31.4(12.2)	98.2(1.1)
MDA-MB-468	32.1(21.9)	100	100	23.0(13.1)	32.6(10.1)
MDA-MB-435	0	66.7(4.8)	0	0	0
M-14	45.3(15.1)	0	0	100	92.6(5.1)
Malme-3M	0	90.6(9.3)	64.8(25.6)	0	0
SK-Mel-28	41.3(3.9)	45.0(12.3)	20.1(7.4)	0	0
UACC-257	0	22.3(13.0)	34.6(1.8)	45.4(7.4)	33.0(6.8)
OVCAR-5	0	0	0	0	0

[a]Standard deviations are shown in parentheses.
[b]NT = Not tested on this cell line.

Table 2: Chemical composition of *Eugenia cartagensis* leaf essential oil.

RI[a]	Compound	Percent Composition
856	*trans*-2-Hexenal	31.2
899	2-Heptanone	2.0
944	α-Pinene	0.7
967	*trans*-2-Heptenal	0.7
981	β-Pinene	0.7
1030	Limonene	trace
1042	*cis*-β-Ocimene	trace
1055	*trans*-β-Ocimene	16.2
1060	γ-Terpinene	0.4
1339	δ-Elemene	1.9
1376	α-Copaene	0.6
1385	β-Bourbonene	0.6
1393	β-Elemene	1.1
1422	β-Caryophyllene	6.3
1430	β-Gurjunene	0.5
1435	γ-Elemene	0.9
1439	α-Guaiene	0.4
1454	α-Humulene	1.6
1463	*epi*-Bicyclosesquiphellandrene	0.2
1484	Germacrene D	12.3
1488	β-Selinene	0.3
1493	Valencene	0.4
1499	Bicyclogermacrene	4.1
1502	α-Muurolene	0.4
1514	γ-Cadinene	0.7
1526	δ-Cadinene	2.3
1533	Cadina-1,4-diene	trace
1559	Germacrene B	6.0
1566	*trans*-Nerolidol	0.6
1577	Spathulenol	0.3
1583	Globulol	0.5
1590	Viridiflorol	0.7
1613	1,10-di-*epi*-Cubenol	trace
1626	1-*epi*-Cubenol	0.6
1630	Unidentified	0.6
1643	*epi*-α-Cadinol	1.1
1647	Torreyol	0.3
1656	α-Cadinol	1.4
2025	Kaurene	1.1

[a] Retention indices on HP-5 ms fused silica capillary column.

adenocarcinoma, information from the ATCC website (http://www.atcc.org) indicates that this cell line may not be of breast origin and may be more melanoma-like.

The chemical composition of *E. cartagensis* leaf essential oil is presented in Table 2. The leaf oil was dominated by sesquiterpene hydrocarbons (40.9%), fatty acid derivatives (33.9%), and monoterpene hydrocarbons (17.9%), with oxygenated sesquiterpenoids (5.7%) and diterpene hydrocarbons (1.1%) making up the remainder. The most abundant components were *trans*-2-hexenal (31.2%), *trans*-β-ocimene (16.2%), germacrene D (12.3%), β-caryophyllene (6.3), germacrene B (6.0%), and bicyclogermacrene (4.1%).

Table 3: Chemical composition of the leaf oil of *Myrcia* sp. "fuzzy leaf".

RI[a]	Compound	Percent Composition
860	*cis*-3-Hexenol	2.4
1338	δ-Elemene	35.5
1376	α-Copaene	trace
1392	β-Elemene	5.2
1419	β-Caryophyllene	1.5
1434	γ-Elemene	trace
1442	α-Guaiene	trace
1450	Unidentified[b]	1.1
1453	α-Humulene	0.6
1464	*allo*-Aromadendrene	0.5
1475	γ-Gurjunene	1.5
1478	γ-Muurolene	1.2
1482	Germacrene-D	3.2
1487	β-Selinene	2.3
1492	Viridiflorene	2.6
1495	α-Selinene	4.9
1499	Bicyclogermacrene	1.1
1502	α-Muurolene	trace
1505	Germacrene A	1.4
1508	Unidentified[b]	0.7
1510	(*E,E*)-α-Farnesene	1.4
1523	Unidentified[b]	1.5
1524	δ-Cadinene	1.4
1556	Germacrene B	1.6
1593	Guaiol	3.7
1612	Unidentified[b]	0.6
1621	10-*epi*-γ-Eudesmol	4.5
1630	Unidentified[b]	10.5
1633	Unidentified[b]	2.4
1643	*epi*-α-Cadinol	0.9
1647	Torreyol	trace
1658	Valerianol	5.8

[a] Retention indices on HP-5 ms fused silica capillary column.
[b] Mass spectra of unidentified compounds available as supplementary material.

Aldehydes, especially α,β-unsaturated aldehydes are known to be cytotoxic agents [4,5]. These materials can alkylate DNA by either conjugate addition [6] or imine formation [7]. The cytotoxic activity of *E. cartagensis* leaf oil is likely to be due, in part, to the high concentration of *trans*-2-hexenal. Although there are no reports of *trans*-β-ocimene being cytotoxic, both germacrene D and β-caryophyllene have been shown to be cytotoxic to a number of tumor cell lines [8].

The leaf essential oil of *Myrcia* "fuzzy leaf" (Table 3) was largely made of sesquiterpene hydrocarbons (65.3%), with lesser amounts of oxygenated sesquiterpenes (14.9%) and fatty acid derived compounds (2.4%). The major components of *Myrcia* "fuzzy leaf" were δ-elemene (35.5%), valerianol (5.8%), β-elemene (5.2%), α-selinene (4.9%), 10-*epi*-γ-eudesmol (4.5%), and an unidentified sesquiterpene alcohol (10.5%), the mass

spectrum of which is consistent with an aromadendrene hydrate, is also active.

β-Elemene has been shown to be cytotoxic to a number of tumor cell lines [9-12] and it is likely that δ-elemene is cytotoxic as well, although this has not been reported. Valerianol, α-selinene, and 10-*epi*-γ-eudesmol have not been reported to be cytotoxic.

The compositions of the leaf essential oils of *Ocotea veraguensis* and *O. whitei* have been reported [3]. Oxygenated sesquiterpenoids (58.8%) comprised a large part of the leaf oil of *O. veraguensis*. The remainder of the oil was composed of smaller amounts of monoterpene and sesquiterpene hydrocarbons (27.5% and 10.1%, respectively) with a very small amount of oxygenated monoterpenoids (2.3%), fatty-acid-derived compounds (1.1%), and others (0.1%). The leaf essential oil of *O. veraguensis* was dominated by bulnesol (29.5%) and *p*-cymene (19.8%). While *p*-cymene has been reported to inhibit bacterial growth [13,14], there are no reports of antineoplastic activity of this compound. Likewise, there have been no reports of bulnesol showing cytotoxic activity.

The leaf essential oil of *O. whitei* [3] was composed largely of monoterpene and sesquiterpene hydrocarbons (22.0% and 31.6%, respectively) as well as oxygenated sesquiterpenoids (33.8%), with smaller amounts of fatty-acid-derived compounds (0.8%), oxygenated monoterpenoids (3.1%), oxygenated sesquiterpenoids (1.5%), and aromatics (0.5%). The most abundant essential oil components of *O. whitei* were spathulenol (15.3%), β-caryophyllene (15.2%), α-pinene (12.7%), and farnesyl acetate (10.1%). Spathulenol has been reported to exhibit cytotoxic activity to KB [15] and Hep 2 [16] tumor cell lines. β-Caryophyllene, α-pinene, and β-pinene have shown cytotoxic activity to a number of tumor cell lines [8].

The chemical composition of the leaf essential oil of *Persea americana* is summarized in Table 4. The most abundant compound in the oil was the phenylpropanoid (*Z*)-isoeugenol acetate (14.8%), followed by the monoterpenoids sabinene (9.9%), 4-terpineol (8.9%), α-phellandrene (7.6%), and 1,8-cineole (7.0%). Eugenol (4.9%), β-caryophyllene [17,18] and isoeugenol [19,20] have shown *in-vitro* cytotoxic activity. It is likely that isoeugenol acetate is also cytotoxic.

Experimental

Plant material: Leaves of *Eugenia cartagensis* were collected on May 19, 2006, from a mature tree located at the Los Llanos field station, Monteverde, Costa Rica (10.3056 N, 84.8370 W, 1200 m above sea level). Leaves of *Myrcia* sp. "fuzzy leaf" were collected on June 4, 2006, from several trees located in the lower montane moist forest in Monteverde, (10.3059 N, 84.8144 W, 1380 m above sea level). Leaves of *Persea americana* were collected on May 23, 2006, from a mature tree located in the Monteverde Cloud Forest Preserve (10.3483 N, 84.7633 W, 1530 m above sea level). The plants were identified by William Haber. Voucher specimens (*Eugenia cartagensis*: Haber number 10989; *Myrcia* sp. ''fuzzy leaf'': Haber number 10880; *Persia americana*: Haber number 9841) have been deposited in the herbarium of the Missouri Botanical Garden. The fresh leaves (*E. cartagensis* 33.1 g; *Myrcia* sp. ''fuzzy leaf'' 48.1 g; *Persia americana* 48.1 g) were chopped and, immediately, hydrodistilled with continuous extraction with $CHCl_3$ using a Likens-Nickerson apparatus. The $CHCl_3$ extract was dried over $CaCl_2$ and evaporated to give 4.26 mg, 28.3 mg and 28.3 mg essential oil for *Eugenia cartagensis*, *Myrcia* sp. ''fuzzy leaf'', and *Persia americana*, respectively.

Cytotoxicity Screening: Each of the human tumor cell lines was grown in a 5% CO_2 environment at 37°C in RPMI 1640 medium with L-glutamine and $NaHCO_3$, supplemented with 10% fetal bovine serum, 100,000 units penicillin and 10.0 mg streptomycin per L of medium, pH 7.3. Cells were plated into 96-well cell culture plates at 2.5×10^4 cells per well. The volume in each well was 100 μL. After 48 h, supernatant fluid was removed by suction and replaced with 100 μL growth medium containing 1.0 μL of DMSO solution of the essential oil (1% w/w in DMSO), giving a final concentration of 100 μg/mL for each well. Solutions were added to wells in four replicates. Medium controls and DMSO controls (10 μL DMSO/mL) were used. Tingenone [21] was used as a positive control. After the addition of compounds, plates were incubated for 48 h at 37°C in 5% CO_2; medium was then removed by suction, and 100 μL of fresh medium was added to each well. In order to establish percent kill rates, the MTT assay for cell viability was carried out [22]. After colorimetric readings were recorded (using a Molecular Devices SpectraMAX Plus microplate reader, 570 nm), average absorbances, standard

deviations, and percent kill ratios ($\%kill_{cmpd}/\%kill_{DMSO}$) were calculated. Cytotoxic activities of the essential oils are summarized in Table 1.

Gas chromatographic – mass spectral (GC-MS) analysis: The leaf essential oils were subjected to GC-MS analysis on an Agilent system consisting of a model 6890 gas chromatograph, a model 5973 mass selective detector (MSD), and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary with a (5% phenyl)-methylpolysiloxane stationary phase, film thickness 0.25 μm, length 30 m, and internal diameter 0.25 mm. Helium was the carrier gas with a flow rate of 1.0 mL/min. The inlet temperature was 200°C and the oven temperature program was as follows: 40°C initial temperature, hold for 10 min; increased at 3°/min to 200°C; increased 2°/min to 220°C, with an interface temp of 280°C. The sample was dissolved in $CHCl_3$ and a splitless injection technique was used. Identification of oil components was achieved based on their retention indices (determined with reference to a homologous series of normal alkanes), and by comparison of their mass spectral fragmentation patterns with the literature [23] and the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.08)].

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Chemical Composition of Leaf Essential Oil of *Hedyosmum arborescens* and Evaluation of Its Anticancer Activity

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The essential oil of *Hedyosmum arborescens* Sw. (Chloranthaceae), a native plant of the Caribbean archipelago, was extracted by hydrodistillation. The chemical composition of the volatile fraction was determined by GC and GC-MS analyses and 50 components were identified. The major components are α-phellandrene (11.4%), bicyclogermacrene (10.6%) and sabinene (9.7%). The anticancer activities of these extracts were assessed against human lung carcinoma cell line A-549 and human colon adenocarcinoma cell line DLD-1. The leaf essential oil of *H. arborescens* was found to be moderately active against both cancer cell lines with GI_{50} values of 158 ± 7 μg/mL for A-549 and 178 ± 9 μg/mL for DLD-1.

Keywords: *Hedyosmum arborescens*, Chloranthaceae, bois-senti, essential oil, anticancer activity, α-phellandrene, bicyclogermacrene.

Hedyosmum arborescens Sw., (Chloranthaceae), a Caribbean native plant [1], is well known in Guadeloupe and Martinique as bois-senti, bois de l'eau and bois fragile [2], whereas it is commonly called cigarbush in the English West Indian islands [3]. This plant is a small resinous tree about 3 to 6 meters high, with numerous very fragile branches, full of pith and swollen at the knots, that grows in degraded tropical forests, in wet glades and on river banks. Its fleshy leaves are thick, elliptical to lanceolate, about 8 to 10 cm long and 1 to 3 cm large [2]. No common use of *H. arborescens* is known. The leaves of the plant give an essential oil, the composition of which has not been previously reported. Thus, the aims of this study were to examine the chemical composition of the leaf essential oil of *H. arborescens* collected in Guadeloupe and to evaluate its anticancer activity.

Leaves of *H. arborescens* extracted by hydrodistillation produced a yellow essential oil with a yield of 0.24% (w/w), relative to the dried plant material. The volatile extract is characterized by a refractive index of 1.5048 (at 20°C) and a density of 0.881 g/mL. The chemical composition of the leaf essential oil is listed in Table 1. Chromatographic analysis showed 51 compounds of which 50 were identified. The oil was composed of terpenic molecules and only one compound was not a terpene: (*E*)-isoeugenol acetate. The oil was constituted of 58.6% monoterpenes, including 12% oxygenated monoterpenes, and 36.5% sesquiterpenes, including 15.3% oxygenated sesquiterpenes. The main components were α-phellandrene (11.4%), bicyclogermacrene (10.6%), sabinene (9.7%), spathulenol (7.5%), β-pinene (6.1%) and *p*-cymene (5.9%). The mass spectrum of the unidentified compound (RI DB-5 = 1595), presented in Table 1, suggests that it is a β-eudesmol derivative.

The anticancer properties of *H. arborescens* essential oil were assessed against cell lines A-549 (human lung carcinoma) and DLD-1 (human colon adenocarcinoma). Both cell lines were subjected to increasing concentrations of the leaf oil for 48 hours.

Table 1: Chemical composition (%) of *Hedyosmum arborescens* leaf essential oil.

Components	RI DB-5[a]	RI Spwax[b]	%
α-Thujene	933	1031	0.7
α-Pinene	938	1024	2.1
Camphene	951	1070	0.2
Sabinene	974	1127	9.7
β-Pinene	976	1111	6.1
Myrcene	991	1172	0.4
α-Phellandrene	1000	1170	11.4
α-Terpinene	1015	1183	0.6
p-Cymene	1025	1284	5.9
Limonene	1030	1200	3.3
1,8-Cineole	1030	1206	0.2
β-Phellandrene	1030	1209	1.4
(*Z*)-β-Ocimene	1045	1251	2.6
(*E*)-β-Ocimene	1057	1257	0.9
γ-Terpinene	1066	1269	1.0
Terpinolene	1097	1296	0.5
Linalool	1111	1565	0.4
(*E*)-Pinocarveol	1141	1657	0.2
(*Z*)-Pinocamphone	1171	1555	0.3
Terpinen-4-ol	1176	1604	3.1
α-Terpineol	1188	1708	0.2
Citronellol	1231	1778	0.3
Thymol methyl ether	1237	1599	5.1
Neral	1240	1688	0.2
Geraniol	1260	1861	0.8
Geranial	1274	1740	0.3
cis-2,3-Pinanediol	1319	2197	0.7
δ-Elemene	1342	1477	0.3
Geranyl acetate	1385	1769	0.2
β-Elemene	1389	1589	1.2
Aromadendrene	1437	1596	0.4
Alloaromadendrene	1460	1640	0.3
9-*epi*-β-Caryophyllene	1462	1630	0.1
Germacrene D	1482	1708	0.9
Bicyclogermacrene	1498	1732	10.6
Curzerene	1498	1874	1.4
(*E,E*)-α-Farnesene	1502	1740	4.6
Germacrene A	1505	1757	0.4
δ-Amorphene	1512	1722	0.2
Elemol	1549	2086	0.7
Germacrene B	1555	1819	0.8
(*E*)-Nerolidol	1565	2053	0.8
Spathulenol	1573	2125	7.5
Globulol	1579	2074	1.4
Viridiflorol	1585	2082	0.8
Carotol	1590	2011	1.6
unidentified[c]	1595	--	0.4
(*E*)-Isoeugenol acetate	1614	--	0.2
γ-Eudesmol	1624	2166	0.2
Isospathulenol	1634	2225	1.4
Selin-11-en-4α-ol	1650	2249	0.5
Total			95.3

[a] Retention indices on apolar DB-5 column.

[b] Retention indices on polar Supelcowax 10 column.

[c] m/z (relative intensity): 204(M^+, 4), 189(4), 175(2), 161(23), 149(79), 136(10), 133(11), 121(20), 108(30), 93(41), 91(42), 81(55), 67(30), 59(100), 55(30), 43(65), 41(55).

Figure 1 shows the percentage of survival of the cells versus the concentration of essential oil. The concentrations of oil for which each cell line's growth was inhibited by 50% (GI_{50}) were calculated from the curve. GI_{50} values were 158 ± 7 μg/mL for A-549 and 178 ± 9 μg/mL for DLD-1. These relatively high GI_{50} values indicate a moderate anticancer activity of *H. arborescens* leaf essential oil. Very few of the compounds found in the oil have been tested for anticancer properties. It has been reported in the literature that derivatives of limonene, such as perillyl alcohol and perillyl aldehyde, inhibit proliferation and migration of breast cancer cells [4] and cause cell cycle arrest in G1 and apoptosis of human carcinoma cell lines [5]. D-limonene is also capable of inducing apoptosis in gastric cancer cells [6]. Terpinen-4-ol can induce caspase-dependent apoptosis in human melanoma cells [7], and has been shown to cause differentiation of human myelocytic cell line HL-60 [8]. Furthermore, spathulenol has a GI_{50} value of 83.8 μM when tested in the KB cell cytotoxicity assay and can moderately inhibit human topoisomerase I [9]. Limonene, terpinen-4-ol and spathulenol are all found in high concentrations in the essential oil, which could explain, in part, the anticancer activity. However, no cytotoxicity assays have been performed for most of the major components of *H. arborescens* leaf essential oil (sabinene, α-phellandrene and bicyclogermacrene).

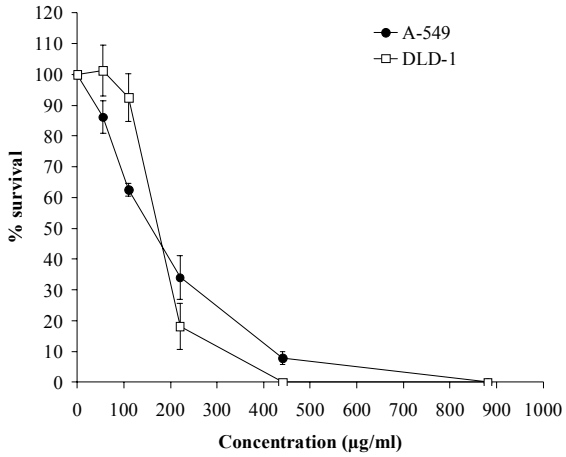

Figure 1: Anticancer activity of *Hedyosmum arborescens* leaf essential oil against cell lines A-549 (human lung carcinoma) and DLD-1 (human colon adenocarcinoma). Values represented are the means of three determinations.

In conclusion, we have determined the chemical composition of the essential oil of *Hedyosmum arborescens*, and have evaluated its anticancer activity. The results show that the essential oil is moderately active against both tumor cell lines tested. The anticancer activity could be explained, in part, by high concentrations of limonene, terpinen-4-ol and spathulenol. However, further studies aimed at determining the anticancer properties of the other major constituents of *H. arborescens* leaf essential oil will be needed in order to fully understand their bioactivity.

Experimental

Plant material and essential oil extraction: Leaves of *H. arborescens* were harvested in May 2002 at Basse Terre (Guadeloupe). A voucher specimen of this plant (Fournet, 1756) has been deposited at the INRA-National Park Herbarium of Guadeloupe. Fresh leaves were extracted by hydrodistillation during two h in a Clevenger apparatus [10]. The oil was dried over anhydrous sodium sulfate and stored under nitrogen at 4°C.

GC and GC/MS analyses: The essential oil was analyzed by GC on a gas chromatograph [Hewlett-Packard 5890 (FID detector)] equipped with a polar Supelcowax 10 column and an apolar DB-5 column (30 m x 0.25 mm x 0.25 μm). Analyses by GC-MS were performed on a Hewlett-Packard mass spectrometer 5972 at 70 eV, coupled to an HP 5890 equipped with a DB-5 column (same as above). The temperature program was 40°C for 2 min, then 2°C/min to 210°C and held constant for 33 min. For injection (split injector), 5 μL of essential oil was diluted to 500 μL in *n*-hexane and 5 μL of this diluted solution was used. Identification of volatile constituents was made on the basis of their retention indices [11] and their mass spectra, which were compared with data references [12].

Cell culture: Human lung carcinoma cell line A-549 and colon adenocarcinoma cell line DLD-1 were purchased from the American Type Culture Collection (ATCC). Cells were maintained at 37˚C in a 5% CO_2 atmosphere. Both cell lines were grown in minimum essential medium containing Earle's salts and L-glutamine (Mediatech Cellgro, VA) and supplemented with 10% fetal bovine serum (Hyclone), vitamins (1X), penicillin (100 I.U/mL) and streptomycin (100 μg/mL), essential amino acids (1X) and sodium pyruvate (1X) (Mediatech Cellgro, VA).

Cytotoxicity assay: Exponentially growing cells (5×10^3 cells per well in 100 μL of culture medium) were seeded in 96-well microplates (Costar, Corning Inc.) and allowed to adhere for 16 h before treatment. Increasing concentrations of essential oil in ethanol (Sigma-Aldrich) were then added (100 μL per well). In order to avoid solvent toxicity, the final concentration of ethanol in the culture medium was maintained at 0.5% (v/v). The cells were incubated for 48 h in either the presence or absence of essential oil. Cytotoxicity was determined using the resazurin reduction test, as described by O'Brien [13]. Fluorescence was measured on an automated 96-well Fluoroskan Ascent Fl™ plate reader (Labsystems) using excitation and emission wavelengths of 530 nm and 590 nm, respectively. Cytotoxicity is expressed as the concentration of essential oil capable of inhibiting cell growth by 50% (GI_{50}).

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Volatile Leaf Constituents and Anticancer Activity of *Bursera simaruba* (L.) Sarg. Essential Oil

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Leaf volatile components of *Bursera simaruba* (L.) Sarg., a native tree from tropical America used in traditional medicine, were extracted by hydrodistillation. The essential oil was analyzed by GC-MS. We have identified 38 compounds in this oil, of which limonene (46.7%), β-caryophyllene (14.7%), α-humulene (13.2%) and germacrene D (7.6%) are the major components. The anticancer activity of the essential oil was tested on human lung carcinoma cell line A-549 and human colon adenocarcinoma cell line, DLD-1. *B. simaruba* leaf essential oil was found to be active against both tumor cell lines, with a GI_{50} of 42 ± 2 μg/mL for A-549 and 48 ± 2 μg/mL for DLD-1. The evaluation of the cytotoxic properties of the major constituents of the oil indicates that α-humulene is possibly responsible for this activity.

Keywords: *Bursera simaruba*, essential oil, anticancer activity, limonene, β-caryophyllene, α-humulene, germacrene D.

Bursera simaruba (L.) Sarg. (Burseraceae) is commonly called gommier, gommier rouge or gommier-barrière in the French West Indies. It is also well known as *almacigo* in Central and South America and as *gumbo-limbo* or West Indian birch in British Caribbean territories and Florida. This species has 50 other vernacular names and is indigenous to these areas [1, 2]. *B. simaruba*, a very common tree of dried groves, reaches a height of 5 to 10 meters (sometimes up to 25 meters) and possesses a brilliant brown reddish bark that peels off in paper thin strips. The tree trunk diameter ranges from 20 to 80 cm. Its 10 to 25 cm long leaves are deciduous, glabrous, oblong to elliptical and fragrant when crushed [3]. Many ethnobotanical studies indicate that the bark is a common topical remedy for skin affections like sores, measles, sunburns, insect bites and rashes. It is also taken internally for urinary tract infections and pain, colds, flu, sun stroke, fevers and to purify the blood. Bark infusions are drunk like tea [4]. The cytostatic properties of aqueous, alcoholic and ketonic extracts of *B. simaruba* have been proven [5]. The fruit essential oil composition of *B. simaruba* from Costa Rica was also reported: α-terpinene (26.2%), γ-terpinene (20.4%), α-pinene (18.2%) and *p*-cymene (15.9%) were the major components [6]. The widespread use of *B. simaruba* in traditional medicine prompted us to explore this plant for new biological activity and we chose to investigate its anticancer properties. So far, to the best of our knowledge, no study on the anticancer activity of this plant's essential oil has been reported. In this article, we establish the chemical composition of *B. simaruba* leaf essential oil and report the results of its testing for anticancer activity.

Leaves of *B. simaruba* extracted by hydrodistillation produced a dark yellow essential oil, the chemical composition of which is listed in Table 1. The volatile extract contained 51.4% monoterpenes (which include 0.25% oxygenated monoterpenes) and 44.1% sesquiterpenes (which include 4.6% oxygenated sesquiterpenes). Therefore, this essential oil is mainly composed of hydrocarbon compounds of which limonene is the main constituent, representing nearly half of the total percentage of the oil (46.7%). The other major components are β-caryophyllene (14.7%), α-humulene (13.2%) and germacrene D (7.6%). Some chemical compounds (4.5%) could not be identified since they were present in too small amounts.

Table 1: Chemical composition (%) of the leaf essential oil of *Bursera simaruba*.

Components	RI DB-5[a]	RI Spwax[b]	%
(*E*)-2-Hexenal	855	1226	0.17
α-Pinene	939	1024	0.64
Sabinene	974	1126	0.25
β-Pinene	976	1110	0.49
Myrcene	992	1174	1.35
p-Mentha-1(7),8-diene	999	--	0.07
α-Terpinene	1015	1183	0.11
p-Cymene	1025	1282	0.15
Limonene	1031	1201	46.69
β-Phellandrene	1031	1206	0.11
(*E*)-β-Ocimene	1057	1267	0.19
γ-Terpinene	1066	1255	0.24
Terpinolene	1098	1296	0.85
Terpinen-4-ol	1176	1600	0.17
α-Terpineol	1187	1708	0.08
α-Copaene	1375	1495	0.11
β-Bourbonene	1382	1521	0.21
β-Elemene	1389	1589	0.15
β-Caryophyllene	1414	1589	14.70
β-Copaene	1426	1585	0.10
α-Humulene	1452	1666	13.25
γ-Muurolene	1479	1690	0.54
Germacrene D	1482	1708	7.60
Bicyclogermacrene	1498	1732	0.30
α-Muurolene	1503	1728	0.25
Germacrene A	1510	1758	0.12
δ-Amorphene	1514	--	0.18
γ-Cadinene	1516	1758	0.20
δ-Cadinene	1526	1758	0.89
(*E*)-Nerolidol	1565	2053	0.19
Caryophyllene oxide	1577	1971	0.76
Humulene epoxide II	1600	2027	0.51
1-*epi*-Cubenol	1624	--	0.16
τ-Muurolol	1639	2170	0.70
τ-Cadinol	1639	2184	0.32
α-Muurolol	1643	2194	0.40
α-Cadinol	1652	2229	1.55
unidentified[c]	2027	--	0.79
Total			95.54

[a]Retention indices on apolar DB-5 column.
[b]Retention indices on polar Supelcowax 10 column.
[c]m/z (relative intensity): 69(100), 41(75), 81(34), 93(30), 107(16), 204(8).

The anticancer properties of *B. simaruba* leaf essential oil were assessed against a human lung carcinoma cell line (A-549) and a human colon adenocarcinoma cell line (DLD-1). The cancer cell lines were submitted to growing concentrations of *B. simaruba* essential oil for 48 h. The results, presented in Figure 1, show the percentage of survival of the cells versus the logarithm concentration of essential oil. The concentrations of oil inhibiting each cell line's growth by 50% (GI_{50}) were calculated from the curve. The GI_{50} values for A-549 and DLD-1 were 42 ± 2 μg/mL and 48 ± 2 μg/mL, respectively. Therefore, the low GI_{50} values obtained for both cell lines tested signify that *B. simaruba* essential oil possesses strong anticancer properties.

It has been shown that derivatives of limonene, such as perillyl alcohol and perillyl aldehyde, inhibit proliferation and migration of breast cancer cells [7]

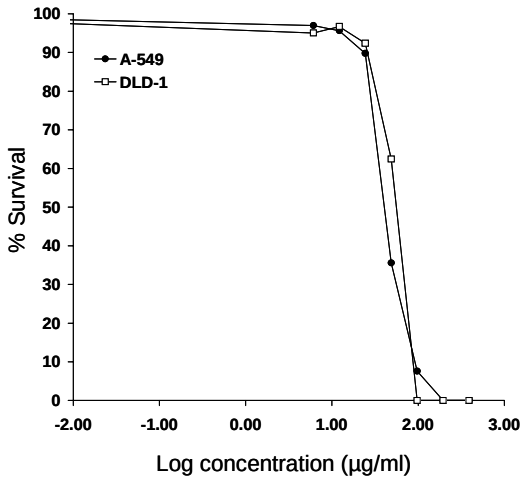

Figure 1: Anticancer properties of essential oil against human lung carcinoma (cell line A-549) and human colon adenocarcinoma (cell line DLD-1). Values represented are means of three determinations.

and cause cell cycle arrest in G1 and apoptosis of human carcinoma cell lines [8]. D-limonene is also capable of inducing apoptosis in gastric cancer cells [9]. The anticancer activities of limonene, the major components of the oil, were evaluated against A-549 and DLD-1 cell lines. The results show that limonene was inactive against A-549 and DLD-1, indicating that it is probably not responsible for the oil cytotoxicity. Another oil constituent, α-cadinol, has been reported to exhibit some selective cytotoxicity against colon cancer [10]. However, it is found in too low concentration in *B. simaruba* leaf essential oil (1.5%) to be responsible for the toxicity observed. In previous work, we reported the anticancer activity of α-humulene suggesting that it could be implicated in the activity of *B. simaruba* essential oil [11]. Indeed, the GI_{50} values of α-humulene against A-549 and DLD-1 cell lines were 62 ± 2 μM and 71 ± 2 μM, respectively [11]. The α-humulene concentration in *B. simaruba* leaf essential oil was determined using a multiple point internal standard method. The α-humulene concentration in the oil was 91 ± 1 mg/mL. Therefore, the α-humulene concentration calculated at the GI_{50} values for A-549 and DLD-1 was 48 ± 1 μM and 55 ± 1 μM, respectively. This result suggests that α-humulene can explain, in part, the cytotoxicity of the *B. simaruba* leaf essential oil. However, we do not exclude that other compounds in the oil could be active against the tumor cell lines.

In conclusion, we have determined the chemical composition of *B. simaruba* leaf essential oil and evaluated its anticancer activity. Our results clearly show that this essential oil is active against both

tumor cell lines tested (A-549 and DLD-1) and that α-humulene is responsible, in part, for the cytotoxic properties of the oil. In future studies, we will identify the unknown compounds present in this essential oil and determine their anticancer activity.

Experimental

Plant material and essential oil: Leaves of *Bursera simaruba* were collected at Fouillole, Pointe-à-Pître, Guadeloupe, in July 2002. The specimen was identified by Dr Félix Lurel (Département de biologie végétale, Université des Antilles et de la Guyane). A voucher specimen of this plant has been deposited at the Guadeloupe INRA-National Park herbarium. Essential oil was obtained from freshly harvested leaves (1041.6 g) by hydrodistillation during three h in a Clevenger apparatus [12]. The oil was dried over anhydrous sodium sulfate and stored under nitrogen at 4°C. The density of the essential oil was 0.390.

Gas chromatographic analyses: The essential oil was analysed by GC on a gas chromatograph [Hewlett-Packard 5890 (FID detector)] equipped with a polar Supelcowax 10 column and an apolar DB-5 column (30 m x 0.25 mm x 0.25 μm). Analyses by GC-MS were performed on a Hewlett-Packard mass spectrometer 5972 at 70 eV coupled to an HP 5890 equipped with a DB-5 column (same as above). The temperature program was 40°C for 2 min, then 2°C/min to 210°C and held constant for 33 min. For injection (split injector), 5 μL of essential oil was diluted to 500 μL in *n*- hexane and 5 μL of this diluted solution was used. Identification of volatile constituents was made on the basis of their retention indices and their mass spectra, which were compared with data references [13, 14].

Quantification of α-humulene: The α-humulene was analyzed by GC-MS using the same method as above. Peak identification was based on retention indices and mass spectra. An α-humulene standard was purchased from Fluka (GC purity ≥ 98%). For quantification, an eight point calibration curve was established by measuring peak areas versus response with a tetradecane internal standard (Aldrich, GC purity ≥ 99%). The calibration curve had a correlation coefficient (r^2) of 0.992 and the quantity of α-humulene in *B. simaruba* essential oil was expressed with a relative standard deviation (RSD) of 1.51%.

Cell culture: Human lung carcinoma cell line A-549 and colon adenocarcinoma cell line DLD-1 were obtained from the American Type Culture Collection (ATCC). Both cell lines were cultured in minimum essential medium containing Earle's salts and L-glutamine (Mediatech Cellgro, VA), to which were added 10% fetal bovine serum (Hyclone), vitamins (1X), penicillin (100 I.U./mL) and streptomycin (100 μg/mL), essential amino acids (1X) and sodium pyruvate (1X) (Mediatech Cellgro, VA). Cells were kept at 37˚C in a humidified environment containing 5% CO_2.

Cytotoxicity assay: Exponentially growing cells were plated in 96-well microplates (Costar, Corning Inc.) at a density of 5 x 10^3 cells per well in 100 μL of culture medium and were allowed to adhere for 16 h before treatment. Increasing concentrations of essential oil in ethanol (Sigma-Aldrich) were then added (100 μL per well). The final concentration of ethanol in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. The cells were incubated for 48 h in either the presence or absence of essential oil. Cytotoxicity was assessed using the resazurin reduction test [15]. Fluorescence was measured on an automated 96-well Fluoroskan Ascent Fl™ plate reader (Labsystems) using excitation and emission wavelengths of 530 nm and 590 nm, respectively. Cytotoxicity was expressed as the concentration of either oil or α-humulene inhibiting cell growth by 50% (GI_{50}).

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Antibacterial and Cytotoxic Activity of *Nepeta cataria* L., *N. cataria* var. *citriodora* (Beck.) Balb. and *Melissa officinalis* L. Essential Oils

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The aim of the present study was to investigate the susceptibility of bacteria that play a role in respiratory tract and skin infections to the essential oils of catnip (*Nepeta cataria*), lemon catnip (*N. cataria* var. *citriodora*) and lemon balm (*Melissa officinalis*) with regard to their chemical composition. In addition, we wanted to assess whether antibiotic-resistant and -sensitive strains differ in their susceptibility to the oils and if there are cross resistances between standard antibiotics and essential oils. To evaluate the safety of topical application, cytotoxicity of the oils was studied in human keratinocyte and bronchial epithelial cell lines and irritation threshold concentrations were determined *in ovo* using the HET-CAM-test. The composition of the essential oils was analyzed by GC and GC-MS. Their MICs and MBCs were determined by a broth microdilution method against both reference strains from culture collections and clinical isolates with different susceptibility to standard antibiotics. Cytotoxicity was assessed by the MTT assay. Except for *P. aeruginosa* (MIC ≥2 %), all reference strains tested were susceptible to catnip and lemon balm oils with MIC values ranging from 0.016 % to 0.25 % (v/v). The clinical isolates were as susceptible to the oils (± 1 serial dilution) as the corresponding reference strains, regardless of their origin and resistance to standard antibiotics. The oils were cytotoxic to both keratinocytes and bronchial epithelial cells at CC_{50} values from 0.0012% to 0.015% (v/v). Lemon balm oil, whose main components were monoterpene aldehydes, exhibited the highest antibacterial and cytotoxic activity, followed by lemon catnip oil, which contained mainly monoterpene alcohols, and catnip oil, which was characterized by nepetalactones. Our results provide a rationale for the use of catnip, lemon catnip and lemon balm oils in the complementary topical treatment of respiratory tract infections, as the oils show a high antibacterial activity against respiratory tract pathogens, including clinical isolates with reduced susceptibility to standard antibiotics. However, cytotoxicity must be considered in topical therapy.

Keywords: *Nepeta cataria*, catnip, *Melissa officinalis*, lemon balm, essential oil, antibacterial activity, respiratory tract infection, cytotoxicity.

Nepeta cataria L. (catnip) and *Melissa officinalis* L. (lemon balm) are traditional medicinal plants from the family Lamiaceae. The lemon scented chemotype *N. cataria* var. *citriodora* (Beck.) Balb. can be distinguished from *N. cataria* by the composition of its essential oil, but not by morphological properties. In addition, because of its physical resemblance and its lemon scented essential oil, lemon catnip is reported to occur as an adulterant in the herbal drug and essential oil of lemon balm [1,2]. While catnip oil is mainly composed of nepetalactones, stereoisomeric iridoid lactones with attracting properties to feline predators [3a-3c], lemon catnip oil contains mainly the monoterpene alcohols citronellol, geraniol and nerol, or their acetates, in addition to small amounts of monoterpene aldehydes [2,4,5]. Whereas Regnier *et al*. [4] found nepetalactones in lemon catnip oil, other authors could not confirm these results [2,5]. In contrast, lemon balm oil is characterized by the monoterpene aldehydes geranial, neral and citronellal [2,6]. Both catnip oils and lemon balm oil contain the

sesquiterpenes β-caryophyllene and caryophyllene oxide.

Lemon balm oil is used in aromatherapy for psychovegetative disorders, whereas for catnip oil no use in modern medicine is reported. The herbs of both species have been applied as mild sedatives and spasmolytics and they are reported to relieve chronic bronchitis and to be useful as diaphoretics for the treatment of colds. In addition, catnip has been applied topically as a cataplasm to reduce swelling in bruises and to promote wound healing, especially to prevent scar formation [7]. On the other hand, essential oils are known to exert cytotoxic activities on eukaryotic cells [8], which are caused by their ability to interact with biological membranes [9a,9b]. For lemon balm oil, an inhibitory effect on several tumor cell lines has been found at concentrations of 0.05 to 0.001% [6c] and citral, one of the main constituents of the oil, has shown cytotoxicity to skin fibroblasts and epithelial cells at the CC_{50} of 0.005 to 0.016%, depending on incubation time [8c]. In addition, citral was found to act as an inductor of apoptosis in tumor cell lines [9c] similar to other essential oils and their components [9d,9e].

Reports on the traditional use of catnip and lemon balm for the treatment of colds and coughs suggest that essential oils derived from these plants may be useful to treat respiratory tract infections. Recently, antibacterial and antifungal activity have been reported for both lemon balm oil [6b,10] and catnip oil [3b,5a], but their activity against clinically relevant respiratory tract pathogens has not been investigated so far, although this group of bacteria has shown high susceptibility to several other essential oils [8a,11].

For treatment of respiratory tract infections essential oils are either inhaled or they are both inhaled and absorbed percutaneously, i.e. when applied as an ointment to the chest or when used in bathing preparations. The part that is absorbed, and also after oral administration of essential oils, is eliminated from the body to a certain extent by exhalation, thus producing a local effect on the airways. Therefore, the oils used in this way come in close contact to epithelia of the respiratory tract and skin. Against this background, it is necessary to assess their cytotoxic potential, in order to adjust the dosage and application form so that the risk of adverse effects due to direct cytotoxicity is minimized.

Chemical characterization of essential oils tested

Essential oils are lipophilic, multi-component systems with a characteristic pattern of mainly monoterpenes, sesquiterpenes and phenylpropanoids. The specific combination of these compounds determines their different biological activities. To confirm the identity and pharmaceutical quality, the chemical composition of each essential oil was quantitatively and qualitatively analyzed by GC- and GC-MS methods. The results are listed in Table 1. The oil components were identified by comparing their mass spectral data and retention indices (relative to *n*-alkanes co-injected) with those of authentic reference substances and literature data [2,3a,4,6,12].

Lemon balm oil consisted mainly of β-caryophyllene (24.0%), geranial (20.3%), neral (14.9%) and citronellal (6.5 %), whereas the main components of lemon catnip oil were nerol/citronellol (31.1%), 4aα,7α,7aα-nepetalactone (20.4%), geraniol (19.9%), geranial (4.9%), 4aα,7α,7aβ-nepetalactone (4.4%), neral (3.7%), β-caryophyllene (3.7%) and caryophyllene oxide (2.3%). Catnip oil contained mainly 4aα,7α,7aα-nepetalactone (77.7%), β-caryophyllene (7.6%), *trans*-β-ocimene (3.3%), and caryophyllene oxide (1.8%). In summary, according to our findings, catnip oil is mainly composed of stereoisomeric nepetalactones and sesquiterpene hydrocarbons and contains only small amounts of monoterpene alcohols and aldehydes, whereas lemon catnip oil exhibits large amounts of monoterpene alcohols beside sequiterpenes and smaller quantities of stereoisomeric nepetalactones and monoterpene aldehydes. In contrast, lemon balm oil is characterized by monoterpene aldehydes (geranial/neral ratio: 4:3) and sesquiterpenes. The analytical data obtained are in agreement with those of the literature [2,3a, 3c-5b,6b,6c].

Antibacterial activity

MIC/ MBC of respiratory tract pathogens and skin commensals: Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of catnip, lemon catnip and lemon balm essential oils of different bacterial species are given in Table 2. All Gram-positive strains were susceptible to all the essential oils tested, exhibiting MIC values of 0.008% to 0.25%. The most susceptible one was *Streptococcus pneumoniae* with MIC values of 0.008 % to 0.03 %. Regarding Gram-negative bacteria, the enterobacteria *E. coli* and *K. pneumoniae* displayed only low sensitivity

Table 1: Main components of catnip oil, lemon catnip oil and lemon balm oil (in % of oil).

Compounds	Retention index OV-1 (RI)	Catnip oil	Lemon catnip oil	Lemon balm oil	Identification
α-Pinene	930	0.56	0.69		a,b,c,
Sabinene	940	0.61	0.75		a,c
6-Methyl-5-hepten-2-one	957		0.25	1.80	a,d
β-Pinene	961	0.26	0.12		a,b,c
cis-β-Ocimene	1026	0.93		0.29	a,b
trans-β-Ocimene	1036	3.33	0.09	2.84	a,b
Linalool	1083		0.38	0.94	a,b,c
trans-Chrysanthemal [t]	1124		0.29	0.65	a
Citronellal	1130		0.81	6.55	a,b,c
Nerol oxide	1141		0.23		a,b
s-*cis*-Verbenol[t]	1144			0.69	a
Menthol isomer	1158			1.17	a,b
Nerol/ citronellol	1215		31.09	1.50	a,b,c
Neral (citral b)	1217	0.10	3.71	14.95	a,b,c
Geraniol	1236		19.57	1.67	a,b,c
Geranial (citral a)	1246	0.14	4.88	20.34	a,b,c
4aα7α7aα-Nepetalactone	1331	77.7	20.37		a,b,d
4aα7α7aβ-Nepetalactone	1357	0.20	4.45		a,b,d
4aβ7α7aβ-Nepetalactone	1360	0.67	0.59		a,b,d
Geranyl acetate	1362			1.32	a,d
Dihydronepetalactone	1369	0.60	0.10		a,b,d
β-Caryophyllene	1414	7.64	3.73	24.0	a,b,c
β-Farnesene	1444	0.46	0.32		a,b
α-Caryophyllene	1447	0.59	0.28	1.87	a,b,c
Germacrene D	1475			10.1	a,b
Caryophyllene oxide	1567	1.76	2.31	0.63	a,b,c
Humulene oxide	1587	0.11	0.18		a,b

Identification: a: GC/MS data, **b:** RI, **c**: coinjection of authentic reference substance, **d:** literature data; [t]tentative identification based on mass spectral data

and *P. aeruginosa* was not affected by any of the oils tested, even at the highest concentration of 2%. In contrast, the Gram-negative respiratory tract pathogens, *H. influenzae* and *M. catarrhalis* (MIC 0.016−0.06%), were among the most sensitive of all strains tested. *A. lwoffii* was remarkably susceptible, too (MIC 0.03-0.25%). Whereas lemon catnip oil and especially lemon balm oil were bactericidal at the MIC against most strains, MBC values of catnip oil (especially against staphylococci) were one or two dilution steps above the MIC-values. Regarding the antibacterial activity, the oils can be ranked in the order: lemon balm oil > lemon catnip oil > catnip oil; however, the enterobacterial strains did not entirely fit into this ranking.

Antibacterial activity against clinical isolates: In addition to reference strains from culture collections, clinical isolates of *S. aureus*, MRSA, *S. pyogenes*, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (n = 12 for each strain, except MRSA: n = 3) were tested for their susceptibility to catnip, lemon catnip and lemon balm oils. The bacteria were derived from clinical specimens, such as throat, nose or ear swabs, sputum and wound swabs (*S. aureus*). The isolates displayed different degrees of resistance to standard antibiotics: All but two isolates of methicillin sensitive *S. aureus* were resistant to penicillin G and ampicillin, and six were resistant to ≥3 of 18 standard antibiotics tested. In contrast, the isolates of MRSA displayed multiresistance to ≥11 of 21 antibiotics tested, mainly to β-lactams, but also to macrolides and quinolones. Only two isolates of *S. pyogenes* from blood culture and a central venous catheter tip, were resistant to ≥2 antibiotics. *S. pneumoniae* isolates displayed susceptibility towards standard antibiotics, except for one strain, which was resistant to macrolides and tetracycline, and one showed intermediate susceptibility to penicillin and levofloxacin. Whereas half of the tested isolates of *M. catarrhalis* were resistant to ampicillin, none of the isolates of *H. influenzae* showed decreased susceptibility to this antibiotic. The results obtained with the essential oils under study are summarized in Table 3. Remarkably, all clinical isolates were sensitive to the oils regardless of their origin and the pattern of antibiotic susceptibility. The MIC/MBC values of our clinical isolates were homogenous and did not differ from those of reference strains by more than one serial dilution. These results suggest that there is no cross resistance between essential oils and common antibiotics. The most active substance was lemon balm oil, which, however, was only in the range of 1 to 2 dilution steps compared to the other two oils tested.

Time kill assay: To investigate time and concentration dependency of the antibacterial activity of the essential oils tested, a time kill assay was performed with *H. influenzae* and *S. pneumoniae*. The results obtained with concentrations of 0.06% (v/v) are shown in Figures 1 and 2. Lemon balm oil

Table 2: Antibacterial activity of catnip oil, lemon catnip oil and lemon balm oil against respiratory tract pathogens and skin commensals. Concentrations of essential oils are given in % (v/v).

Bacterial reference strains	Catnip oil		Lemon catnip oil		Lemon balm oil	
	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive						
Staphylococcus aureus ATCC 6538	0.13	1	0.13	0.25-0.13	0.06	0.06
S. aureus ATCC 25923	0.13	0.25	0.13-0.06	0.13-0.06	0.06	0.13
S. aureus ATCC 29213 (β-lactamase +)	0.13	0.25	0.13	0.13	0.13-0.06	0.13-0.06
S. aureus (MRSA) NCTC 10442	0.13	2-1	0.13	0.13	0.06	0.13
S. epidermidis ATCC 49134	0.5-0.25	2-1	0.13	0.25	0.06	0.13-0.06
S. saprophyticus ATCC 15305	0.25	1	0.13	0.25-0.13	0.06	0.13
Streptococcus pyogenes ATCC 12344	0.13	0.25-0.13	0.06	0.13	0.06	0.06
Streptococcus pneumoniae ATCC 33400	0.03	0.13	0.016	0.03	0.016-0.008	0.016
Gram-negative						
Escherichia coli ATCC 11229	1-0.5	2-1	1-0.5	>2	2	2
E. coli ATCC 25923	0.5	1	0.5	0.5	0.5	0.5
Klebsiella pneumoniae ATCC 10031	1-0.5	1	0.5-0.25	1-0.5	2	2
Pseudomonas aeruginosa ATCC 15442	≥2	>2	≥2	>2	≥2	>2
Acinetobacter lwoffii ATCC 15309	0.25	0.5-0.25	0.06	0.13-0.06	0.03	0.03
Moraxella catarrhalis DSM 9143	0.03	0.03	0.03	0.03	0.016	0.016
Haemophilus influenzae ATCC 33391	0.06-0.03	0.13-0.06	0.06-0.03	0.06	0.03-0.016	0.03
H. influenzae ATCC 49766 (β-lactamase+)	0.06-0.03	0.25-0.13	0.016	0.06-0.03	0.03	0.03

Table 3: Antibacterial activity of catnip oil, lemon catnip oil and lemon balm oil against clinical isolates from respiratory tract and skin. Concentrations of essential oils are given in % (v/v).

Clinical isolates	Catnip oil		Lemon catnip oil		Lemon balm oil	
	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive bacteria						
Staphylococcus aureus (n=12)	0.13	1-0.5	0.25-0.13	0.25	0.13	0.13
MRSA (n=3)	0.13	0.25-0.13	0.25	0.25	0.13	0.13
Streptococcus pyogenes (n =12)	0.25	0.25	0.13-0,06	0.13	0.06	0.06
Streptococcus pneumoniae (n =12)	0.13	0.25	0.06-0.03	0.06	0.03	0.06
Gram-negative bacteria						
Moraxella catarrhalis (n=12)	0.06-0.03	0.06	0.03-0.016	0.03-0.016	0.016-0.008	0.016
Haemophilus influenzae (n=12)	0.06	0.25-0.13	0.03	0.06	0.016	0.03

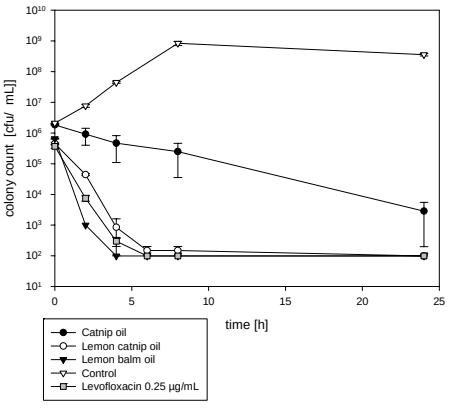

Figure 1: Time kill assay against *H. influenzae*, concentration of essential oils: 0.06% (v/v).

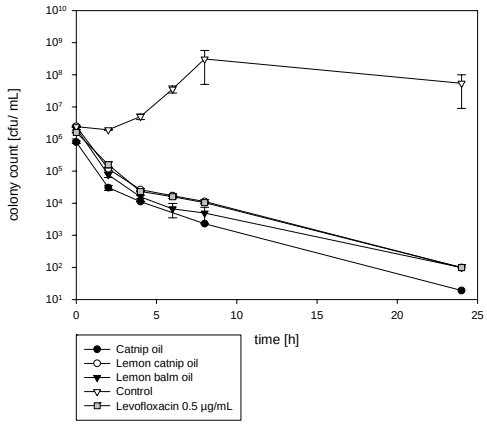

Figure 2: Time kill assay against *S. pneumoniae*, concentration of essential oils: 0.06% (v/v).

and lemon catnip oil exhibited bactericidal activity (log 3 reduction) against *H. influenzae* (Figure 1) within 2 h and 4 h, respectively, comparable to the effect of levofloxacin (0.25 µg/mL). Catnip oil displayed only bacteriostatic activity within 4 h, and gave a ≥ log 2 reduction within 24 h. The essential oils tested were nearly equally effective against *S. pneumoniae* (Figure 2): they reduced the colony number by approximately 2 log steps during 4 h and had a bactericidal effect after 24 h. Levofloxacin (0.5 µg/mL) gave a nearly identical time kill curve as lemon catnip oil. If oil concentrations of 0.13% were applied, lemon balm oil exhibited bactericidal activity within 2 h, and lemon catnip oil within 6 h, respectively, whereas the bactericidal effect of catnip oil occurred still after >8 h (data not shown).

Cytotoxic activity to human keratinocytes and bronchial epithelial cells

The CC_{50} values of the test oils obtained by the MTT cytotoxicity assay are displayed in Table 4. In the standard test, the cells were exposed to the different oil concentrations for 48 h. In addition, for catnip oil

and lemon balm oil CC_{50} values were determined in separate experiments after 4 h and 24 h of incubation.

HaCaT and BEAS-2B cells were comparably susceptible to the respective oils, and cytotoxicity decreased in the same order as in the antibacterial tests: lemon balm oil > lemon catnip oil > catnip oil. Whereas the CC_{50} of catnip oil was approximately 0.015% (v/v) for both cell lines, lemon catnip oil exerted an equally toxic effect, yet at the concentration of 0.003-0.004% and lemon balm oil at 0.001-0.002% (v/v). As expected from literature data [8b,8c,8e,8f], the cytotoxic effect increased with incubation time so that maximum cytotoxicity resulted after 48 h. At 4 h of incubation the CC_{50} values of both catnip and lemon balm oil were still within the range of 0.01-0.05% , but subsequently the toxicity of lemon balm oil increased at a higher rate with time than the toxicity of catnip oil.

Table 4: Cytotoxicity of lemon balm oil, lemon catnip oil and catnip oil to human keratinocytes (HaCaT) and human bronchial epithelial cells (BEAS-2B). CC_{50} values of the essential oils are expressed in % (v/v).

Essential oil	Incubation time [h]	HaCaT CC_{50}	BEAS-2B CC_{50}
Lemon balm	4	0.0096	0.0391
	24	0.0023	0.0030
	48	0.0017	0.0012
Lemon catnip	48	0.0025	0.0038
Catnip	4	0.0211	0.0450
	24	0.0185	0.0207
	48	0.0156	0.0151

Irritation potential in the HET-CAM test

Catnip oil, lemon catnip oil and lemon balm oil did not cause symptoms of irritation when applied to the CAM at concentrations of 25%. Subsequently, the irritation threshold concentration (ITC) was determined for catnip oil and lemon balm oil. The endpoint of evident hemorrhage within 5 min after application was obtained with both oils at a concentration of 35% (v/v). In order to compare the irritation potential of catnip and lemon balm oil to an essential oil which is well established for topical application, tea tree oil was included in the test. Its ITC was also determined as 35% (v/v).

Therapeutic considerations based on antibacterial activity and cytotoxicity data

Today, many respiratory tract pathogens are becoming increasingly resistant to common antibiotics [13,14]. Specific problems are methicillin resistant staphylococci (*S. aureus*, MRSA; *S. epidermidis*, MRSE) and vancomycin resistant enterococci (VRE). Other problems are penicillin and macrolide resistance in *S. pneumoniae*, as well as β-lactam resistance in *H. influenzae* and *M. catarrhalis*, mostly due to the formation of β-lactamases. Finally, an emerging threat is posed by ESBL (extended spectrum β-lactamase) producing enterobacteria, such as *E. coli* and *K. pneumonia*, which may be involved in hospital acquired pneumonia. For the development of resistances a correlation to prescription habits and antibiotic consumption has been demonstrated. [15]. Consequently, whenever possible, alternatives to antibiotics should be used, at least for the prevention of bacterial superinfections and topical adjuvant therapy. As shown in several studies, for example with tea tree oil in the decolonization of MRSA [16], essential oils might be among these alternative agents.

In our study, we have investigated the susceptibility of clinical isolates of the most common respiratory tract pathogens to catnip and lemon balm oils in comparison to laboratory reference strains. The good antibacterial activity of these oils, especially to the three bacterial species that are most frequently isolated from clinical specimens from the respiratory tract, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* [17], is remarkable. For *S. pneumoniae* and *H. influenzae* the data are in good agreement with literature data obtained with other oils [8a,11], whereas the susceptibility of *M. catarrhalis* to essential oils has not been investigated so far.

MIC/MBC values of catnip and lemon balm oils of clinical isolates did not differ in more than one serial dilution step from reference strains. Furthermore, even clinical isolates with multiple resistances to standard antibiotics displayed the same sensitivity as non-resistant isolates. These observations indicate that neither natural resistance to catnip and lemon balm oils, nor cross resistances to common antibiotics is present in the bacterial strains tested.

Essential oils differ in their mode of action from the common antibiotics and act probably, like biocides, on several target sites. Electron microscopic and biochemical studies of several bacteria (*S. aureus*, *E. coli*, *Bacillus cereus*) treated with different concentrations of essential oils (e.g. tea tree oil and components, oregano oil, thymol, carvacrol, eugenol) have shown that their antibacterial activity might be due to alterations in cytoplasm membrane physiology and integrity, leading to disturbances in homeostasis of pH and inorganic ions (e.g. K^+, phosphate),

respiration and energy dependent processes [18-24]. Electron microscopic investigations of *S. aureus* treated with different concentrations of tea tree oil have demonstrated that not only the cytoplasmic membrane is influenced by tea tree oil, but also structures within the cell, together with septum formation during cell division [22]. Other investigations suggested that essential oils may interfere with surface adherence and biofilm formation in staphylococci, possibly due to an altered composition of proteins at the bacterial surface and/or the capsular polysaccharide adhesins [25]. As *S. pneumoniae* expresses adhesins as well, and *H. influenzae* utilizes pili for adhesion to epithelia, an alteration of the bacterial surface structures would be likely to contribute to a decrease in their pathogenic potential. Recently pneumocci were shown to undergo autolysis when exposed to essential oils. This effect was attributed to the activation of its major autolytic enzyme *N*-acetylmuramoyl-L-alanine amidase [11c], which is also responsible for the characteristic susceptibility of pneumococci to optochin and bile salts.

The strikingly different susceptibility of Gram-negative bacteria to essential oils is probably related to the structure of the outer membrane. The outer membrane lipopolysaccharides (LPS) of *Haemophilus*, *Moraxella* and *Neisseria* spp. lack the hydrophilic *O*-polysaccharide chains (O-antigens), which are characteristic of enterobacteria and *P. aeruginosa* and is, therefore, probably more permeable to lipophilic substances like essential oils [26a,26b]. For *P. aeruginosa*, it has been demonstrated, that the outer membrane is responsible for its intrinsic resistance to tea tree oil and that permeabilization of the outer membrane may significantly increase its susceptibility to essential oils [26c]. However, besides their promising activity against pathogenic bacteria from the respiratory tract, the oils tested exhibited also cytotoxic activity *in vitro* to cells of human skin (keratinocytes) and bronchial epithelium, pointing to the fact that essential oils interact quite unspecifically with biological membranes [9a,9b,21]. It seems quite likely that mammalian cells that do not possess a cell wall are less protected against the action of lipophilic compounds, like essential oils, than bacteria. Based on the CC_{50} classification system of Halle and Göres [26d] the cytotoxicity of lemon balm oil and lemon catnip oil can be rated as moderate, and that of catnip oil as low, which is in agreement with its low systemic toxicity *in vivo*. The LD_{50} values obtained by i.p. administration in mice were 1330 mg/kg for catnip oil and 1550 mg/kg for nepetalactone [26e].

The results obtained in the HET-CAM test and cytotoxicity assay revealed that the essential oils tested were not only cytotoxic to human keratinocytes and bronchial epithelial cells, but also irritating to the chorioallantoic membrane (CAM) of the fertilized hen egg. These findings correspond very well with the assessment of the International Fragrance Research Association (IFRA), which has classified pure lemon balm oil as irritating to skin [27a]. To date, the safety of application has not been investigated for catnip oil and lemon catnip oil, as no toxicology data apart from the above mentioned LD_{50} values are available. Interestingly, tea tree oil, the essential oil of *Melaleuca alternifolia* (Myrtaceae), gave similar results in the HET-CAM test and cytotoxicity assay as lemon balm and catnip oil: ITC-value: 35%; CC_{50}-value: 0.03% for human fibroblasts and epithelial cells. Furthermore, pure tea tree oil is known as a skin and eye irritant, labelled as R36/R37 [9e, 27b]. On the other hand, several clinical trials with human volunteers and patients revealed that pharmaceutical preparations containing 5 to 10% TTO were either non-irritating or only slightly so to skin and mucous membranes [16b,28a]. These discrepant findings underscore the difficulties in transferring results from *in-vitro* cytotoxicity studies using isolated cells to the *in vivo* situation (see also [8c]). Consequently, skin irritation/tolerance tests in animals are necessary, in order to find out up to which concentration an essential oil with irritating and cytotoxic potential can be applied to skin and mucous membranes of humans or animals. Regarding catnip and lemon balm oil, the results obtained in the HET-CAM test suggest, that a concentration of 1% to 5% of either essential oil may be tolerated, when applied to undamaged skin, because both essential oils did not cause any symptoms of irritation when applied to the CAM in concentrations up to 25% (v/v). Essential oils are most frequently applied as inhalants for their secretolytic properties. Although only few *in vivo* evidence data are available, some essential oils and their components, such as citral and geraniol, have been shown to increase significantly volume output and soluble mucus content of respiratory tract fluid and to decrease specific gravity of mucus [28b,28c]. *In vitro* investigations of the antibacterial effects of essential oils in the vapor phase showed that the minimum inhibitory doses of citral and geraniol to *H. influenzae* and *S. pneumoniae* by gaseous contact were between

3.13 to 12.5 mg/L air [11b] and remarkably below the MIC values obtained in the aqueous phase. However, *in vivo* data about antimicrobial effects of essential oils and their components upon inhalation and, especially clinical studies, are missing. Some case reports about successful adjuvant inhalant treatment of pulmonary tuberculosis and chronic bronchitis with other essential oils [28d-28f] are quite promising and signal the need of further research in this field. For inhalation therapy, it is recommended to use essential oils in concentrations barely detectable by odor, at which the substances will probably not exert cytotoxic effects [28b,28c]. This recommendation is also true for the essential oils tested concerning the *in-vitro* cytotoxicity against bronchial epithelial cells.

Experimental

Essential oils: Catnip (*Nepeta cataria*) oil (d 1.063) was kindly provided by ALVA (Wallenhorst, Germany) and Paul Kaders (Hamburg, Germany). Lemon catnip oil (d 0.897) was obtained by hydrodistillation of dried plant material of *Nepeta cataria* var. *citriodora* for 4 h. Commercially available plants of *N. cataria* var. *citriodora* (Dehner Gartencenter, Rain, Germany) were grown in the botanical garden of Heidelberg University and harvested during flowering and dried at room temperature. A voucher specimen was deposited at the plant collection of IPMB, Heidelberg University. Lemon balm (*Melissa officinalis*) oil (d 0.891) was purchased from Primavera (Sulzberg, Germany).

GC-MS method: GC-MS-analysis was performed on a Hewlett Packard 5980 Series II gas chromatograph coupled to a Thermo Finnigan SSQ 7000 mass spectrometer. The GC column was a 30 m x 0.25 mm (i.d.) capillary column coated with OV-1 (0.25 µm film thickness) and with He as carrier gas (head pressure 14 psi). Temperature program: The initial column temperature of 40°C was kept for 2 min. Subsequently the column was heated to 130°C at 6°C/min and then at 10°C/min to 300°C. Injector temperature: 250°C. Electron energy was 70 eV. Alternatively, the analysis was carried out on a Perkin Elmer Clarus 500 gas chromatograph coupled to a Perkin Elmer Clarus 500 mass spectrometer. The GC column was a 30 m x 0.25 mm (i.d.) polar BP-21 column (0.25 µm film thickness) and with He as carrier gas (flow rate: 1 mL/min). Temperature program: The column temperature was heated from 60°C at 5°C/min to a final temperature of 220°C, which was kept for 10 min. Substances were identified by their retention times in relation to those of co-injected homologous *n*-alkanes, from which retention indices were calculated, in combination with their mass spectral data, which were compared to those of NIST and CAS databases, or data from authentic reference substances.

GC method: GC was performed using a Varian 3400 gas chromatograph and a PeakSimple software (version 3.0). The GC column was a 30 m x 0.25 mm (i.d.) glass capillary column coated with OV-1 (0.25 µm film thickness) and with He as the carrier gas (head pressure 14 psi). Temperature program: The initial column temperature of 60°C was kept for 2 min. Subsequently, the column was heated to 170°C at a rate of 3°C/min, and in a second step to 300°C at a rate of 10°C/min. Injector temperature: 250°C; detector FID, temperature: 300°C; injection volume: 2 µL of a 0.05% (v/v) solution of the oils in *n*-hexane. Substances were identified by their retention indices and optionally by co-injection of authentic reference substances. GC-signal area percentages were calculated by the method normalization.

Bacteria and cell lines: Bacterial reference strains were derived from type culture collections (DSMZ, Germany; ATCC, UK; NCTC, UK). Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, ATCC 29213 (β-lactamase positive) and ATCC 6538; methicillin resistant *S aureus* (MRSA) NCTC 10442, *Staphylococcus epidermidis* ATCC 49134, *Streptococcus pyogenes* ATCC 12344, *Streptococcus pneumoniae* ATCC 33400. Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10031, *Pseudomonas aeruginosa* ATCC 15442, *Acinetobacter lwoffii* ATCC 15309, *Moraxella (Branhamella) catarrhalis* ATCC 25238, *Haemophilus influenzae* ATCC 33391 and ATCC 49766 (β-lactamase positive). Clinical isolates of *S. aureus*, MRSA, *S. pyogenes*, *S pneumoniae*, *H. influenzae* and *M. catarrhalis* were obtained in the routine laboratory of the Hygiene Institute, University Hospital Heidelberg, Germany, from clinical specimens. Strains were identified and subjected to antibiotic susceptibility testing by routine methods. The strains were kept in SkimMilk (Becton Dickinson, Heidelberg, Germany) at -27°C until use.

Cell lines: HaCaT human keratinocytes were kindly provided by Dr N. E. Fusenig, DKFZ Heidelberg, BEAS-2B human bronchial epithelial cells were obtained from Dr R. Bals, University Hospital

Marburg and kindly provided by Prof. Dr A. Dalpke, Hygiene Institute Heidelberg.

Cultivation of bacteria: Prior to testing the bacteria were cultivated aerobically at 37°C on either blood or chocolate (*H. influenzae*) agar plates (Beckton-Dickinson, Heidelberg, Germany), the fastidious bacteria were incubated in a 5% CO_2 containing atmosphere (CO_2-Gen, Oxoid, Wesel, Germany). For susceptibility testing, the non-fastidious bacteria were cultivated in Iso-Sensitest broth (Oxoid, Wesel, Germany), streptococci and *M. catarrhalis* in brain heart infusion broth (Merck, Darmstadt, Germany) and *Haemophilus influenzae* in Mueller-Hinton broth (Becton-Dickinson, Heidelberg, Germany) enriched with 2% lysed horse blood, 15 µg/mL NAD+ and 5 mg/mL yeast extract.

Broth microdilution method: The antibacterial activity of catnip oil, lemon catnip oil and lemon balm oil was tested by determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) with a modified broth microdilution method, according to the DIN 58940-8 [29], as described previously [30]. Briefly, a serial dilution of the essential oil was prepared in physiological saline solution with Tween 80 (Merck) as emulsifier in a 96-well-microtiter plate, the bacterial inoculum ($5x10^5$ cfu/mL) was prepared in nutrient broth and added to the wells. After incubation at 37°C for 18-20 h, MIC was determined as lowest concentration of the essential oil that inhibited visible bacterial growth (turbidity, precipitation). MBC was determined by subcultivation of medium from wells without visible growth.

Time kill assay: A time kill assay with catnip oil, lemon catnip oil and lemon balm oil against *H. influenzae* ATCC 33391 and *S. pneumoniae* ATCC 33400 was performed according to the NCCLS guidelines [31a]. The essential oil was prepared in duplicate at several concentrations (MIC, 2 x MIC, 4 x MIC) in the appropriate medium with 0.5% Tween 80 and the mixture was inoculated with an overnight culture of the test strains adjusted to approximately 10^6 cfu/mL. Medium with 0.5% Tween 80 was used as growth control, and levofloxacin (0.25 µg/mL and 0.5 µg/mL, respectively) as positive control. Immediately after inoculation and after 2, 4, 6, 8 and 24 h of incubation at 37°C, aliquots were withdrawn from the test tubes and diluted with physiological saline solution according to the expected colony count. Different dilutions were spread onto either blood or chocolate agar plates and the colonies were counted after incubation for 24 to 48 h at 37°C in order to calculate the cfu in the test medium at the corresponding time points.

Cultivation of cell lines and cytotoxicity assay: HaCaT human keratinocytes were cultured in DMEM + GlutaMax (Gibco-Invitrogen, Karlsruhe, Germany) supplemented with 10% heat inactivated fetal bovine serum (Biochrom Berlin, Germany), 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. BEAS-2B cells were grown in RPMI 1640 (Gibco-Invitrogen), supplemented with 2 mM glutamine, 10% heat inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were kept at 37°C in a humidified atmosphere with 5% CO_2 in 25 cm^2 cell culture flasks (Greiner). Upon formation of a confluent monolayer, the cells were subcultured using trypsin-EDTA (Gibco-Invitrogen). Cytotoxicity was assayed, as described previously, [31b] by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay [31c]. Cells were seeded into 96-well plates (10^4 / well) and allowed to adhere for 48 h at 37°C. Subsequently the medium was replaced by fresh medium containing the respective essential oil dilutions to give final oil concentrations of 0.13-0.001% (v/v). Ethanol at a final concentration of 1% was used to solubilize the oils, and included as a negative control. The cells were either incubated for 48 h with the test oils, or in a modification of the test for 4 h or 24 h, respectively, before MTT (Sigma-Aldrich, Taufkirchen, Germany) in PBS was added to each well (final concentration 0.05 mg/mL) and incubated for a further 2−3 h. Subsequently the medium was discarded and the blue MTT-formazan produced by living cells was extracted using DMSO with 10% SDS and 1% acetic acid. Absorbance at 570 nm was measured with an EIA-reader (BioRad, Munich, Germany) and CC_{50}-values (CC_{50}: 50% cytotoxic concentration) were calculated from dose response curves.

HET-CAM-irritation test: The HET-CAM-test was performed, as described previously [30]. Briefly, different concentrations of the oils (in olive oil) were applied to the chorioallantoic membrane (CAM) of fertilized hen eggs and the reactions of the CAM's blood vessels were observed during 5 min after application for signs of irritation/ tissue damage. Irritation threshold concentrations were determined.

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Chemical Composition, Antiradical and Antifungal Activities of Essential Oil of the Leaves of *Cinnamomum zeylanicum* Blume from Cameroon

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The aim of the present study was to investigate the essential oil of *Cinnamomum zeylanicum* from Cameroon for its chemical composition, antiradical and antifungal activities against some common fungi causing spoilage of stored food product. The essential oil, obtained by hydrodistillation of fresh leaves, was analysed by GC and GC/MS. The oil contains 11 components among which eugenol (89.1%), linalool (4.3%), benzoate benzyl (3.1%) and cinnamaldehyde (1.5%) were the main components. Determination of antiradical activity of the oil was studied by the DPPH (diphenyl picryl hydrazyl) method. The antiradical activity of *Cinnamomum* essential oil (SC_{50} = 4.5 mg/L) was higher than that of butylated hydroxy toluene (BHT), which was used as the reference compound (SC_{50} = 7 mg/L). The growth inhibitory effect of *C. zeylanicumn* essential oil on *Aspergillus flavus* and *Fusarium moniliforme* was determined on potato dextrose agar. After 9 days of incubation on essential oil-supplemented medium, the growth of *A. flavus* and *Fusarium* was totally inhibited by 500 ppm of *Cinnamommum zeylanicum* oil. Results obtained in the present study indicate the possibility of exploiting *C. zeylanicum* essential oil to prevent diseases such as diabetes and cancer, to slow down ageing, and also to combat strains of *A. flavus* and *Fusarium moniliforme* responsible for biodeterioration of stored food products.

Keywords: *Cinnamomum zeylanicum,* yield, chemical composition, eugenol, antiradical activity, antifungal activity.

Plant essential oils and their components have been known to exhibit biological activities, especially antimicrobial, since ancient time. With the growing interest of the use of either essential oils or plant extracts in the food and pharmaceutical industries, screening of plant extracts for these properties has become of increasing importance [1].

Cinnamomum zeylanicum (Lauraceae) is a potential source of essential oils in Cameroon and other tropical areas [2]. This plant has been used for many purposes since ancient times and the leaves and the bark are used in various food applications [2]. The essential oil has previously demonstrated high fungicidal activity against *Colletotrichum musae*, *Lasiodiplodia theobromae* and *Fusarium proliferatum* [3]. We have investigated the essential oil extracted from fresh leaves of *Cinnamomum zeylanicum* from Cameroon and report herein its antiradical and antifungal activities against *Aspergillus flavus* and *Fusarium moniliforme*.

The yield of essential oil from fresh leaves of *C. zeylanicum* was 1.40% (w/w). As the results show (Table 1), the main components of the essential oil are eugenol (89.1%), linalool (4.3%), benzyl benzoate (3.1%) and cinnamaldehyde (1.5%). Previous studies have reported the chemical profile of *C. zeylanicum* essential oils from different localities: Sri Lanka [3,4], Bangalore and Hyderabad [5]. In

Table 1: Essential oil composition of fresh leaves of *Cinnamomum zeylanicum* identified by GC and GC/MS.

Compound	Retention index	Percentage
α-Pinene	935	0.1
Limonene	1026	0.5
Linalool	1088	4.3
Cinnamaldehyde	1251	1.5
Eugenol	1351	89.1
α-Copaene	1388	0.1
α-Cedrene	1413	0.3
β-Caryophyllene	1434	0.5
α-Humulene	1468	0.2
Caryophyllene oxide	1596	0.2
Benzyl benzoate	1852	3.1

general, the profile obtained from the GC analysis of the essential oil used in this experiment was similar to those described by other authors, although the eugenol content was slightly different. The eugenol content was 76.6%, 81.4% and 84.5%, in the samples from Sri Lanka, Bangalore and Hyderabad, respectively, while a content of 89.1% was obtained in the present experiment. This confirmed the fact that the extracts obtained from a plant can vary according to agronomic conditions, the harvest time and the type of processing followed [6].

Table 2: Scavaging capacity of BHT, eugenol and *C. zeylanicum* expressed as SC_{50}.

Compounds	SC_{50}, mg/L
BHT	7
Eugenol	1.8
C. zeylanicum essential oil	4.5

From Table 2, it can be observed that *C. zeylanicum* exhibited very strong radical scavaging capacity (RSC). This RSC was higher than that of BHT used as the reference compound. This result is consistent with the results of other researchers, who found that cinnamon oil obtained from Sri Lanka had strong antiradical capacity [4]. The oil of *C. zeylanicum* showed a higher RSC than those of *Plectranthus grandis* and *P. ornatus* [7], *Laurus nobilis* and *Foeniculum vulgare* subsp. *piperitum* [8] and *Clausena anisata* [9]. This strong RSC of *C. zeylanicum* oil is probably due to its higher yield of eugenol. This compound, which is used as a flavoring agent in cosmetic and food products, has both pro-oxidant and antioxidant activities [10].

C. zeylanicum leaf oil was fungistatic against *Aspergillus flavus* and *Fusarium moniliforme* (Figure 1). There were significant differences in the mycelial growth of oil-supplemented samples compared with the control (ANOVA and Duncan

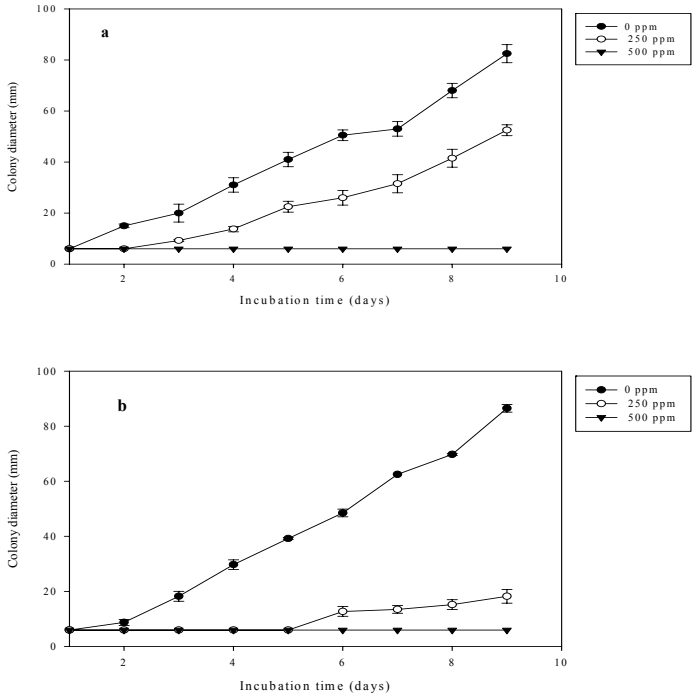

Figure 1: Effect of different concentrations of *C. zeylanicum* essential oil in PDA medium on *A. flavus* (a) and *F. moniliforme* (b).

Multiple Range Test, $P < 0.05$). At 500 ppm, fungal development was completely inhibited over 9 days of incubation. Essential oil at 250 ppm inhibited development of *Fusarium moniliforme* during the first five days and that of *Aspergillus flavus* during the two first days. Our results are consistent with the results of other researchers, who found that cinnamon oil had strong and consistent inhibitory effects against various pathogens [3,11]. The antimicrobial activity has been attributed to the presence of some active constituents in the oil. Our GC/MS study revealed eugenol to be the major constituent of *Cinnamomum zeylanicum* oil. Eugenol, reported by different workers to be the main component of cinnamon leaf, is also responsible for the antifungal effect of this oil [11]. It has been reported that total inhibition of *Penicillium citrinum* was achieved by adding 2000 ppm of eugenol and thymol to the liquid medium [12]. Earlier study found eugenol to be the active compound responsible for fungal inhibition produced by clove essential oil [11], but the authors raised the possibility that interactive effects of other compounds present in smaller quantities may also contribute. In this respect, GC/MS analysis revealed the presence of linalool and cinnamaldehyde in the essential oil used in our experiment. Earlier studies also suggested the antimicrobial activity of cinnamaldehyde [11] and linalool [13]. Although in

minor percentages, these compounds together with the major compound identified, for example eugenol, can be considered as the antifungal constituents of the oil of *C. zeylanicum*.

The present study showed the antiradical and antifungal activities of the essential oil of *Cinnamomum zeylanicum* leaves against *A. flavus* and *F. moniliforme*. Its use in granaries could help to prevent the growth of these fungi, which are known for their ability to alter the nutritional and organoleptic qualities of stored food products.

Experimental

Plant material: Fresh leaves from *Cinnamomum zeylanicum* were collected from the Botanical Garden of Limbe (southwest Cameroon) in April 2006 and identified at the National Herbarium of Yaounde (Cameroon), where voucher specimens are deposited. The leaves were steam-distilled for about 5 h using a Clevenger apparatus. Oils recovered were dried over anhydrous sodium sulfate and stored at 4°C until used.

Analysis of essential oils: The essential oil obtained was analyzed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS).

Gas chromatography: The oil was analyzed on a Varian CP-3380 GC with flame ionization detector fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB5, film thickness 0.25 μm); temperature program 50°-200°C at 5°C/min, injector temperature 200°C, detector temperature 200°C, carrier gas N_2 1 mL/min. The linear retention indices of the components were determined relatively to the retention times of a series of *n*-alkanes and the percentage compositions were obtained from electronic integration measurements without taking into account relative response factors.

Gas chromatography/mass spectrometry: GC/MS analyses were performed using a Hewlett-Packard apparatus equipped with an HP1 fused silica column (30 m x 0.25 mm, film thickness 0.25 μm) and interfaced with a quadrupole detector (GC- quadrupole MS system, model 5970). The column temperature was programmed from 70°-200° at 10°C/min; injector temperature was 200°C. Helium was used as the carrier gas at a flow rate of 0.6 mL/min; the mass spectrometer was operated at 70 eV.

Identification of the components: The identification of the constituents was assigned on the basis of comparison of their retention indices and their mass spectra with those given in the literature [14,15].

Determination of antiradical activity: The antiradical activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) [16] free stable radical scavenger, which was dissolved in ethanol to give a 100 μM solution. To 2.0 mL of the ethanolic solution of DPPH was added 100 μL of a methanolic solution of an antioxidant reference (BHT, eugenol) at different concentrations. The oil was tested using the same method. The control, without antioxidant, is represented by the DPPH ethanolic solution containing 100 μL of methanol. The decrease in absorption was measured at 517 nm after 30 min at room temperature. The actual decrease in absorption induced by the test compound was calculated by subtracting that of the control. The concentration required for 50% reduction (SC_{50}) was determined graphically. All the spectrophotometric measurements were performed with a SAFAS UV-mc^2 spectrophotometer, equipped with a multi-cell/multikinetics measuring system and with a thermostated cell-case.

Antifungal activities

Fungal strains: The strains of *Aspergillus flavus* and *Fusarium moniliforme* used as test microorganisms were obtained from the Microbiology Laboratory of the National School of Agro-Industrial Sciences (University of Ngaoundere, Cameroon). The microorganisms were grown on Sabouraud dextrose agar (Difco, Detroit, MI) plates at 25°C for 5 days. Ten mL of 1% Tween 20 was added for collection of the spores. Conidia were harvested by centrifugation at 1000 x g for 25 min and washed with 10 mL of sterile distilled water. The spore suspension was stored in sterile distilled water at 4°C until used.

Antifungal assay: Antifungal assay was performed using the agar disc diffusion method [17]. Potato dextrose agar (PDA) medium with different concentrations of essential oils (250, 500, 750 or 1000 mg/L) were prepared by adding the appropriate quantity of essential oil to the melted medium, followed by manual rotation of the Erlenmeyer flask to disperse the oil in the medium. About 20 mL of the medium was poured into glass Petri–dishes (9 cm x 1.5 cm). Each Petri–dish was inoculated at the centre with a mycelial disc (6 mm diameter) taken at the periphery of an *A. flavus* colony grown on PDA for

48 h. Control plates (without essential oil) were inoculated following the same procedure. Plates were incubated at 25°C and the colony diameter was recorded each day. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of essential oil in which no growth occurred.

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Antifungal and Anti-insect Activities of Three Essential Oils on *Aspergillus flavus* Link and *Sitophilus zeamais* Motsch

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Combinations of equal volumes of essential oils of *Ocimum gratissimum, Lippia rugosa* and *Xylopia aethiopica* were studied for their inhibiting activity on the mycelial growth of *Aspergillus flavus* by the determination of the minimum inhibiting concentrations (MIC), and on *Sitophilus zeamais* by the determination of the LV_{50} (volume that kills 50% of insects) and LV_{90} (volume that kills 90% of insects) values. All the combinations led to an increase in the inhibition of the mycelial growth of *A. flavus* with a synergy between these oils. The interesting combination of *O. gratissimum* and *L. rugosa* increased the inhibition of the mycelial growth of *A. flavus*; the observed MIC (600 ppm) was significantly lower than that predicted (900 ppm). Concerning anti-insect activity, a slight reduction in the LV_{90} on *S. zeamais* was observed for the combination of the three oils. The binary combinations showed higher LV_{50} and LV_{90} values than those predicted. There was no synergistic anti-insect activity between the three essential oils.

Keywords: synergy, combination, essential oil, *Aspergillus flavus*, *Sitophilus zeamais.*

The invasion of various food products by insects and moulds is often the cause of their losses in quality and quantity. The uses of synthesized chemicals like pesticides and fumigants are a great contribution to the fight against these pests, but have also enormous environmental and health problems due to toxic residues and their carcinogenicity [1]. The use of substances of vegetable origin, such as essential oils, is considered more and more as an alternative, as they are largely accessible and without danger to agriculture and the environment [2,3]. The combination of preservatives in the protection of the foodstuffs also seems an alternative being able to ensure good safety while reducing the amount of each substance in the application. The individual use of some aromatic compounds in the protection of foodstuffs requires them to be applied in high concentrations, which often exceeds the threshold of acceptable flavour to the consumer [4]. A combination of aromatic compounds, like thymol, carvacrol, eugenol, citral and geraniol, increased the inhibition of the mycelial growth of certain fungi stocks, with a synergy between these compounds when thymol was added at low doses [4]. This current study presents the anti-insect and antifungal activities of the balanced combination of three essential oils (*Ocimum gratissimum* (*O.G.*), *Lippia rugosa* (*L.R.*) and *Xylopia aethiopica* (*X.A.*) on the mycelial growth of *Aspergillus flavus* and on *Sitophilus zeamais*.

The essential oil yields obtained for the three plants were 0.47, 0.61 and 3.0% v/w, respectively, for *L. rugosa*, *O. gratissimum* and *X. aethiopica*. The chemical compositions of the three oils have been published [5-7]. The main constituents of *X. aethiopica* [5] oil were β-pinene (18.3%), terpinen-4-ol (8.9%), sabinene (7.2%), α-phellandrene (7.1%), α-terpineol (4.1%), α-pinene (3.2%) and *trans*-β-ocimene (3.1%). The oil of *O. gratissimum* [6] contained thymol (53.9%), γ-terpinene (17.8%), *p*-cymene (3.9%), myrcene (2.5%), β-caryophyllene (2.8%), α-terpinene (2.0%), limonene (2.0%). *L. rugosa* oil [7] components were geraniol (51.5%), nerol (18.6%), geranial (10.4%), linalool (4.6%), and myrcene (1.6%).

Minimum inhibiting concentration (MIC) of the various formulations of essential oils: The results obtained (Table 1) show a significant influence of the time of incubation on different MICs from oils alone and combined ($R > 0.8$; $p = 0.000$). Thus the MIC increased from the first day to the eighth day of incubation, respectively for *O. gratissimum, L. rugosa* and *X. aethiopica* from 400 to 800 ppm, 400 to 1000 ppm, and 4800 to 11,200 ppm. The balanced combination of oils of *O.G. + L.A.* inhibited the mycelial growth of *A. flavus* from 400 to 600 ppm; while for others combinations *O.G. + X.A., L.R. + X.A., O.G. + L.R. + X.A.,* MICs ranged from 400 to 1000 ppm, 600 to 1400 ppm and 400 to 1000 ppm, respectively. The MIC increased with the time of incubation, as had been observed by others researchers [8,9], and could be explained by the evaporation of some compounds in the culture medium during the incubation period.

The combinations, in general, increased inhibition of mycelial growth. The higher fungal activity of *O. gratissimum* is likely due to thymol, which has been established on other strains [4,7]; the activity of *L. rugosa* oil could be explained by the higher concentrations of geraniol and geranial [7]. *X. aethiopica* oil, containing mainly monoterpene hydrocarbons, was less active. The combination with either *L. rugosa* or *O. gratissimum* improved its activity. The combination of *O. gratissimum* + *L. rugosa* increased inhibition of mycelial growth of *A. flavus*; the observed MIC (600 ppm) was significantly lower than the predicted value (900 ppm). There is some interesting synergy in antifungal activity with this combination. Combinations of aromatic compounds have been shown to synergistically increase the inhibition of microbe growth [10-15].

LD_{50}, LV_{50}, LD_{90} and LV_{90} values of various essential oils: From the probit analyses [16,17], the calculated regression line equations of the 2, 12, 22, and 24 h data for the oils and the combinations were determined and the LD_{50} values calculated (Table 2). Using LD_{50} and LD_{90}, the lethal volumes were calculated for 24 h (Table 3). No significant difference ($P < 0.05$) is observed between the LV_{50} of the three oils. On the other hand, a variation in the level of their LV_{90} values is seen, but only the values of *O. gratissimum* (32 µL) and *L. rugosa* (52.7 µL) present a significant difference ($P < 0.05$). The balanced combination of oils of *O. gratissimum* and *L. rugosa* gave a LV_{50} of 11.50 µL, which is not different from their respective LV_{50} values (11.3 µL). However, the LV_{90} of this combination (19.9 µL) is significantly different from the respective values of the two oils (32.2 µL and 52.7 µL). One thus notes a reduction of 12.3 µL in the LV_{90} compared to the essential oil of *O. gratissimum* and 32.8 µL compared to that of *L. rugosa.* The combinations of *O. gratissimum* + *X. aethiopica* and *L. rugosa* + *X. aethiopica* gave LV_{50} values of 25.9 and 33.2 µL, which were significantly higher than the values of the three oils. There is also a difference between the LV_{90} (60 µL) of the mixture *X. aethiopica* + *L. rugosa* and their individual values (52.7 and 38.5 µL).

The balanced combination of *O. gratissimum* + *L. rugosa* did not show any interaction because the LV_{50} and LV_{90} predicted by the regression equation (10.3 and 19.1 µL) and are not significantly different from those obtained in experiments (11.5 and 19.9 µL). On

Table 1: MIC (ppm) of three essential oils and their combination, according to the time of incubation.

	Incubation time (days)							
Essential Oils	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
O.G.	400	400	400	600	600	800	800	800
L. R.	400	400	600	600	800	800	800	1000
X. A.	4800	8800	9200	10000	10400	10800	10800	11200
O.G. + L.R.	400	400	400	400	600	600	600	600
O.G. + X.A	400	600	600	800	1000	1000	1000	1000
L.R. + X.A.	600	800	1000	1000	1200	1200	1400	1400
O.G. + L.R. + X.A.	400	600	600	800	800	800	1000	1000

O.G.:Ocimum gratissimum, L.R.: Lippia rugosa and *X.A.: Xylopia aethiopica*

Table 2: LD_{50} and LD_{90} of various essential oils and their combinations for various times (2, 12, 22, and 24 hours).

Times (h)	Slope ± SE	R^2	LD_{50} (ppb)	LD_{90} (ppb)
Ocimum gratissimum				
2	1.74 ± 0.38	0.69	396	2145
12	2.05 ± 0.31	0.74	151	641
22	1.93 ± 0.50	0.84	96	442
24	2.87 ± 0.75	0.85	70	195
Lippia rugosa				
2	0.65 ± 0.52	0.80	127015	12144809
12	1.43 ± 0.24	0.86	144	1131
22	1.89 ± 0.53	0.91	72	341
24	1.98 ± 0.53	0.91	70	313
Xylopia aethiopica				
2	1.15 ± 0.45	0.51	3440	45103
12	2.07 ± 0.26	0.71	175	728
22	2.58 ± 0.57	0.81	91	285
24	2.78 ± 0.50	0.84	80	232
Ocimum gratissimum + Lippia rugosa				
2	1.00 ± 0.70	0.77	4124	79220
12	3.86 ± 0.68	0.71	111	239
22	4.33 ± 0.55	0.92	72	143
24	5.53 ± 0.65	0.93	71	122
Ocimum gratissimum + Xylopia aethiopica				
2	2.04 ± 0.70	0.43	724	3080
12	3.78 ± 1.27	0.49	205	447
22	4.90 ± 1.24	0.56	166	303
24	5.06 ± 1.22	0.79	158	283
2	0.84 ± 0.88	0.74	24684[c]	838863[c]
12	2.76 ± 0.54	0.86	248	722
22	4.59 ± 1.12	0.88	199	378
24	5.19 ± 1.55	0.87	201	354
2	0.16 ± 0.41	0.78	1.10e13[c]	1.76e21[c]
12	2.61 ± 0.38	0.71	126	369
22	2.67 ± 0.50	0.86	77	234
24	2.58 ± 0.50	0.88	74	231

the other hand, for the combinations *O. gratissimum* + *X. aethiopica,* the predicted LV_{50} and LV_{90} (12.2 µL and 20.9 µL) are considerably lower than those obtained (25.9 µL and 47.4 µL). In the case of *L. rugosa* + *X. aethiopica*, the predicted LV_{50} and LV_{90} (10.9 µL and 20.4 µL) are lower than those obtained in the experiments (33.2 µL and 60 µL). Concerning the balanced combination of three oils, the LV_{50} (4.15 + 2.95 + 4.6 = 11.7 µL) predicted and obtained (12 µL) do not differ. However, the LV_{90} predicted (6.46 + 5.3 + 7.3 = 19.06 µL) and obtained (38.4 µL) are different. From these results we could conclude that there is no synergistic anti-insect activity between the three essential oils.

Table 3: LV_{50} and LV_{90} values of various essential oils and their combinations.

Essential oils	LV_{50} (µL)	LV_{90} (µL)	LV_{50} (µL) calc	LV_{90} (µL) calc.
O.G.	11,3[a]	32,2 [a,b]		
L.R.	11,3[a]	52,7 [c,d]		
X.A.	13 [a]	38,5 [b,c]		
O.G. + LR	11,5[a]	19,9 [a]	10.3	19.09
O.G.+ X.A	25,9b	47,4 [b,c]	12.16	20.93
L.R.+ X.A.	33,2 [b,c]	60 [d]	10.95	20.36
O.G.+ L.R.+ X.A.	12 [a]	38,4 [b,c]	11.7	19.06

The values followed by the same letter in the same column are not significantly different (P > 0.05).

Experimental

Plant collection: Fresh leaves of *O. gratissimum,* and *L. rugosa* were collected in the locality of Dang-Bini in March 2005 and the dry fruits of *Xylopia aethiopica* were bought at the market of Ngaoundéré. Essential oils were obtained by hydrodistillation using Clevenger type equipment for five hours and stored at 4°C until their use for the bioassays.

GC/MS chemical analysis: GC/MS analysis utilized an HP-5MS column (5% phenyl methyl siloxane), 30 m long and 250 µm in diameter. The carrier gas was helium; the temperature program applied was from 40°C to 230°C at a rate of 5°C/min and then maintained at 230°C for 5 min. The pressure of the carrier gas was 49.9 KPa with a flux of 74.1mL/min. The ion-source temperature was 230°C and the ion scan range was 50-350 amu. The mass spectrum of each compound was compared with those of the Wiley 275 L library [18,19].

Insects: Insects used for the test were reared in the *in vivo* collection at the Storeprotect laboratory at the University of Ngaoundéré in Cameroon. They were derived from a strain collected at Ngaoundéré in November 2003.

Microbial stock: The mould, *Aspergillus flavus* is a pure aflatoxinogenic stock provided by the Laboratory of Microbiology at the University of Ngaoundéré in Cameroon.

Quantitative evaluation of the inhibiting activity of oils on the growth of A. flavus: The MICs (Minimum Inhibiting Concentrations) of essential oils were determined according to the standard method [20]. Sabouraud medium was prepared and, after sterilization, suitable quantities of essential oils were added in order to obtain concentrations of 200, 400, 600, 800, 1000, 1200, 1400, 1600 ppm, up to 11,200 ppm. The mixtures were homogenized and poured into Petri dishes (9 cm). A fragment of mycelium, 6 mm in diameter, was taken from the periphery of a 2 days old pre-culture of the stock and deposited in the centre of the dish and the unit was incubated at 25 ± 2°C. Controls without essential oil were inoculated in the same way. The experiment was repeated three times for each concentration. The diameters of mycelia growth were recorded each day for 8 days.

Insecticidal activity: In preliminary tests, several doses were chosen between those having no killing

effect on the experimental population to the minimal one killing 100%, in order to establish the LD_{50} of each essential oil. With a micropipette (Rainin Magnetic-assist), the precise volume of essential oil was added to acetone and diluted to 5 mL. From this, 0.5 mL of solution was uniformly applied to a 9 cm disk of filter paper (Whatman N°1) and placed in a Petri dish. Twenty adult insects, less than one month old, were introduced into the dish and 5 min later the dish was covered. A control with acetone alone was made. For each preparation, 5 replications were made. The number of dead insects was determined 2, 12, 20 and 24 h after the application.

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Biological Activities of Selected Essential Oils

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The manuscript reviews the broad spectrum of biological activities associated with essential oils. From the analyses of the data it is evident that essential oils could have tremendous application in the therapeutic, food, agrochemical and poultry industries.

Keywords: essential oils, biological activities.

Essential oils are the volatile components of plants usually extracted by steam distillation using a Clevenger type apparatus [1a]. Essential oils were used in ancient Rome, Greece and Egypt and throughout the Middle and Far East as perfumes, food flavours, deodorants and pharmaceuticals [1b]. The present review selects articles across the broad spectrum application of essential oils that highlight their versatility for use in various industries. The compounds present in essential oils can serve as prototypes for the development of therapeutic agents [1c]. Several reports exist on the biological activities of essential oils [1d], thus in the present article the authors have selected examples that have the greatest immediate application for use to highlight their versatility.

Pharmaceuticals: Essential oils have a wide range of pharmaceutical application; these include antimicrobial, anti-inflammatory, anti-malarial, cytotoxic, nematicidal and anti-oxidant properties[1d].

Antifungals: From the data presented in Table 1, the essential oil of *Hyptis ovalifolia* could have immediate application for development as an anti-fungal agent based on a comparative analysis of MIC (minimum inhibitory concentrations) values with commercial agents on *Trichophyton rubrum* [1c]. A similar trend in lower MIC values of the essential oil relative to the positive controls highlights the greater efficacy of *Achillea biebersteinii* essential oil over the commercial standard shown in Table 2.

Table 1: Antifungal activities of *H. ovalifolia* on the dermatophyte; *Trichophyton rubrum*.

Dermatophyte	MIC values (µg/mL)
Trichophyton rubrum	7.8
Terbinafine[a]	10.0

[a]Positive control.

Table 2: Antifungal activities of *Achillea biebersteinii*.

Fungal species	MIC values (µg/mL)
Fusarium acuminatum	15.62
F. oxysporum	15.62
Rhizopus species	31.25
Sclorotinia minor	15.62
Amphotericin B[a]	
F. acuminatum	62.50
F. oxysporum	62.50
Rhizopus species	125.0
Sclorotinia minor	125.0

[a]Positive control.

Antioxidants in the food industry: The synthetic anti-oxidants, such as butylhydroxyanisole (BHA) and butyl hydroxytolune (BHT) are used as preservatives in foods and food packaging. These anti-oxidants are used to delay the deterioration of food flavours and odours and increase the shelf life of many foods [2a]. However, interest is growing internationally for herbal products, such as essential oils, to replace the synthetic anti-oxidants based on their emerging deleterious side effects. For example, Takahashi *et al*. revealed that when BHA is administered in the diet of rats it induced papillomas and squamous cell carcinomas in their fore-stomach [2b]. One of the essential oils that has demonstrated significant potential as a replacement for the synthetic anti-oxidants based on its preservation effects is rosemary (*Rosmarinus officinale*) [2c]. In

addition, the antioxidant properties of the essential oils of oregano, dittany, thyme, marjoram and lavender have been reported [2d].

Antibacterials: In the cases of antibacterial activity, the MIC values of essential oils seem to be generally larger than those of commercial standards and thus indicating that the oils are of a lower order of toxicity to pathogens**.** However, based on the side effect profiles of known anti-bacterial agents, essential oils could serve as replacements since some are known to be of low toxicity, as highlighted in Table 3 for *Cinnamomum zeylanicum*, an edible oil [2f].

Table 3: Antibacterial activity based on the diameter of inhibition zones, with MIC values in parentheses, for *Cinnamomum zeylanicum* essential oil on *Bacillus subtilis, Klebsiella pneumoniae* and *Escherichia coli.*

Material	Bacterial species	Zones of inhibition (mm) at 50 µg/mL
C. zeylanicum	*B. subtilis*	22.8 ± 0.2 (>1.6 mg/mL)
	K. pneumoniae	18.6 ± 0.5 (3.2 mg/mL)
	E. coli	21.0 ± 0.2 (>1.6 mg/mL)
Streptomycin	*B. subtilis*	26.9 ± 0.5
	K. pneumoniae	20.9 ± 0.9
	E. coli	21.2 ± 0.1

[a]Positive control at 25 µg/mL.

Essential oils in the agrochemical industry: The need to replace synthetic pesticides, such as the organophosphate group of insecticides, is reflected in the widespread contamination of the environment [2e]. Various formulations of plant extracts, including the essential oils, have demonstrated promise in replacing the persistent agrochemicals. For example, the essential oil of *Hyptis verticillata* has demonstrated acaricidal action on *Boophilus microplus* and insecticidal activity on *Cylas formicarius elegantulus*, two economically important pest species [2g]. Thus, a 48 hour LD_{50} value (dose of either essential oil or insecticide required for killing 50% of the test insects) of 0.4 µL/g insect compared with a value of 0.13 µL/g insect was reported for dimethoate on adult *Cylas formicarius* [2g]. *Hyptis verticillata* oil disrupted the oviposition and hatching of *Boophilus microplus* eggs; however, it was not very toxic to the adult ticks [2g]. Essential oils are also applicable in the fumigation process of stored product pests. For example, the oil of *Ocimum basilicum* at 12.5% (w/w) inflicted 99% mortality after 24 hours, relative to a control value of 0.0% [2h].

The mosquito larvicidal activities of some essential oils have been documented. For example, those of *Ocimum gratissimum*, *O. americanum*, *Lippia sidoides* and *Citrus citratus* gave LC_{50} values (concentrations of essential oils required for killing 50% of the test populations) of 60 ppm, 67 ppm, 63 ppm and 69 ppm, respectively, on the larvae of *Aedes aegypti* [2i].

Application in the broilers industry: Essential oils containing carvacrol, such as thyme oil, when included at concentrations ranging from 20 to 200 ppm, induced weight gain and feed intake in chickens [2j]. Essential oils are known to play a part in the selective uptake of dietary amino acids. For example, cinnamaldehyde and eugenol, two of the main components of clove oil, when fed at dietary concentrations of 1000 ppm and 850 ppm, significantly impaired the absorption of alanine in rat jejunum [2d].

The present manuscript provides information on the wide range possibilities of the application of essential oils for commercial development.

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Antifungal Activity of the Volatile Phase of Essential Oils: A Brief Review

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Interest in the antifungal activity of essential oils has increased markedly in recent years. The volatile (vapour) components of several essential oils have been demonstrated to have potent antifungal activity, often in excess of that displayed in direct contact assays. A lack of consistent methodology and reporting, however, hinders direct comparison of publications. A variety of mechanisms have been suggested for the activity of these active volatiles against hyphate fungi. This paper briefly reviews some of the more recent data and identifies areas that require standardization and further study.

Keywords: Essential oil, volatiles, antifungal.

Interest in the bioactivity of essential oils and antimicrobial activity in particular has increased significantly in recent years. Most of these studies have examined the direct effect of essential oils on a range of microorganisms. However, unlike the majority of antimicrobial agents, essential oils also have a potentially bioactive vapour (volatile) phase, some of which have been demonstrated to have antimicrobial activity that acts in the absence of direct contact. Surprisingly, it has been noted that the inhibitory effect of these oils on fungi can be greater when the oil volatiles are used rather than when the fungi come into direct contact with liquid oil [1-3].

In response to the growing problem of antibiotic resistance, there has been increasing interest in the potential use of these volatiles as disinfectants and preservatives and their possible therapeutic applications, particularly for respiratory and superficial fungal infections [4-11].

It is believed that the use of essential oil volatiles has several benefits over direct application of the oils themselves, namely reduced toxicity (compared to direct contact) and ease of application, whether in an enclosed airspace or via inhalation. Inhalation of essential oil volatiles may have particular relevance in the treatment and/or prevention of lung infections; indeed inhalation of oil volatiles has already been used for symptomatic relief of conditions, including bronchitis and sinusitis, and some oil volatiles have been shown to reduce the symptoms of asthma [12-14].

Although the antifungal activity of essential oil volatiles was first reported in 1959, specific antifungal activity associated with these volatiles has, until recently, focused on inhibition of either food spoilage or post-harvest plant pathogens, with little known about their potential activity against medically important fungi [7, 15-26].

Antifungal Activity

The vapour phases of essential oils serve a variety of functions in plants. They are utilised by plants as a means to attract pollinating insects, are believed to play a role in communication between plants and act as a natural defence mechanism against pathogens and predators, whether microbial, insects or herbivores [21,27,28].

Essential oil volatile compounds are defined as low molecular weight lipophilic molecules that have a tendency to volatise at relatively low temperatures [21,29]. In comparison to their plant counterparts, essential oils contain a high concentration of these volatile agents [22]. Volatile essential oils contain a

Table 1: Examples of oils within major activity groupings and average MFD (μg oil/mL air).

Major Component of oil	Example of plants in this group	Average MFD (μg/mL air)
Phenol	Clove (*Eugenia caryophyllata*)	1.56
	Oregano (*Oreganum vulgare*)	
Aldehyde	Lemongrass (*Cymbopogon citrates*)	3.13
	Cinnamon bark (*Cinnamonum zeylanicum*)	
Alcohol	Citronella (*Cymbopogon nardus*)	12.5
	Lavender (*Lavandula angustifolia*)	
Ketone	Spearmint (*Mentha spicata*)	25
	Caraway (*Carum carvi*)	
Ester	Valerian (*Valeriana officinalis*)	50
	Helichrysum (*Helichrysum italicum*)	
Ether/oxide	Rosemary "camphor" (*Rosmarinus officinalis*)	50
	Myrtle (*Myrtus communis*)	
Hydrocarbon	Frankincense (*Boswellia carteri*)	≥100
	Lemon (*Citrus limonum*)	

Adapted from Inouye *et al*, 2006 [9].

complex mixture of compounds that are mostly composed of monoterpenes and sesquiterpenes [30]. Major compounds include alcohols, aldehydes, esters, ketones, phenols, oxides, coumarins and phenylpropenes [30].

A wide range of essential oil volatiles have been demonstrated to have activity against a range of both hyphate fungi and yeast, including both animal and plant pathogens [3,7-10,22,26,31-35]. A potential correlation between the major components of essential oils and the oil/oil volatiles antifungal activity has led to the suggestion that these oils should be grouped by major active compounds rather than plant species [9,36].

Inouye *et al*. (2006) recently reported the vapor activity of 72 essential oils against *Trichophyton mentagrophytes* which demonstrated that those essential oils with phenol as the major component displayed the most potent vapour activity [9]. In descending order of activity, from most to least potent, they suggest that the order of potency of essential oil vapours can be determined as those rich in phenol > aldehyde > alcohol > ketone > ester (= ether/oxide) > hydrocarbon. Examples of oils in each of these groups and average MFD (minimum fungicidal dose) are shown in Table 1.

Some variation in this potential link between most abundant constituent and activity has been reported [17,37]. For example, Kalemba and Kunicka (2003) reported that, in their study, ketone oils are in fact more active than alcohol oils [37]. While this delineation of activity is, therefore, not definitive, as some variation in activity does occur within groups, it does provide a general ranking of likely activity.

Similarly, the role of synergism between essential oil components remains unclear. Lis-Balchin *et al*. (1998) suggested that any synergism exhibited with less abundant components of the oils is unlikely to be of significance. However, others report that synergism between components does play an important role [22,38]. For example, it has been demonstrated that application of the major constituents of basil oil (linalool and eugenol), when applied individually, did not produce the fungicidal activity demonstrated when the two compounds were applied simultaneously in the same ratios as present in native basil oil [22]. This area clearly warrants further study, but it is likely that factors such as method of evaporation utilized (especially in relation to speed of evaporation) and viscosity are also important as these factors play an important role in determining the final concentration of individual components within the air space [22]. Indeed, it has been noted that rate of evaporation does have an impact in determining the antifungal activity, and, therefore, potential use for an essential oil volatile [8,22].

Methodology and Reporting

As with direct contact studies, methodology and reporting of volatile activity against fungi is inconsistent [39]. While the majority of studies in this area have utilised the reliable micro-atmosphere assay (also known as the 'reverse Petri plate' or fumigation method), the reporting of effective concentrations in relation to air space, evaporation speed, exposure times, microbial strains and definitive source of the essential oils is variable, making comparison of reports difficult. For example, several essential oil volatiles have been shown to have activity against the fungus *Aspergillus flavus*, but reports vary in their recording of the plant species that was the source of the oil; some reports cite botanical names (for example, *Ocimum gratissimum* (East Indian Basil), *Thymus vulgaris* (thyme) and *Chrysanthemum coronarium* (garland chrysanthemum), while others only provide the common name (for example oregano, rosemary and mustard) [17-19,23].

When noted, the activity of volatiles is most commonly reported as either μL of oil/L of airspace or ppm (part per million: mg/L). However, in many

instances, the volume of oil utilised is noted but the air space is not [7,19-22,26,41,42].

From reports published to date, it appears unlikely that the inoculation form of the fungus (spore suspension or fungal plug inoculation) affects the results of the study, however, the variation in fungal growth conditions between studies has the potential to have a significant effect on outcome [5,7,19,22, 42,43]. Choice of media for example may play an important role in determining susceptibility of the fungi as it has previously been reported that growth on different media can significantly alter fungal susceptibility to antifungal agents, such as essential oil volatiles [23]. Exposure time also varies between reports, but its role in fungal susceptibility is less clear. While some studies report volatile exposure for up to 42 days, others report that exposure time is irrelevant after the first few hours [5,18,44]. Despite, or perhaps because of, this variation in exposure time, little information is available for most essential oils examined to date about the minimum exposure time required for fungal growth inhibition. For example, it has been reported that a 2 hour and a 24 hour exposure of bacteria to volatiles resulted in similar growth inhibition. This is most likely due to the maximum concentration of the active components being released into the airspace and absorbed into agar within the first hour and that producing a high vapour concentration in a short time may result in the most efficient antimicrobial activity [8,44]. Determination of accurate exposure times will be of vital importance for development of potential therapeutic applications.

Mechanism of Action

The exact mechanism of action of essential oil volatiles on fungi remains unclear. However, a number of effects and hypothesis have been reported: these include inhibition of sporulation, disruption of cell wall and membranes, germination and hyphal elongation [7,8,19,26,44). Not surprisingly, due to the differing effects on specific fungi of individual oils, it is suggested that the mechanism of action of essential oil volatiles may differ significantly from that of oils added directly into the growth medium [32].

In general, the majority of reports agree that essential oil volatiles result in significant morphological changes to the hyphae, most noticeably a reduction in hyphal diameter and hyphal wall thickness, possibly related to interference by essential oil components in the enzymatic reactions of cell wall synthesis leading to incorrect assembly of wall components, such as chitin, glucans and glycoproteins [26,31,45,46]. Plasma membrane disruption, mitochondrial structure disorganisation, decreases in both lipid and saturated fatty acid content, increases in unsaturated fatty acids and Mg^{2+}, Ca^{2+} and K^{+} leakage from exposed cells/hyphae have also been reported [26,31].

It is unclear how the volatiles are inhibiting fungal growth, with some reports demonstrating that the volatiles are acting directly on the mycelia, while others suggest that the volatiles are acting on fungal growth indirectly by being absorbed into the growth medium and diffusing through to the mycelia [47,48]. Other authors believe that it is a combination of both that results in the demonstrated antifungal activity of essential oil volatiles, while others suggest the mode of action is directly related to the amount of each compound absorbed to solid phase components (membrane, granules etc) and that, in low doses, fungicidal activity is directly related to the characteristics of the individual compounds, while at high concentrations, compounds from essential oils are fungicidal by a common mechanism [22,32].

For example, it has been demonstrated that oil volatiles are preferentially absorbed onto the lipophilic surface of mycelia and that the greater the surface area of mycelia the stronger the inhibitory effect of oil volatiles [44,48]. Inouye and others hypothesise that compounds within the essential oil volatiles irreversibly cross link with components in the fungal cell membrane causing the leakage of intracellular components [31,47]. It is also possible that respiratory suppression of aerial mycelia may be involved [50,51].

Oil volatiles have been demonstrated to inhibit sporulation of fungi [19,44]. It has been suggested that this inhibition of sporulation, as with cell wall damage, is also associated with alterations to the cell membrane or cell wall damage, leading to increased permeability and subsequent loss of cytoplasmic content (perhaps during synthesis) [51]. Based on analysis of the antisporulation activity on *M. gypseum* of extracts whose main constituent was lapachol, it has been proposed that this inhibition is due to components either damaging the cell wall or altering the membrane permeability of the microconidia, which results in loss of cytoplasm, which in turn would lead to cell death [51].

By measuring the absorbance of volatile components within the agar it has been demonstrated that compounds that are water soluble and stable are incorporated into the agar quickly and in high amounts [21,44]. In one study, for example, it was shown that in one hour of exposure, 70% of the ethanol volatiles were absorbed into the agar, with only 0.5% remaining in the headspace, while only 30% of cinnamaldehyde volatiles were found within the agar and 0.05% was found within the headspace, suggesting that oil volatiles may inhibit fungal growth after being absorbed into the agar [21]. However, unpublished results from this laboratory indicate that when sterile agar is exposed to oil vapours prior to inoculation, the growth inhibition is significantly less than when the oil vapours are in direct contact with the fungus. This implies that the essential oil vapours are acting in combination, directly and indirectly, on the fungi to produce growth inhibition. While this review has concentrated on hyphate fungi, the possible anticandidal mechanism of action of individual essential oil components has recently been reviewed by Pauli (2005) [32].

Conclusion

There can be no doubt that essential oil volatiles have great potential for use in fungal control and/or treatment, however, there is a need for consistent methodology and reporting before this potential can be fully realized. Essential oil volatiles have the advantage that they can treat large areas and do not require direct contact with liquid oils. An added bonus is that such complex substances are unlikely to lead to the development of resistance. *In vivo* studies to determine the applicability, efficacy and safely of essential oils volatiles in the prevention and treatment of fungal infections are now required to determine correlation between *in vitro* and *in vivo* results.

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The Medicinal Use of Essential Oils and Their Components for Treating Lice and Mite Infestations

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Recent studies have demonstrated that essential oils, and in particular, pennyroyal, tea tree and anise, have potent insecticidal and acaricidal (mite-killing) activity. The individual components of essential oils are now being investigated in order to give a rational basis to discover which essential oils may prove to be the most effective all-round agents for killing headlice and their eggs, and treating scabies, and for eliminating house dust mites, a major cause of asthma.

Keywords: essential oils, monoterpenes, insecticidal, acaricidal.

Essential oils have been used for centuries as insecticides and insect repellents, for the treatment and prevention of infestations by lice, and in particular headlice [1,2]. They have also been suggested to be acaricidal, with a potential use in treating scabies, mange in animals or for reducing infestations of house dust mites which cause allergic reactions, such as asthma [3]. Constituents of plant volatile oils have long been known to affect the behavioural responses of pests, with the monoterpenoid components appearing most useful as insecticides or anti-feedants [4]. There is however a surprising difference in the susceptibility of different insect species to different essential oils, and it is therefore not possible to extrapolate from studies done using other species to assume a similar activity in lice. Both lice and mites are evolutionarily highly adapted to their environment, which this may have resulted in such changes. For example, limonene (a major component of lemon oil) and camphor are lethal to house flies and other species of insect [5], whereas they are not particularly toxic to lice [2].

1. The use of essential oils for treating lice

There are three species of louse affecting humans, the head louse, *Pediculus humanus capitis*, the body louse, *Pediculus humanus corporis* and the pubic louse *Pthirus pubis* (Anoplura: Pediculidae) All are blood-sucking ectoparasites, but there has been considerable discussion as to whether head and body lice are distinct species, or sub-species of *P. humanus* [1]. DNA analysis of patients with dual infestations has now shown that head and body lice generally form genetically distinct populations [6]. Migration of lice from head to body was thought not to occur until recently, when Burgess [1] reported several instances of migration of lice from a heavily infested head to clothing on the upper body.

Head lice are a common problem in most countries, irrespective of wealth or status of patients, and particularly amongst schoolchildren where they are easily passed from head to head. A recent random survey of primary schoolchildren in Wales found that more than one child in ten was infected [7]. Body lice live on clothing and are comparatively rare, especially in wealthy countries, as they are destroyed when clothing is washed. Head lice can only survive for about a day away from the host, whereas body lice are more robust and can survive for much longer periods away from the body. Head lice are considered to be merely a social problem; however, body lice can transmit the agents of serious diseases such as typhus, trench fever and epidemic relapsing fever, and these can cause epidemics in developing countries in areas with unsanitary conditions [8].

Essential oils, especially tea tree oil, have often been proposed as alternative pediculosis control agents in

both scientific and lay media articles but despite this, little research has been carried out into their use, either to evaluate their efficacy or to investigate which essential oils are the most active [9]. There are concerns about the toxicity of synthetic insecticides; so many parents are using essential oil based or other 'natural' remedies to try to treat head lice infestations [10]. Patient acceptability of essential oils is high as they are pleasant to use; however there is some debate over whether these methods are effective or indeed safe, as they have not been tested for toxicity. Although most essential oils are in fact of low (or known) toxicity they can cause irritation or sensitization.

In recent years, research into the use of essential oils for treating lice has increased, and several studies have shown that a number are effective pediculicides *in vitro*. Some examples include thyme (*Thymus vulgaris*), tea-tree (*Melaleuca alternifolia*), and lavender (*Lavandula officinalis*) oils [11], as well as clove bud (*Syzygium aromaticum* [syn. *Eugenia caryophyllata*]) [12]. In a screening study of 54 plant essential oils against female *Pediculus humanus capitis,* eucalyptus (*Eucalyptus globulus*), marjoram (*Origamum marjorana*), pennyroyal (*Mentha pulegium)*, cade (*Juniperus oxycedrus*), cardamom (*Eletaria cardamomum*), myrtle (*Myrtus communis*), rosewood (*Aniba rosaeodora*), sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*) oils were found to be at least, if not more, effective than delta-phenothrin and pyrethrum, two commonly used pediculicides [13]. Tea Tree oil, and its two major constituents of, 1,8-cineole and terpinen-4-ol, were shown to inhibit acetylcholinesterase at IC_{50} values (concentrations required to give 50% inhibition) of 0.04 and 10.30 mM, respectively. These findings supported the hypothesis that the insecticidal activity of tea tree oil is attributable, in part, to its anti-cholinesterase activity, and confirm that terpinen-4-ol is the major active component [14]. Essential oils and their constituents therefore provide a good starting point for investigating the development of novel pediculicides.

1.1. Methods of testing essential oils for pediculicidal activity: Although adult headlice are rather fragile, and are easily killed by occlusive agents (for example paraffins) headlice infestations can still be difficult to eradicate because of the impermeability of the louse eggs to insecticidal agents. Therefore treatment must take this into account, and essential oils appear to have an important part to play in the ovicidal activity of treatments: it has even been suggested that the efficacy of some proprietary malathion-containing lotions owe much of their efficacy to the presence of terpenoid perfume ingredients such as α-terpineol [1]. The method of testing is an important consideration when deciding how to conduct these studies, and ovicidal activity should also be investigated separately, although this does not seem to be the case in many reports. The lice used in testing are usually human body lice rather than headlice, but there are sound reasons for this. The resistance status to insecticides is known, and they are more robust than headlice, and so give fewer false positives during testing. Headlice which have been removed from the scalp for more than a few hours will die anyway, regardless of treatment.

Direct contact and fumigation methods have largely been used to test for pediculicidal activity, and both have disadvantages. A study comparing the lethal activity of oils using both a filter paper contact bioassay with a fumigation assay found that potency was different depending on which method of testing was used. For example, eucalyptus, marjoram, pennyroyal, and rosemary oils were more effective in closed containers than in open ones, indicating that the effect of these oils was largely a result of action in the vapor phase, and neither delta-phenothrin nor pyrethrum (often used as positive controls or standard insecticidal agents) exhibited fumigant toxicity [13]. However, measuring vapour concentrations and assessing the contribution of each of a mixture of compounds of different volatility is also less than satisfactory. The direct contact, filter paper, assay and a newer 'dip' method, will be briefly described here.

1.1.1. Filter paper disc pediculicide assay. Essential oils and their constituent monoterpenes have successfully been tested for pediculicide activity using a simple technique which involves adding the essential oil, diluted in a non-insecticidal solvent such as ethanol, to a filter paper disc held in a glass Petri dish and allowing the sample to spread out and fully saturate the paper [2]. The ethanol is allowed to evaporate completely before lice are placed on the filter paper, the Petri dish then covered, and placed in an incubator. The lice will only be in direct contact with the test sample via their legs, which is a disadvantage of the method highlighted by Burkhart and Burkhart [15] and Yang *et al* [13], who suggested that the paper disc assay favours volatile

compounds which could be absorbed through the spiracles. However, for essential (volatile) oils the method is adequate, as they will produce a vapour which would then be in contact with the whole louse.

1.1.2. Dip method pediculicide assay. A dip method is now used at one of the leading institutions for medical entomology, Insect R&D, Cambridge, UK, whereby the lice are held on a piece of gauze and dipped into the test solution ensuring that the louse is completely immersed in the sample. About 20 lice are used in each assay, and carefully placed onto a piece of fine meshed gauze to which the lice cling. This can then be placed in a small Petri dish, and using forceps, the gauze with the lice attached dipped in the test solution for 10 seconds, blotted on paper tissue and returned to the Petri dish. The lice are left in an incubator at 30°C and 70% relative humidity for one hour. After incubation, the lice are washed in shampoo diluted 1:15 with warm water to give as near a real-life scenario as possible, by adding diluted shampoo to the Petri dish and gently shaking. The lice and gauze are tipped into a small tea strainer and rinsed with warm water, blotted on a paper tissue, placed in a new Petri dish, returned to the incubator and left overnight. The following morning, the number of dead, morbid and alive lice can be scored for each test sample and the percentage mortality calculated.

1.1.3. Estimation of morbidity and mortality: In both assays, lice classified as 'morbid' are not moving around, but could be moving their antennae, head, gut or legs. Lice have the ability to reach an apparently morbid state, but recover just a few hours later [15] and are therefore not scored at regular time intervals but left undisturbed in the incubator overnight. Once a louse has reached a morbid state after an overnight incubation, it is unlikely to recover and the figures for both dead and morbid lice can be included in the percentage mortality figures. If the control group has a mortality rate above 14%, Abbott's Correction formula [16] is applied to the results to take account of the high control mortality.

1.1.4. Assessment of ovicidal activity: Ovicidal activity can be assessed using a protocol used routinely by Burgess [17]. Adult lice are provided with nylon gauze on which to lay eggs over a two-day period. The lice are removed and the gauze, with eggs attached, is incubated as usual and tests carried out one or two days later. In the report by Priestley *et al* [2] the sheets of gauze were cut into squares of about 2 cm^2, which carried approximately 300 eggs (200 minimum). These were immersed in the diluted terpenoid solutions for 10 min, the gauze removed, then blotted and dried of solvent. A control batch of eggs exposed to solvent should be run concurrently with each batch of tests, to correct for solvent activity, and an untreated control batch periodically to ensure that solvent treatment continued to have no significant effect on the background mortality rate. After treatment, batches of eggs were incubated in separate glass Petri dishes, under normal maintenance conditions, until all the nymphs in the control batches had hatched and died. For calculation of percent mortality, all hatched nymphs were classified as having survived the treatment, and those failing to hatch or only partially hatching as having been killed by the treatment.

1.2. Effects of individual essential oil components on lice: As essential oils are very variable in composition, and individual constituents have different insecticidal potencies, the logical starting point for such an investigation is the evaluation of a range of isolated monoterpenoids. Structural features relating to pediculicidal and ovicidal activity can then be determined, and used to both predict the potency of an oil from its composition, and to standardize the constitution of an oil for maximum effect and minimum toxicity. Few studies have attempted to systematically assess the contribution of monoterpenoids, although a few have been assessed as part of other studies, *e.g.* Yang *et al* [12]. One recent report describes a range of common individual compounds which were tested in an *in vitro* toxicity model (filter paper disc assay) against both human lice and their eggs, at different concentrations. Adult lice were observed for lack of response to stimuli over 3h and the LT_{50} (time taken to kill 50% of lice) calculated, and the percentage of eggs failing to hatch was used to generate ovicidal activity data [2]. A ranking was compiled for adult lice (Table 1, Figure 1), and partially for eggs, enabling structure-activity relationships to be assessed for lethality to both, and showed that for activity in both life-cycle stages, different structural criteria were required.

1.2.1. Structure-activity relationships of terpenes on lice: Effects on adult lice. Some general structural features of terpenoids are necessary for pediculicidal activity. Mono-oxygenated compounds (a single alcohol, phenol or ketone functional group), were the most active against adult lice whereas non-oxygenated terpenoids were mainly inactive, and di-

oxygenated compounds had little or no activity. Flat, compact terpenoids were more effective than extended or bulky structures, and bicyclic compounds, which are more bulky than linear or monocyclic types, had low efficiency even if mono-oxygenated. Although mono-oxygenation and compact shape appear to be general determinants of activity, more specific structural features are also found in the most active compounds. The six most effective were unsaturated monocyclic structures with a *p*-menthane skeleton, and the four most effective compounds additionally share a methyl group at position 1; a carbon attached to the ring via either a double or single bond at position 4, to which are bonded two methyl groups; and an =O or –OH functional group at position 3 or 4. The methyl group arrangement seen in the top four ranking compounds may also be a key determinant of activity and is also present in the top seven ranking pediculicidal terpenoids. Furthermore, although the phenols thymol and carvacrol have similar structures and correspondingly similar pediculicidal activities, carveol, identical to carvacrol except that it has a double bond between C7 and C8 that disrupts the methyl group arrangement, has relatively low activity.

Effects on lice eggs. The ovicidal activity of mono-oxygenated monocyclic terpenoids was also higher in comparison to other structures. There was, similarly, little or no activity from non-oxygenated terpenoids, a mono-oxygenated bicyclic terpenoid (cineole), or the di-oxygenated monocyclic terpenoid menth-6-ene-2,8-diol. Linalyl acetate again showed low activity in comparison to the alcohols. Unlike the pediculicidal assay (+)- and (–)-terpinen-4-ol performed only moderately well [2].

2. The effects of essential oils on mites

Mites are not insects, but related to the arachnids (spiders); post-larval stages of have eight legs, larval stages have six legs. Many species are microscopic, but it is possible to see some species (e.g. dust mites) under a magnifying glass. Mites are responsible for the skin disease scabies in humans and for various infestations in animals, but the major problem associated with them is the allergenic reaction produced by the house dust mite, which can cause severe asthma. Essential oils have been proposed as a method for treating both mite infections in humans and animals, and for controlling levels of dust mites. Testing for acaricidal activity using house dust mites is even more problematic than testing for lice, because the mites are so small and very mobile, and again it had been found that using fumigant, closed-container methods is most satisfactory (see section 2.2.1).

Table 1: Relative efficacy of essential oil constituents on human clothing louse eggs and adults.[a]

Rank versus lice[b]		Rank versus eggs[b]	
1	(+)-Terpinen-4-ol	1	Nerolidol
2	Pulegone	2	Thymol
3	(–)-Terpinen-4-ol		Geraniol
4	Thymol	4	Carveol
5	α-Terpineol	5	Menthol
6	Menthone		α-Terpineol
7	Carvacrol	7	Citral
8	Linalool		Citronellic acid
9	Perillaldehyde		Linalool
			(+)-Terpinen-4-ol
10	Geraniol		(–)-Terpinen-4-ol
11	Citral		
12	Carveol	12-19	Cineole
13	Menthol		α-Pinene
14	Thujone		α-Terpinene
15	Geranyl acetate		Limonene
16	Linalyl acetate		Menth-6-ene-2,8-diol
			β-Pinene
			Linalyl acetate
			Menthone
17-28	Camphene		
	Camphor		
	Cineole		
	Citronellic acid		
	Limonene		
	Menth-6-ene-2,8-diol		
	Methane-3,8-diol		
	Myrcene		
	Nerolidol		
	α-Pinene		
	β-Pinene		
	α-Terpinene		

[a]From: Priestley *et al*, 2005 [2] (with permission).

[b]The rankings for lice and eggs are based on the percentage mortality, and the lists are adjacent to facilitate comparison of relative activity of compounds on the different life stages.

2.1 The use of essential oils for treating scabies and mange: Scabies, unlike headlice, is primarily a disease of poverty and is an unpleasant condition caused by the mite *Sarcoptes scabiei* var *hominis*. It is not only distressing, causing severe itching especially at night, but (not always justifiably) is considered a 'dirty' disease, caused by lack of hygiene and overcrowding. Essential oils have a folklore use for treating scabies, and in Australia in particular, tea tree oil is widely used. Resistance to existing acaricidal compounds is increasing, and treatment failures with lindane, crotamiton, and benzyl benzoate, as well as likely emerging resistance to permethrin and oral ivermectin have already been reported. A study comparing the activity of tea tree oil *in vitro* with some of its individual active components suggested that tea tree oil has a

potential role as a topical acaricide for use in scabies, and confirmed terpinen-4-ol as the main active component [18]. The study was carried out using scabies mites collected from a patient, which were used within 3 hours of collection. The mites were placed in continuous direct contact with tea tree oil products and control acaricides, and were observed at regular intervals. Tea tree oil (5%), and terpinen-4-ol, were highly effective in reducing mite survival times and were comparable to 5% permethrin and ivermectin. *In vivo* effectiveness was also observed [18]. Another clinical study of 268 prison inmates with scabies used a formulation containing oil of *Lippia multiflora* (20% v/v in light liquid paraffin) and found it to be superior to benzyl benzoate at the same concentration, although multiple applications were needed in both cases [19].

Lavender and other essential oils have been suggested as possible treatments for psoroptic mange in sheep and other animals [20]. The mite *Psoroptes cuniculi*, was tested for its susceptibility to some natural terpenoids by direct contact and by inhalation. Lavender oil and linalool, among others, were found to be effective [21]. In this study, it was also possible to discern a correlation between chemical structure

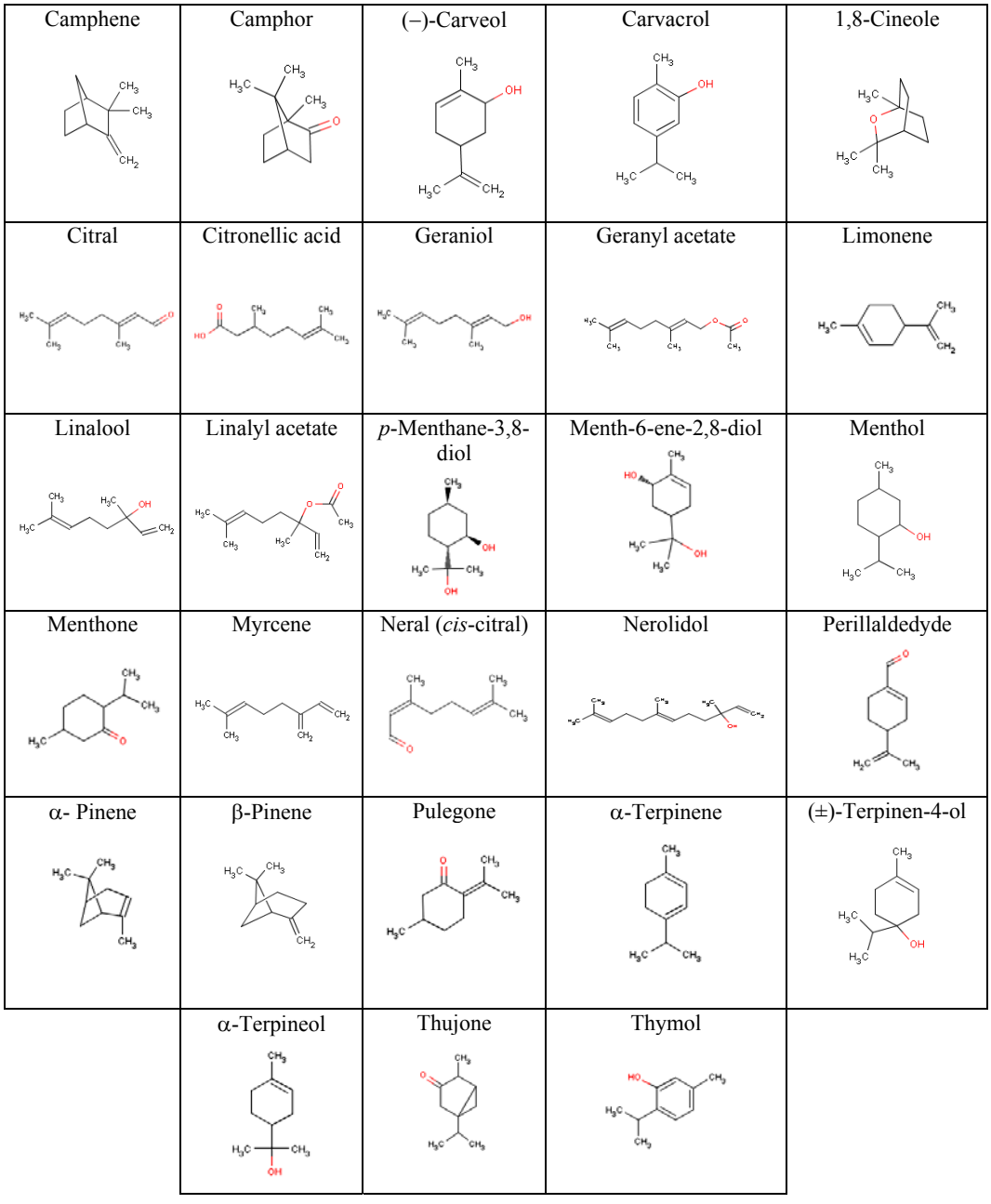

Figure 1: Structures of terpenes tested.

and acaricidal activity, and the results corresponded with those found in the experiments with lice outlined above [2], in that molecules possessing free alcoholic or phenolic groups showed the most potent acaricidal activity.

2. 2. The use of essential oils for eliminating house dust mite infestations: House dust mites, *Dermatophagoides pteronyssinus* (European) and *Dermatophagoides farinae* (American) (Acari: Pyroglyphidae) induce allergic reactions in some individuals which can lead to severe asthma. It has been suggested that essential oils may find an application in their control, by for example adding them to the water used to wash bed-linen or soft furnishings [22]. Surprisingly, where acaricidal activity is concerned, there is little reliable evidence that dust mites are actually susceptible even to the agents used as standard acaricides, and reports are conflicting. For example, a study comparing the activities of eucalyptus and laurel essential oils with that of benzyl benzoate in laboratory conditions indicated that benzyl benzoate was less effective than previously thought, and may need more frequent application than stated in the manufacturer's instructions [23]. Although in this test, eucalyptus and laurel essential oils were shown to have little acaricidal activity, another study found that adding eucalyptus oil and benzyl benzoate to laundry killed mites and reduced the incidence of allergens [22]. These discrepancies suggest that dust mites are either able to somehow acquire resistance to some essential oils, or that assay methods are not reproducible. A new *in vitro* assay for dust mites was developed to try and overcome this problem [3]. However, regardless of the method used to expose the mites to the test agent, closed containers were more effective than open methods, confirming results found when testing lice

2.2.1. Essential oils with acaricidal effects on dust mites: A summary of the most important essential oils against dust mites is given in Table 2. The most active compounds on mites correlate well with those which are most toxic to lice. For example, tea tree, lavender and lemon (*Citrus limon*) oils were recently tested against *D. pteronyssinus*, and the most active found to be tea tree oil, which correlated with its effects on lice [3] and with previous reports on the scabies mite [18]. Lavender oil was moderately effective, and lemon oil had a lesser effect, which fits with the results shown in Table 1 for their major consitutents. The acaricidal effects of tea tree, pennyroyal, ylang ylang (*Cananga odorata*), citronella (*Cymbopogon nardus* and *C. winterianus*), lemon grass (*Cymbopogon citratus* and *C. flexuosus*), and rosemary have also been tested on house dust mites, and the most effective found to be pennyroyal, which consists mainly of pulegone and again reflects its action against lice (see Table 1) [24].

Clove bud oil-derived eugenol and its congeners (acetyleugenol, isoeugenol, and methyleugenol) have been assessed for activity against adults of both *Der. farinae* and *Der. Pteronyssinus,* using both direct contact application and fumigation methods for comparison. The standard compounds benzyl benzoate and *N*,*N*-diethyl-*m*-toluamide (DEET) were also tested and were much less active than methyleugenol, isoeugenol or eugenol. In fact, very low activity was observed with DEET. There were some differences in responses to individual compounds between *Der. Farinae* and *Der. pteronyssinus*, but not in their rank order of potency. The typical poisoning symptom of eugenol and its congeners was a similar death symptom of the forelegs extended forward together, leading to death without knockdown, whereas benzyl benzoate and DEET caused death following uncoordinated behaviour. Once again, compounds were much more effective in closed rather than in open containers, indicating that the mode of delivery of these compounds was largely due to action in the vapor phase [25]. Another test which compared essential oil components to synthetic acaricides, found that the acaricidal activity of *p*-anisaldehyde (from anise seed *Pimpinella anisum* oil), was superior to benzyl benzoate and *N*,*N*-diethyl-*m*-toluamide (DEET) [26].

The results of all these studies demonstrate that essential oils, and in particular, pennyroyal, tea tree and anise, have potent insecticidal and acaricidal activity, and that toxicity of a particular oil or constituent to mites closely follows that of lice. In fact, it appears that there is more correlation here than between different species of insects, possibly due to their adaptation to similar environments. Recent studies looking at individual components of essential oils is giving a rational basis to focusing of which essential oils may prove to be the most all-round effective pediculicides and substantiates results obtained from testing whole oils. It also supports the anecdotal use of tea tree oil as a headlice treatment and for treating mite infestations such as scabies, and identifies the active constituent as terpinen-4-ol as the most effective compound against both adult lice

(although less effective against eggs) and the scabies mite. As a result, the insecticidal and acaricidal activities of an essential oil can be predicted from its composition to some extent, and the studies described here also demonstrate that natural head louse remedies made from essential oils should be standardized to produce consistent results. They also support the use of mixtures of oils in some instances: for example the addition of nerolidol - or an essential oil rich in this compound - which is particularly lethal to eggs (but ineffective against adult lice), could be used to enhance ovidical activity. Terpenes or oils which are toxic or irritant can also be avoided, and more innocuous substances substituted if equivalent activity can be found.

Great care must be taken when applying essential oils directly to the skin, as some cause irritation or sensitization in the concentrations required for efficacy. To teat scabies for example, non-toxic oils such as tea tree and anise would be suitable, but pennyroyal and other more toxic oils or compounds may be more useful in treating house dust or other mites where humans are not exposed directly to them. Being volatile, essential oils can easily be removed from the environment after use. However their volatile nature may also aggravate respiratory conditions, including the asthma they are intended to alleviate and skin sensitization is a possibility. This may not be as much of a problem as perceived: rosemary oil, which is known to provoke sensitisation and irritancy in some individuals, was recently found to suppress interleukin-13 induction by house dust mite allergen and may, at least partially, prevent allergic airway inflammation induced by house dust mite [27].

Table 2: Summary of essential oils and components shown to have significant acaricidal activity.

Essential Oil	Main active component(s)	Test species	Ref
Tea tree *Melaleuca alternifolia*	Terpenen-4-ol	Scabies mite: *Sarcoptes scabiei* var *hominis*	[18]
		House dust mite *Dermatophagoides pteronyssinus* and *Der. farinae*	[3]
Bush tea *Lippia multiflora*	No individual components tested, but contains 1,8-cineole, linalool, thymol, carvacrol and α-terpineol	Scabies mite: *S. scabiei* var *hominis*	[19]
Lavender *Lavandula officinalis*	Linalool	Mange mite: *Psoroptes cuniculi*	[21]
		House dust mite: *Dermatophagoides pteronyssinus*	[3]
		House dust mite *Der. pteronyssinus*	[3]
Pennyroyal, *Mentha pulegium*	Pulegone	House dust mites: *Der. pteronyssinus*, *Der. farinae*	[24]
Clove bud oil *Eugenia caryophyllata*	Methyleugenol, isoeugenol, eugenol, benzyl benzoate acetyleugenol.	House dust mites: *Der. Pteronyssinus*, *Der. farinae*	[25]
Anise *Pimpinella anisum*	*p*-anisaldehyde benzyl benzoate	House dust mites: *Der. pteronyssinus*, *Der. farinae*	[26]

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A Review of Aromatic Herbal Plants of Medicinal Importance from Nigeria

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Nigeria is blessed with a rich source of aromatic flora, many of which have not been previously investigated for their chemical constituents and biological potentials. This flora constitutes a rich source of potential spices or flavoring, ingredients of formulae intended for pharmaceutical administration, and for perfumery. Interestingly, essential oil constituents such as 1,8-cineole, precocene, 6,10,14-trimethylpentadecan-2-one, eugenol, β-caryophyllene, α-pinene, α-terpineol and even hitherto uncommon compounds such as zerumbone and rare terpenoid esters have been isolated and characterized from these plants. In addition, some of the studied volatile oils have exhibited biological activities of importance such as antimicrobial and cytotoxicity. The majority of these aromatic plants occur either as perennial or annual herbs which are suitable for cultivation purposes in herbal gardens, traditional medicinal centers, parks, research institutes and forest reserves. This paper presents a review of some of the endemic aromatic and medicinal plants of Nigeria with a view to ascertaining their suitability as raw materials for the pharmaceutical and perfumery applications.

Keywords: Aromatic plants, Nigeria, essential oil, antimicrobial, cytotoxicity.

Plants, apart from providing foods, have also been the focus for deriving natural products, which have been exploited for their medicinal, pharmaceutical and industrial applications. Such compounds have modulated several physiological changes in humans and have contributed to the promotion of health. Even in the age of combinatorial chemistry, natural products have an important place in pharmaceutical development and are much more successful than artificially designed compounds. Exploitation of local raw materials by pharmaceutical and allied industries for drug production and conversion to materials of daily uses will be a viable approach to reduce dependence on imported drugs thereby conserving the scarce foreign exchange of developing nations like Nigeria. However, detailed information on the chemistry of some of the medicinally important compounds from these plants is currently unavailable.

Essential-oil-bearing plants rank high both in quality and frequency among the plants that are widely used world wide in different forms as whole herbs, powders, extracts and vapors for pharmaceutical, chemotherapeutic and perfumery purposes [1]. Such plants are widely distributed in Nigeria, and the fragrant principles they contain will be readily acceptable as raw materials. The uses to which these aromatic plants are put are usually attributed to the constituents of their essentials oils, which can be readily isolated. Essential oils are widely used in medicines, perfumery, as preservatives, for agricultural purposes and acupuncture. They generally possess strong and persistent odors, usually characteristic of the plant in which they are found. They have been exploited for many purposes, including antimicrobial, antiparasitic and insecticidal.

The isolation of essential oils from plant sources involves simple technology such as hydrodistillation to complex ones, such as solid phase microphase extraction. The oils are normally stored in well-capped, airtight containers and under refrigeration. The oils are analyzed for their constituents by means

of gas chromatography and gas chromatography coupled with mass spectrometry. Conventional techniques such as nuclear magnetic resonance, ultraviolet and infra-red spectroscopy are also employed to ascertain correctly the identity of the compounds.

Essential oil components from traditional herbal medicines are extremely useful because the components can be used to produce potential drugs for health care. The components can also be used as biological and pharmacological tools against cancer, diabetes, ulcers, and other illnesses. Essential oils are also used in commercial industries for flavors, fragrances, dyes, cosmetics, and pesticides. In exploring natural products, one can discover various new and complex structures that could benefit drug design. There is a significant number of diverse chemical structures within the tropical forests of the world yet to be discovered. Expanding natural products and biological research could potentially lead to useful compounds.

Studies on the chemical composition of the oils revealed the presence of monoterpenes, sesquiterpenes, diterpenes, aromatics, and fatty acids. The anti-inflammatory properties of some of the oils were determined by the abundance of monoterpenes and sesquiterpenes, while the oxygenated compounds contributed to the antibacterial effects. For example, oxygenated sesquiterpenoids were the most abundant class of the leaf oil of *Cassia alata* and the floral oil of *Datura metel*, which contributed to the antibacterial effects.

This paper reviews some of the interesting chemical constituents and biological activities of some essential oils from plants endemic to Nigeria. The constituents of a majority of these essential oils are being reported for the first time in the literature.

A. Annonaceae

(i) Name: *Annona reticulata* L [2]

Local name: Custard apple

Uses: Eaten fresh and used to flavor ice cream and as condiments in soup preparation; the oil has been shown to be active against some intestinal microbes (unpublished data)

Main constituents: (*E*,*E*)-farnesyl acetate (19.0%), *ar*-turmerone (12.0%), benzyl benzoate (10.9%), γ-terpinene (7.4%), elemol (6.3%).

(ii) Name: *Polyalthia longifolia* Thw. [3]

Uses: The plant is used for the treatment of skin diseases, fever, diabetes and hypertension.

Main constituents: The leaf oil was almost exclusively composed of sesquiterpenes: allo-aromadendrene (19.7%), caryophyllene oxide (14.4%), β-caryophyllene (13.0%), β-selinene (7.9%), α-humulene (7.0%), *ar*-curcumene (6.8%).

while the stem bark was composed of: α-copaene (8.7%), α-muurolol (8.7%), β-selinene (8.6%), viridiflorene (8.1%), α-guaiene (7.8%), allo-aromadendrene (7.4%), δ-cadinene (7.0%).

(iii) Name: *Xylopia aethiopica* (Dunal) A. Rich [4]

Local name: Eru awola

Uses: Sold in herbal markets nationwide as spices in food preparation, antimicrobial, anti-malarial, anti-inflammatory and for treating cough. Decoction of the fruits is useful for amelioration of dysentery. Also used in perfumery.

Main constituents: β-santalol (14.5%), α-cadinol (13.0%), benzyl benzoate (10.0%), dodecanoic acid (10.0%), elemol (9.2%).

We also described the isolation and characterization of an anti-HIV and cytotoxic compound, known as zerumbone, for the first time in the essential oil.

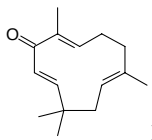

Figure 1: Zerumbone.

B. Araucariaceae

(i) Name: *Araucaria cunninghamii* Sweet Grown [5]

Uses: For sweetening and as laxative

Main constituents: α-pinene (14.8%), terpinen-4-ol (14.7%), shyobunol (8.9%), spathulenol (8.6%).

C. Asclepiadaceae

(i) Name: *Gongronema latifolium* Benth. [6]

Uses: Tea made from the leaf is used to maintain healthy blood sugar levels. The oil is used as an antioxidant and anti-inflammatory.

Main constituents: linalool (19.5%), (*E*)-phytol (15.3%), aromadendrene hydrate (9.8%), (*E*)-β-ionone (7.0%).

D. Asteraceae

(i) Name: *Eclipta indica* L. [7]

Uses: Known for its antimicrobial potential

Main constituents: 2-tridecanone (89.7%), caryophyllene oxide (3.9%), β-caryophyllene (2.6%).

(ii) Name: *Tagetes erecta* L. [8]

Local name: African marigold

Uses: Ornamental plants used as spices in drink formulation. Medicinally, the oil extract is used locally as an antioxidant, nutritional supplement and as opthamological agents.

Main constituents: The leaf oil was characterized by the abundance of: piperitone (50.7%), piperitenone (13.2%), (*E*)-β-ocimene (6.7%).

while the flower oil has: 1, 8-cineole (23.1%), α-pinene (11.8%), α-terpineol (10.7%), piperitone (8.0%).

(ii) Name: *Tithonia diversifolia* (Hemsl) A. Gray [9]

Local name: sunflower

Uses: Used in the treatment of malaria, diabetes, sore throat and liver pains. It has also been employed in the treatment of ulcer

Main constituents: from the leaf oil: α-pinene (32.9%), β-caryophyllene (20.8%), germacrene D (12.6%), β-pinene (10.9%), 1, 8-cineole (9.1%).

and from the flower oil: germacrene D (20.3%), β-caryophyllene (20.8%), bicyclogermacrene (8.0%).

E. Burseraceae

(i) Name: *Boswelia dalzelii* Hutch [10]

Uses: Resins are burnt as incense for spiritual purposes, while the leaves are known for their antimicrobial and anti-inflammatory potential.

Main constituents: α-pinene (45.7%), α-terpinene (11.5%).

F. Caesalpinaceae

(i) Name: *Brachystegia eurycoma* Harms [11]

Uses: As an anthelmintic, toxic to the vector of *Schistosoma*, while the seeds are used as spices and consumed as condiments and food additives. The seeds are excellent sources of protein and carbohydrate and contain linoleic acid, which is one of the three essential fatty acids.

Main constituents: 1, 8-cineole (23.1%), acorenone (10.0%), β-caryophyllene (5.6%), geranyl acetone (4.5%).

(ii) Name: *Brachystegia nigerica* Hoyle et A. Jones [12]

Uses: The plant is rich in fatty acids, oil and protein and is used in ethnomedicine for the treatment of malaria, dysentery and cancer-like symptoms.

Main constituents: α-pinene (17.7%), β-selinene (12.5%), α-gurjunene (8.8%), β-caryophyllene (7.5%), limonene (7.0%).

(iii) Name: *Dialium guineense* Willd. [13]

Uses: Known to be rich in mineral elements, sugars, and tartaric, citric, malic and ascorbic acids. Used in the management of fever, diarrhea, and palpation, and as an antibacterial. From the medicinal point of view, extracts from the plants growing in Nigeria have been shown to posses both antimutagenic and molluscicidal activities.

Main constituents: Precocene I (78.8%), β-caryophyllene (5.3%).

G. Compositae

(i) Name: *Centratherum punctatum* Cass. [14]

Uses: Antimicrobial.

Main constituents: β-caryophyllene (16.6%), germacrene D (6.4%), globulol (5.7%), α-copaene (5.3%), sesquisabinene (5.3%).

H. Cupressaceae

(i) Name: *Callitris columellaris* F. Muell [15]

Uses: Cytotoxic effects and as insect repellant.

Main constituents: limonene (17.7-30.0%), α-pinene (13.9-17.2%), bornyl acetate (0.8-27.1%).

(ii) Name: *Callitris intratropica* R. T. Baker & H. G. Smith [16]

Uses: As an antimicrobial, cytotoxic and insect repellant.

Main constituents: α-pinene (35.9-55.6%), limonene (21.6-50.5%), myrcene (6.0-10.1%).

I. Euphorbiaceae

(i) Name: *Acalypha segetalis* Muell Arg. [12]

Uses: Antimicrobial, prevention of bio-deterioration and as a trypanocidal agent.

Main constituents: α-pinene (29.8%), 1,8-cineole (16.2%), (*E*)-phytol (11.8%), δ-3-carene (9.8%).

J. Fabaceae

(i) Name: *Samanea saman* (Jacq.) Merr. [17]

Uses: The seed pods are highly palatable and are used as food supplement. It is also used as a poultice to cure constipation and stomach cancer.

Main constituents: palmitic acid (55.5%), 1,8-cineole (15.9%), oleic acid (7.4%).

K. Gnetaceae

(i) Name: *Gnetum africanum* L. [6]

Uses: The leaves are either eaten raw or are finely shredded and added to soups and stews. It is used for the treatment of an enlarged spleen, sore throats and as a cathartic. It is also an antidote to some forms of poison. The oil exhibited promising antimicrobial effects on *E. coli* ATCC No. 25922

Main constituents: β-caryophyllene (18.1%), (*E*)-phytol (16.5%), 6, 10, 14-trimethyl-2-pentadecanone (9.7%).

L. Irvingiaceae

(i) Name: *Klainedoxa gabonensis* Pierre ex Engl. [12]

Uses: It serves as a source of protein and dietary fiber. It has been employed in the treatment of gonorrhea and sexual dysfunction

Main constituents: in the leaf oil: geranyl acetone (13.8%), β-bourbonene (11.1%), (*E*)-α-ionone (10.5%).

and from the stem bark: linalool (17.4%), 1, 8-cineole (9.9%), 1-octen-3-ol (8.0%).

and from the root oil: 1, 2, 3-trimethyl-benzene (9.8%), 1-ethyl-2-methyl benzene (9.1%), pentyl benzene(9.1%), methyl salicylate (9.1%).

M. Moraceae

(i) Name: *Ficus exasperata* Vahl [18]

Uses: Employed for anti-ulcer, anti-diabetic and antifungal properties.

Main constituents: 1,8-cineole (13.8%), (*E*)-phytol (13.7%), *p*-cymene (11.4%), β-ionone (7.5%), 6,10,14-trimethyl-2-pentadecanone (7.0%), caryophyllene oxide (5.4%).

N. Myrtaceae

(i) Name: *Eucalyptus cloeziana* F. Muell [19]

Uses: Flavoring agent in food preparation and as an antimicrobial

Main constituents: α-pinene (46.6%), 1,8-cineole (15.4%), *p*-cymene (6.4%).

(ii) Name: *Eucalyptus microtheca* F. Muell [20]

Uses: Useful for the treatment of malaria, dysentery and cancer-like symptoms.

Main constituents: 1,8-cineole (53.8%), α-pinene (6.8%), α-terpineol (5.6%), α-fenchyl acetate (5.4%), γ-cadinene (5.0%).

(ii) Name: *Eucalyptus propinqua* Deane & Meane [19]

Uses: As an astringent and as an anti-ulcer agent. Also useful as a scent and for flavoring ice cream and liquid drinks.

Main constituents: 1,8-cineole (61.8%), γ-terpinene (23.3%), *p*-cymene (4.7%).

(iv) Name: *Eucalyptus torreliana* Sm. [21]

Uses: As an anthelmintic, anti-inflammatory and as condiments. The plant possesses potent antimicrobial and cytotoxic activities (Table 1).

Main constituents: from the leaves: 1,8-cineole (33.8%), α-pinene (21.7%), *p*-cymene (10.7%), β-pinene (10.3%).

from the fruits: α-pinene (55.8%), β-pinene (10.8%)

(v) Name: *Eugenia uniflora* L. [22]

Uses: Useful as an anti-inflammatory and against stomach diseases. The oils have been shown to possess considerable cytotoxic and antimicrobial activities.

Main constituents: leaf oil: curzerene (19.7%), selina-1,3,7(11)-trien-8-one (17.8%), atractylone (16.9%), furanodiene (9.6%).

from the fruit oil: germacrone (27.5%), selina-1,3,7(11)-trien-8-one (19.2%), curzerene (11.3%), oxidoselina-1,3,7(11)-trien-8-one (11.0%)

O. Myristicaceae

(i) Name: *Pycnanthus angolensis* (Welw.) Exell. [23]

Uses: The uses range from the incorporation in condiments, soups and seasoning to cattle feeds and medicines. The seeds are important sources of oil and wax. It is known to be useful as an antimalarial. The volatile oils

displayed potent antimicrobial activity against tested organisms.

Main constituents: from the stem bark: α-bergamotene (25.1%), 4-terpineol 916.6%), α-terpineol (15.6%), *trans*-β-bergamotene (12.9%).

and from the leaf oil: spathulenol (82.0%), caryophyllene oxide (14.0%).

P. Poaceae

(i) Name: *Hypparrhenia rufa* (Nees) Staph. [24]

Uses: Not known until chemically analyzed

Main constituents: τ-cadinol (17.4%), β-selinene (11.6%).

We also described the isolation and characterization of some hitherto unknown terpenoid esters:

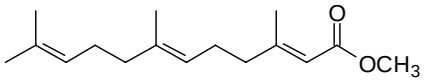

Figure 2: methyl (*E,E*)-farnesoate (1.0%)

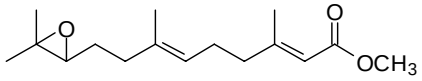

Figure 3: methyl (*E,E*)-10,11-epoxy-farnesoate (12.17%)

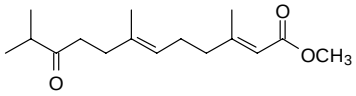

Figure 4: methyl (2*E*, 6*E*) -3,7, 11-trimethyl-10-oxododecadienoate (2.25%)

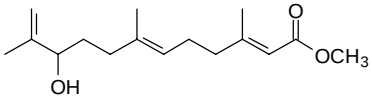

Figure 5: Methyl (2*E*,6*E*)-10-hydroxy,3,7,11-trimethyldodeca-2,6,11-trienoate (4.3%)

Q. Polygalaceae

(i) Name: *Securidata longependuculata* Fers [25]

Uses: The plant is commonly employed for the treatment of inflammatory conditions and as a purgative. It is also useful as an antimalarial, insecticide and as an insect repellent.

Main constituents: methyl salicylate (89.6%).

R. Rutaceae

(i) Name: *Murraya paniculata* (L.) Jack [26]

Uses: Used for the treatment of fractured bones and malaria.

Main constituents: The leaf oil contains: β-cyclocitral (22.9%), methyl salicylate (22.4%), *trans*-nerolidol (11.7%), α-cubebene (7.9%), (-)-cubenol (6.8%);

and the fruit contains: β-caryophyllene (43.4%), (-)-zingiberene (18.9%), germacrene D (8.3%)

S. Taxodiaceae

(i) Name: *Taxodium distichum* (L.) L. C. Rich [27]

Uses: As an antimicrobial and seasoning agent. The oil displayed notable cytotoxic activity (Table 1).

Main constituents: from the fruits: α-pinene (60.5%), thujopsene (17.6%).

from the leaf oil: thujopsene (27.7%), widdrol (12.8%), β-caryophyllene (11.4%).

Table 1: Cytotoxicity of some Nigerian essential oils.

Oil samples	Cell lines[a]	Reference
Eucalyptus torreliana (leaf)	PC-3 (99.4) Hep G2 (99.5) Hs 578T (100) MDA-MB-231 (98.9)	[21]
Eucalyptus torreliana (fruit)	PC-3 (98.5) Hep G2 (87.9) Hs 578T (100) MDA-MB-231 (94.6)	[21]
Eugenia uniflora (leaf)	PC-3 (99.36) Hep G2 (99.71) Hs 578T (100)	[22]
Eugenia uniflora (fruit)	PC-3 (99.55) Hep G2 (959.96) Hs 578T (100)	[22]
Taxodium distichum (leaf)	PC-3 (99.77) Hep G2 (100) Hs 578T (100)	[27]
Taxodium distichum (fruit)	PC-3 (97.58) Hep G2 (95.19) Hs 578T (0)	[27]
Peristrophe bicalyculata (entire plant)	MDA-MB-468 (66.66) MCF-7 (100)	[28]

[a] % inhibition at 100 μg/mL in parentheses; PC-3 = Human prostrate tumor cells; Hep G2 = Human liver tumor cells; Hs 578T = Human breast (ductal) tumor cells; MDA-MB-231 = Human breast (adenocarcinoma) tumor cells; MDA-MB-468 = Human breast (adenocarcinoma) tumor cells; MCF-7 = Human breast (adenocarcinoma) tumor cells.

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The Biology of Essential Oils in the Pollination of Flowers

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Pollination is an essential biological process in higher plant reproduction that involves the transfer of pollen to the female sexual organs of flowers or cones. It plays a critical role in the reproductive success and evolution of most plant species by allowing plants to share genetic material from other members of the same or closely-related species, thus increasing genetic diversity. In many cases, non-plant organisms are involved in carrying out this cross-pollination, including insects, bats, mammals, and birds. In order to attract such pollinators, plants have evolved the ability to produce a mind-boggling array of volatile compounds that have also found abundant use for humans when collected as essential oils. In this review, we focus on the role of essential oil compounds that are produced by flowers as chemical attractants used to draw in their often highly-specific pollinators. We examine in some detail various questions behind the biology of floral scent, including how these compounds are produced in flowers, how they are detected by potential pollinators, and how biotechnology can be used to alter their activity.

Keywords: essential oil, floral scent, insect attraction, linalool, pollination, scent engineering.

I. INTRODUCTION

What is pollination and why is it important? Pollination is a key biological process in higher plant reproduction that involves the transfer of pollen grains (male gametes) to the plant flower carpel, the structure that contains the ovule (female gamete). The receptive part of the carpel is called the stigma in the flowers of angiosperms (flowering plants) and the micropyle in gymnosperms (represented by conifers, ginkgo, cycads, and gnetes). Pollination can be carried out directly, without the aid of any other organisms, as when self-pollination occurs. However, self incompatibility often occurs, in which case the pollen that a flower produces is not compatible at the stigmatic site of the same flower. For successful pollination to occur here, plants have developed cross-pollination strategies. Wind pollination is the primary strategy in the case of grasses and sedges; many willows, poplars, oaks, and alders; and gymnosperms such as pines, spruces, and true firs. The flowers of wind-pollinated plants are often reduced in size and simple in structure. Wind-pollinated flowers are also frequently produced as separate male and female structures (as with male and female cones of pine and with male and female catkins of many willows, poplars, alders, and oaks), or they may be complete flowers with male and female parts produced in the same flower (as with grasses).

Non-plant agents involved in carrying out cross-pollination in nature include insects, bats, mammals, and birds. These pollinators seek food rewards from either pollen/pollinia or from sugar-producing nectaries located in the flowers that they visit. Plants in turn have evolved rather interesting strategies to attract these pollinators [1]. They include flowers that produce differently colored, often hairy "nectar guides" on their petals (as in *Iris*); plants that produce ultraviolet pigments that insects see as "bulls-eyes"; various colored petals and/or sepals whose flavonoid and anthocyanin pigments attract specific pollinators; flowers that open only at night when moth type pollinators are active in flight (as with yucca flowers visited by hawkmoths); flowers that produce a rotten meat smell (due to indoles, skatole, or amines) that attract flies or beetles, as in the case of skunk cabbage and other aroids; flowers that produce

pheromones (sex hormones) that attract specific insect pollinators; and finally, flowers that mimic female insects of a given species in shape and form so that "pseudocopulation" and pollination ensue, as in case of many orchid species. In many of these cases, the flowers produce essential oils as olfactory cues that attract specific insect pollinators because of their highly evolved sensing systems.

In this review, we shall focus on the role of essential oil compounds that are produced by flowers as chemical attractants used to draw in specific kinds of pollinators.

What are essential oil compounds? An essential oil is any concentrated, hydrophobic liquid containing volatile aroma compounds produced by plants. They are also known as either volatile or ethereal oils, or simply as the "oil of" the plant material from which they were extracted, such as oil of cloves or lemon grass oil. Essential oils are synthesized in various organs or tissues of plants, including leaves and stems (e.g., fennel, parsley, tarragon, rosemary, basil, mints, sage, wintergreen, spicebush, eucalyptus, pine, lemon grass, bay, oregano), seeds (e.g., almond, anise, celery, cumin), berries (e.g., juniper, allspice), bark (e.g., cinnamon, sassafras), fruit peel or rind (e.g., grapefruit, lemon, citron, orange, lime), roots (e.g., valerian), rhizomes (e.g., ginger), and flowers (e.g., chamomile, clove, geranium, jasmine, lavender, orange, and rose).

The flowers of many plant species attract pollinators by producing different complex mixtures of essential oil compounds within the various floral organs (i.e., stigma, style, ovary, filaments, petals, sepals and/or nectaries) or in special scent gland tissues (called osmophores) most commonly located on the epidermal cells of the petals. It is the combinations of the constituents of this scent mixture that give each flowering plant species a unique fragrance [2,3]. A few examples of the chemical structures of fragrance molecules emitted from flowers are shown in Figure 1. For the purpose of this review, floral essential oil compounds will also be referred to as olfactory compounds, aroma compounds, volatile compounds, or simply as scent compounds.

II. WHAT ARE THE DIFFERENT KINDS OF ESSENTIAL OIL COMPOUNDS THAT FLOWERS PRODUCE?

The individual compounds that make up each floral scent are widely distributed among the flowers of many different species. This likely reflects the fact that the major biosynthetic pathways that lead to the production of such compounds are present in all plants [4]. More than 1700 individual aroma compounds have been identified so far from over 990 taxa belonging to 90 families and 38 orders [5]. Table 1 illustrates the diversity of plant taxa in which scent composition has been characterized.

Table 1: Families of plants and numbers of taxa producing a characterized scent (from [5])

Plant family	Number of taxa producing scent
Amaryllidaceae	17
Apiaceae	11
Araceae	55
Arecaceae	40
Asteraceae	13
Cactaceae	21
Caryophyllaceae	20
Fabaceae	18
Lecythidaceae	13
Magnoliaceae	26
Moraceae	15
Nyctaginaceae	20
Oleaceae	13
Orchidaceae	417
Ranunculaceae	14
Rosaceae	24
Rubiaceae	10
Rutaceae	21
Solanaceae	21

Table 2: Classes of compounds and numbers of compounds found in essential oils of flowers (from [6]).

Compound class	Number of compounds
Aliphatics	
C1 through C25	528
Benzenoids and Phenylpropanoids	
C6-C0 through C6-C7	329
C5 Branched-Chain Compounds	
Saturated	40
Unsaturated	53
Total	93
Miscellaneous Cyclic Compounds	
Carbocyclic	60
Heterocyclic	51
Total	111
Nitrogen Compounds	
Acyclic	42
Cyclic	19
Total	61
Sulfur Compounds	
Acyclic	37
Cyclic	4
Total	41
Terpenoids	
Monoterpenes	
Acyclic	147
Cyclic	148
Total	295
Sesquiterpenes	
Acyclic	44
Cyclic	114
Total	158
Diterpenes	
Acyclic	4
Cyclic	2
Total	6
Irregular Terpenes	
Apocarotenoid	52
C8 through C18	45
Total	97

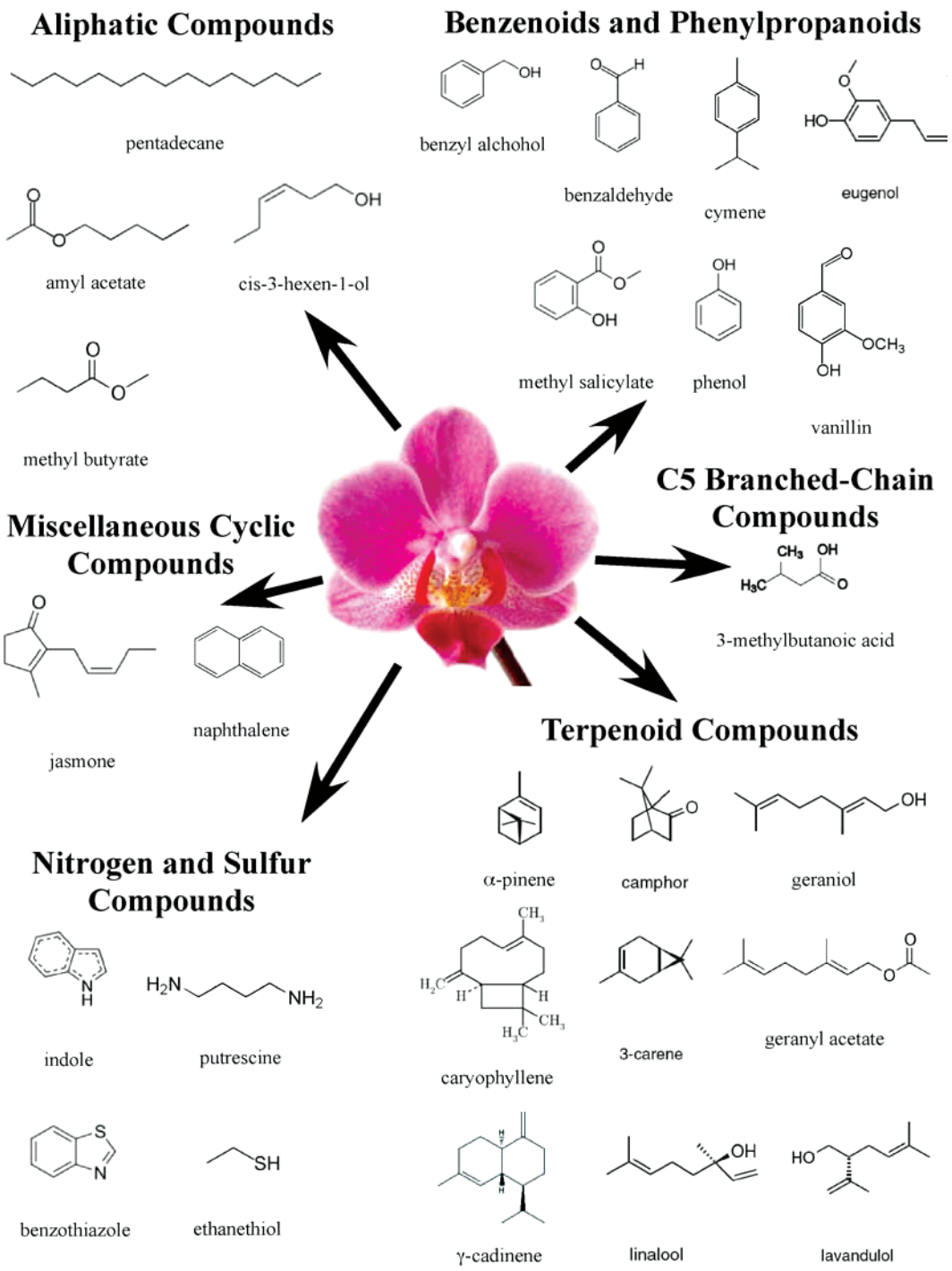

Figure 1: Examples of the chemical structures of some common floral scent compounds.

Aroma compounds produced by plants can be classified by functional groups. These groups include alcohols (e.g., menthol, eugenol, hexanol, furaneol), aldehydes [e.g., benzaldehyde (marzipan, almond) acetaldehyde (pungent), hexanal (green, grassy) cinnamaldehyde (cinnamon), citral (lemon grass, lemon oil), furfural (burnt oats), vanillin (vanilla), octanal, nonanal], amines (e.g., indole, skatole), esters (e.g., lutein fatty acid esters from marigold), ethers (nerolin = methyl β-naphthyl ether), terpenes (e.g., linalool in many flower species, citronellol in rose, geraniol, β-ionone; caryophyllene, nerol).

Almost all of these compounds are also found in floral scent mixtures. However, rather than using functional groups as criteria, essential oils/volatile

compounds found in flowers are usually grouped according to specific classes of chemical compounds, as shown in Table 2. These are grouped according to their supposed biosynthetic origin (see Section III). The two largest groups are the terpenoid compounds (556 members) and the aliphatic compounds (528 members).

Case study: Rose flowers contain over 300 essential oil compounds that contribute to the attraction of pollinators: Two major species of rose are cultivated for the production of rose oil, obtained mainly from the flower petals: *Rosa damascena*, the damask rose, which is widely grown in Bulgaria, Turkey, Russia, India, Iran and China and *R. centifolia*, the cabbage rose, which is more commonly grown in Morocco, France and Egypt. Most rose oil is produced in Bulgaria, Morocco, Iran and Turkey. Recently, China has begun producing rose oil as well. Rose flower extracts contain over 300 volatile compounds which make up their floral scent mixtures and work together to attract potential pollinators.

Of all the compounds that have been identified in rose oil, the most common are: citronellol, geraniol, nerol, linalool, phenyl ethyl alcohol, farnesol, stearoptene, α-pinene, β-pinene, α-terpinene, limonene, *p*-cymene, camphene, β-caryophyllene, neral, citronellyl acetate, geranyl acetate, neryl acetate, eugenol, methyl eugenol, the rose oxides [(4*R*,2*S*)-(−)-*cis*-rose oxide, (4*S*,2*R*)-(+)-*cis*-rose oxide, (4*S*,2*S*)-(+)-*trans*-rose oxide, (4*R*,2*R*)-(−)-*trans*-rose oxide], α-damascenone, β-damascenone, benzaldehyde, benzyl alcohol, rhodinyl acetate, β-ionone, and phenyl ethyl formate.

The key compounds that contribute to the distinctive scent of rose oil, however, are β-damascenone, β-damascone, β-ionone, and the rose oxides. Even though these compounds exist in less than 1% quantity of rose oil, they make up for slightly more than 90% of the odor content due to their low odor detection thresholds [7]. The odor detection threshold is generally considered to be the lowest concentration of a certain odor compound that is perceivable by the human sense of smell. It also applies to insect pollinators that are in search of a food reward from the flowers they visit, and the threshold appears to be much lower for most insects. The threshold of a chemical compound is determined in part by its shape, polarity, and molecular weight, as well as the receptors that perceive it. However, the olfactory mechanisms responsible for a compound's different detection threshold are not well understood.

III. HOW AND WHERE ARE ESSENTIAL OIL COMPOUNDS PRODUCED BY FLOWERS?

(a) ***How are essential oils made?*** Although there are some 1700 volatile compounds identified so far, most of them are produced by only a few major biochemical pathways. These include the isoprenoid, lipoxygenase, and phenylpropanoid /benzenoid pathways. Several model plants having strong floral scents, such as *Clarkia breweri, Antirrhinum majus* (snapdragon), *Petunia hybida, Rosa* spp (rose), *Stephanotis floribunda*, and *Nicotiana suaveolens*, have been used to isolate and characterize the enzymes and genes involved in the biosynthesis of floral volatiles [8].

All terpenoids originate through the condensation of the five-carbon building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are universal and derived from two alternative pathways localized in different cellular compartments. In the cytosol, IPP is synthesized from the classical mevalonic acid (MVA) pathway, which starts with the condensation of acetyl-CoA. However, in plastids, IPP is formed from pyruvate and glyceraldehyde-3-phosphate via the methylerythritol phosphate (MEP) pathway [4, 9, 10]. Metabolic crosstalk between these two different IPP pathways has also been reported, especially in the direction of plastids to cytosol [11,12].

In both cellular locations, IPP and DMAPP are used by prenyltransferases in condensation reactions to produce prenyl diphosphates. For example, in plastids, head-to-tail condensations of IPP and DMAPP catalyzed by the prenyltransferase, geranyl diphosphate (GPP) synthase, yield GPP, the precursor of all monoterpenes [13]. In the cytosol, condensation of two IPP molecules with one DMAPP by the action of farnesyl diphosphate (FPP) synthase generates FPP, the C15 diphosphate precursor of sesquiterpene biosynthesis [14]. The genes encoding such enzymes have been isolated from diverse plant species, and they all appear to be related to one another, as well as to other prenyltransferases from animals, fungi, and bacteria [4,15,16].

After the formation of such prenyl diphosphate precursors, the various monoterpenes and sesquiterpenes are generated through the action of a

large number of enzymes named terpene synthases [17]. Many of the terpene volatiles found in floral scent mixtures are direct products of such terpene synthases, while others are formed through alteration of the primary terpene skeletons by hydroxylation, dehydrogenation, acylation, and other reactions [8]. Similar mechanisms control the formation of diterpenes and irregular terpenes.

Volatile fatty acid derivatives make up most of the aliphatic compounds, including saturated and unsaturated short-chain alcohols, aldehydes, and esters. They represent the second largest class of floral volatiles and originate primarily from membrane lipids through the action of the lipoxygenase pathway. Such fatty acid derivatives are primarily derived from the degradation of C18 fatty acids (linolenic and linoleic acids) [6]. After being transformed to a hydroperoxide by lipoxygenase, they are cleaved into C12 and/or C6 components by hydroperoxide lyase [18]. Depending on the C18 substrate, hydroperoxide lyase produces either 3-*cis*-hexenal or hexanal, which are also common constituents of floral volatiles [19]. These short-chain aldehydes can undergo further processing by alcohol dehydrogenase and acyltransferase to be converted to the corresponding alcohols (3-*cis*-hexenol or hexanol) or 3-hexenyl acetate [20]. Recently, a good number of the genes involved in the lipoxygenase pathway have been identified; however, the expression of these genes has not yet been characterized in floral tissues [21].

Phenylpropanoids constitute a third large class of secondary compounds in plants and are derived from phenylalanine via a complex series of branched pathways. While most of the phenylpropanoids are not volatile, those that are reduced at the C9 position (to aldehydes, alcohols, or alkane/alkenes) or those that have alkyl additions to the hydroxyl groups of the phenyl ring or the carboxyl group are volatile [6]. In addition, many benzenoid compounds that lack the three-carbon chain and originate from *trans*-cinnamic acid as a side branch of the general phenylpropanoid pathway, are also volatile. These volatile phenylpropanoids/benzenoids are among the common components of floral scent [19].

The first committed step in the biosynthesis of most phenylpropanoid compounds is catalyzed by the well-known and widely distributed enzyme, L-phenylalanine ammonia-lyase (PAL). PAL catalyzes the deamination of L-phenylalanine (Phe) to produce *trans*-cinnamic acid [22]. The subsequent formation of benzenoids from cinnamic acid requires the shortening of the side chain by a C2 unit, for which several routes have been proposed. The side chain shortening could happen via a CoA-dependent β–oxidative pathway, CoA-independent non-β-oxidative pathway, or by a combination of both pathways [23]. While little is known about the genes responsible for most of the metabolic steps leading to phenylpropanoids/benzenoids, hydroxylation, acetylation, and methylation are quite common chemical modifications.

A large portion of floral volatiles contain a methylated hydroxyl group (a methoxyl group). As an example, methyl eugenol and methyl chavicol are the results of the 4-hydroxyl methylation of eugenol and chavicol, respectively, catalyzed by two separate, but very similar enzymes, eugenol and chavicol *O*-methyltransferases (OMTs), which use *S*-adenosyl-L-methionine (SAM) as the methyl donor [24]. Indeed, OMTs and other methyltransferases are quite active in the production of many essential oil compounds. Likewise, acyltransferases catalyze the acylation of alcohols with acetyl moieties, as well as with larger acyls such as butanoyl or benzoyl acyls, leading to the formation of volatile esters [4]. These acyltransferases often show wide substrate specificity for both the acyl moiety and the alcohol moiety. Similarly, oxidoreductases play an important role in interconversion of volatile alcohols and aldehydes. Such chemical modifications are different for each essential oil compound and their complexity is outside the scope of this review. However, the activity of the enzymes that catalyze such modifications is a key aspect to the complex mixtures of volatile compounds emitted from flowers.

(b) Spatial and temporal emission of floral essential oils: It has been found that floral aroma compounds are synthesized *de novo* in the tissues from which they are emitted, and their production in plants is under both spatial and temporal control. Within the flowers, the petals are the principal emitters of volatiles, although various other parts of the flower may also participate in volatile emission. For example, different parts of the petals, stamens, and pistils, as well as pollen and nectar, may emit different compounds [25-28]. While the same floral scent compounds are often emitted from all parts of the flower, they are not necessarily emitted in the same amounts, and in some cases specific compounds are emitted from specific floral organs

[29,30]. In addition, some species, such as orchids, emit the majority of their volatile compounds through highly specialized "scent glands" called osmophores [31]. However, in many species (e.g. *Clarkia* spp.), such scent glands are not present, yet the flowers still produce a very strong aroma.

Osmophores may be found within any part of the floral inflorescence as part of the petals, sepals, bracts, or anthers. Although they may vary in shape, they tend to have some common features. They form on the epidermal cells and generally face toward the adaxial (inner) side of the perianth, displaying a bullate, rugose, pileate, conical, or papillate shape [32,33]. Studies using transmission electron microscopy revealed that the cells of the glandular layers are supplied with abundant rough and smooth endoplasmic reticulum, many mitochondria, and lipoid droplets that appear to contain essential oils to be released, as well as lipids such as fatty acids and triacylglycerides [34].

Glandular trichomes present on floral organs may also be a source of floral volatiles. A well-known example is that of the glandular hairs that are distributed over the shoot vegetative and reproductive organs of members of the Lamiaceae (nettle family) [35]. The volatiles produced in these trichomes protect the plants against herbivores and attract pollinators to the flowers. Two types of glandular hairs in these plants include "short-term glandular hairs", which start and end secretion rapidly (serving to protect *young* organs); and "long-term glandular hairs", in which secretory materials accumulate gradually under an elevated cuticle (serving to protect *mature* organs).

As far as temporal control is concerned, the expression of genes encoding scent biosynthetic enzymes peaks one to two days ahead of the enzyme activity and actual emission of the corresponding compound. The temporal changes in the activities of the enzymes responsible for volatile formation suggest that the biosynthesis of volatiles is regulated largely at the level of gene expression [6,20,23,36, 37]. However, it is still unclear as to what extent transcriptional, post-transcriptional, translational, and post-translational events contribute to this process.

Emission of floral volatiles from some plant species also changes rhythmically during a 24 hour period, whereas other flowers may continuously emit volatiles as a constant rate. In addition, some plants emit one set of compounds during the day and another set at night [38]. Moreover, it has been shown that within the flower, different compounds are emitted in a rhythmic manner during a 24 hour period, while other compounds are not. This suggests that different mechanisms regulate the biosynthesis and emission of each volatile [39]. The rhythmic release of scent is almost always correlated with the corresponding temporal activity of the most efficient flower pollinator and is controlled by either a circadian clock or regulated by light [40,41].

Interestingly, the scent of many flowers is markedly reduced soon after pollination. Such post-pollination changes have been characterized mostly in orchids, where the subsequently reduced attractiveness of these flowers increases the overall reproductive success of the plant by directing pollinators to the flowers that remain unpollinated [42]. This is particularly important for plants with a low visitation rate, where reproductive success is mostly pollinator limited [43].

Thus, the timing and magnitude of essential oil production in flowers may vary within different floral organs according to the stage of plant development, timing of the opening of flowers, time of day or night (often according to circadian patterns), environmental factors (e.g., wind velocity and ambient air temperature), as well as the genetic background of the plant species [27,44].

IV. HOW ARE ESSENTIAL OIL COMPOUNDS EMITTED FROM FLOWERS?

Identification of the enzymes responsible for the formation of some floral volatiles has allowed the determination of how the levels of enzymatic activities are distributed in the different floral parts. After being synthesized, scent volatiles have to move to the exterior of the cell and evaporate. Until recently, it was not known whether these compounds were synthesized at the surface or whether they were transported from adjacent cells. *In situ* hybridization and immunolocalization studies on enzymes such as LIS (linalool synthase), IEMT (isoeugenol *O*-methyltransferase), and BAMT (benzoic acid methyltransferase) have demonstrated that the biosynthesis of the volatile products of these enzymes occurs almost exclusively in the cells of the epidermal layer of the petals and other floral organs from which they can easily escape and evaporate [26,40]. Once produced in the epidermal cells, four

major steps are involved in floral volatile emission: (1) trafficking within the epidermal cell; (2) export from the plasma membrane into the epidermal apoplast and subsequent transport across the cell wall; (3) permeation of the cuticle; and (4) evaporation at the surface of the cuticle.

Current understanding indicates that volatile compounds are formed (a) in the epidermal plastids and exported to the cytosol, (b) in association with the ER, or (c) in plastids and further modified in the ER [45]. In all cases, the compounds end up in the cytosol and are likely associated with membrane systems of the ER. To date, no concrete evidence is available for the mechanisms that traffic these compounds toward the plasma membrane; however, participation of the Golgi apparatus is likely, as it is often active in the trafficking compounds or their storage in the vacuole. In addition, direct vesicular transport or protein-mediated movement across the aqueous environment is also a possibility. Alternatively, there is one report of direct contact between the membranes of the ER and those of the plasma membrane that may create a lipophilic pathway for intracellular trafficking of floral scent compounds [46].

Export from the plasma membrane into the periclinal cell wall involves transfer of the relatively non-polar scent molecules from a lipophilic environment (the plasma membrane) to an aqueous compartment (the cell wall). The low solubility of scent molecules in an aqueous environment is thought to substantially hamper their transport cross the cell wall [47]. Again, the mechanisms behind this level of transport have not been investigated; so, this step is the second unknown in the overall scent export process. As one possibility, parts of the plasma membrane could detach in a process similar to exocytosis, to form vesicles of amphiphilic lipids [45]. Vesicular transport across the cell wall may then be directed by gradients of either bilayer constituents or scent molecules. In addition, specialized proteins, such as adenosine triphosphate binding cassette (ABC) transporters, may be involved both in the export from the plasma membrane and transport across the cell wall. Similarly, either lipid transfer proteins (LTPs) or other lipid-binding proteins could be involved in the transport of scent compounds across the epidermal cell wall.

As far as transport across the floral cuticle is concerned, there are currently no published reports on the cutin composition of floral tissues that can be compared with general models for cutin structure from vegetative organs. Consequently, only postulated mechanisms are available for the movement of volatiles across this membrane: (a) a non-polar pathway for the transport of lipophilic compounds and water [48], and (b) a polar pathway important for the transport of larger hydrophilic compounds [49]. Although the transport of scent compounds across the cuticle has not been well investigated, it is likely that these lipid-like molecules will move exclusively along the non-polar pathway.

Once at the surface of the floral organ, the essential oil compounds can easily evaporate and enter the airborne environment. However, most of the steps involved in the export of scent products clearly require energy. Consequently, these steps impose transport barriers that generate a build-up of scent products in the corresponding compartments [45]. It is likely that a critical concentration is built up that results in a concentration gradient from inside to outside, and it is this gradient that drives the transport of these compounds across the cell wall and cuticle. Such transport may also be facilitated by specific proteins, especially when moving compounds across the aqueous environment of the cell wall.

V. WHAT TYPES OF ORGANISMS ARE ATTRACTED TO ESSENTIAL OIL COMPOUNDS?

There is a wide range of aroma compounds that plant flowers may produce. Their variation in abundance within each floral scent mixture presents flower-visiting animals with an almost unlimited array of odor blends to be learned and recognized while foraging. Floral scent mixtures may contain from one to more than 100 compounds; however, most species emit between 20 and 60 independent compounds [50]. The amount of floral compounds produced varies from low picograms to more than 30 micrograms per hour [51]. For example, the flowers of many beetle and moth pollinated plants produce the highest quantities of scent compounds, while most hummingbird-pollinated plants produce little if any. The quality and quantity of floral sent composition varies within and between plant species, and such variation allows the sensory mechanisms of potential pollinators to perceive differences between species, sometimes from a great distance.

Flowers attract pollinators through highly-regulated visual and olfactory stimuli. The role of floral scent volatiles in attracting as well as eliciting landing, feeding, and in some cases mating behaviors on the flower varies with each flower-animal interaction [52,53]. Such pollinators may be invertebrates (insects) or vertebrates, and the relative importance of floral scent in the act of pollination depends on both the purpose of the animal's visit to the flower and the features of the animal's biology, such as general morphology. Most flowers are visited by a diverse array of potential pollinator species. Only a few of these may actually impact pollination [54]. Likewise, the variety of animal species that may pollinate a given plant species may vary in location. This sets up a selection pressure between the plant and animal, as it is in the best interest of the plant to produce flowers that are visited by the most efficient pollinator species. It is also in the best interest of the animal to find flowers that offer the most rewards. It is this selection pressure that has likely led to the evolution of such diverse arrays of floral scent [55,56].

In most cases, flowers reward pollinators with food, such as nectar, pollen or oils, used in direct consumption or to attract mates. Other materials, such as petals, resins or essential oils may also be taken from the flowers for use in nest building or sexual reproduction. Some flowers are deceitful in attracting animals, whereby they mimic oviposition sites, mates, or food sources of pollinators (see orchid case study below). Other flowers may provide essential breeding sites for their pollinators.

Table 3: Proposed chemical profiles of floral scents linked to primary animal pollinator groups, based on the review by Dobson, 2006 [57].

INVERTEBRATES

A. Generalist	
diverse insects	Fatty acid derivatives, terpenoids, and benzenoids. Usually one dominant.
B. Coleoptera	
tropical scarab beetles	Methoxylated benzenoid compounds common.
other tropical beetles	Fatty acid-derived esters, benzenoid esters, and terpenoids.
beetles of temperate regions	Variable; *N*-compounds frequent
C. Diptera	
food-seeking flies	Fatty acid-derived acids, alcohols, and *N*-compounds common.
midge-like flies	Variable.
male fruit flies	Methyl eugenol or 4-(*p*-hydroxyphenyl)-2-butanone common.
D. Insects associated with decaying organic matter	
beetles and flies on carrion	*S*- or *N*-compounds, fatty acid-derived acids, alcohols, ketones, as well as *p*-cresol (excrement odors).
flies on decaying vegetation	Variable with little data.
flies on decaying fruits	Variable with fatty acid-derived alcohols frequent.
flies on fungi	Fatty acid-derived alcohols, aldehydes, and ketones with occasional *S*-compounds.
E. Thrips	Variable with little data.
F. Bees and Wasps	
food-seeking bees	Variable with terpenoids normally abundant.
fragrance-seeking male bees	Few volatiles, mainly benzenoid and monoterpene compounds.
nectar-seeking wasps	Variable with little data.
fig wasps	Few volatiles, normally with one or two terpenoids dominating.
G. Moths and Butterflies	
micropterigid moths	Fatty acid-derived esters frequent.
yucca moths	Fatty acid-derived hydrocarbons and alcohols as well as sesquiterpenes.
butterflies	Benzenoids (phenylacetaldehyde, 2-phenyl ethanol, benzaldehyde, benzyl alcohol), terpenoids (linalool, *trans*-β-ocimene, *cis*-3-hexenyl acetate, oxoisophorone), *N*-compounds occasional.
nocturnal settling moths	Benzenoids (phenylacetaldehyde, benzaldehyde, esters), terpenoids (linalool, β-ocimene, lilac compounds), sometimes fatty acid-derived esters and *N*-compounds.
nocturnal hovering moths	Abundant benzenoids (esters, especially methyl benzoate), terpenoids (especially linalool), and *N*-compounds.

VERTEBRATES

A. Birds	Weak or no scent.
B. Bats	*S*-compounds common.

There are literally thousands of pollinator species, and most have developed highly sensitive mechanisms for detecting and distinguishing between the complex arrays of volatile mixtures that they may encounter on a daily basis. While there is still surprisingly little information on how each species uses floral scent to efficiently choose which flowers to visit, there appear to be some generalized "pollinator syndromes" that can be described from the species that have been studied in detail. One reference that has attempted to make these generalizations is Dobson (2006) [57]. Table 3 shows a summary of their findings.

Many plant species have animal associations that fall under a generalist pollination syndrome, where the flowers are pollinated by a diversity of insects (beetles, flies, bees, butterflies) that feed on the exposed nectar and pollen [58]. Typical examples of plant families that have animal species displaying this pollination syndrome include, Apiaceae, Arecaceae, Rosaceae and Ranunculaceae. Coleoptera or beetles often visit flowers to feed on pollen, floral tissues, and other floral exudates [59]. They also use flowers as sites of mating and egg laying, and flowers pollinated by beetles are generally placed under the syndrome of cantharophily [58]. Diptera (flies) is also an important order of flower pollinators, where most act as generalists in their associations with flowers [60]. Flies form a major portion of the pollinators at higher elevations and latitudes, where they replace the small bees that are most prevalent at lower altitudes [61].

Flowers that are pollinated by insects associated with decaying and organic matter have traditionally been classified under the syndrome of sapromyophily, but this term is somewhat of a misnomer because the pollinators include not only flies, but also, many types of beetles [57]. Such flowers are characterized by colors that tend to be dull and dark brown and purple, and the pollination is typically performed by deceit. Here, flowers mimic mating and/or egg-laying sites. They emit odors that resemble the smell of decaying protein, dung, urine, mushrooms, cabbage, or onions. There is also increasing documentation of plant species that are pollinated by Thysanoptera or thrips. Thripophily has been proposed as a relatively new pollination syndrome [59]. Thrip-pollinated flowers tend to be of medium size, white to yellow, have floral structures that provide shelter, and are sweetly scented [62].

Perhaps the best known insect pollinators are bees and wasps, Hymenoptera. Pollination by bees, referred to as melittophily, covers plants that vary immensely in floral morphology and color, as well as fragrance, with no obvious trends emerging in scent chemistry [58,59]. Bees in general appear to detect a wide range of floral volatiles, and numerous studies have been made to address the ability of bees, especially honeybees and bumblebees, to discriminate between individual volatiles and different combinations of volatiles [63,64]. Similar statements can be made about wasps; however, there are few documented studies that deal with nectar-seeking wasps as primary pollinators. Most wasps feed on flowers with readily available nectar, and these are typically plant species with generalist-type pollination syndromes, such as species of Apiaceae [58].

Moths and butterflies (Lepidoptera) are primarily nectar-feeding insects, and are also well known for their roles as flower pollinators. Some groups, such as the Micropterigidae moths, have chewing mouth parts and also feed on pollen or in some case fern spores [65]. The proteins consumed by these insects also can provide the necessary energy for Micropterigidae species to survive longer than their counterparts that feed on nectar alone. The three major groups of lepidopteran pollinators that have evolved nectar feeding are the butterflies, settling moths, and hovering moths [59,66]. Since the majority of flower-visiting Lepidoptera have a long proboscis, a common feature of most flowers visited by these species is that they produce nectar in narrow tubes or spurs. For adult butterflies, the floral scents of the flowers that they visit are often described as weak, fresh, and sweet [67]. The nocturnally active Lepidoptera that serve as pollinators are either moths that land when they feed at the flowers (settling moths), which are principally members of Noctuidae, or moths that hover (i.e., hawkmoths) of the Sphingidae family. Flowers pollinated by nocturnal moths are usually characterized as having nocturnal anthesis (the time the flower opens), nectar in floral tubes or spurs, light color to be seen at night, and a generally pleasant and often very strong scent containing acyclic terpene alcohols (e.g., linalool), benzenoid compounds, and some nitrogen-containing compounds.

Vertebrates such as birds and bats are also important pollinators. Pollination by birds, or ornithophily, is carried out in both tropical and temperate parts of the

world. Such bird pollinators fall within mainly ten families [58]. Floral morphology depends on the type of bird pollinator, which may either hover while it feeds (hummingbirds) or perch (honeycreepers, sunbirds, white-eyes, sugarbirds, and honeyeaters) [58,68]. However, birds are not known for their sensitive sense of smell. Accordingly, most of the flowers that birds pollinate are reported to be either weakly scented or devoid of scent [69,70]. Bats, on the other hand, have a highly developed sense of smell, and olfaction is probably the main sensory mechanism used by bats to locate flowers. An estimated 750 plant species rely on bats for pollination [71]. The typical floral syndrome is similar to that of the nocturnal moths, having nocturnal anthesis, whitish or drab colors, copious amounts of nectar, and strong odors that are described as fetid, pungent, fermented, or butter-, cabbage-, or onion-like [72].

VI. HOW ARE ESSENTIAL OIL COMPOUNDS DETECTED BY POTENTIAL POLLINATORS?

Consider for a moment how a foraging insect is able to distinguish between the smell of different flowers, each of which may consist of hundreds of odor volatiles, intermingled among hundreds of other odor-emitting flowers in the environment. Humans can certainly distinguish between scent molecules in the air; however, insects are often considerably better at detecting these compounds. Unlike humans, insects live in an odor world where an ability to accurately distinguish chemicals in the environment is essential for survival. Mates are often located and identified by odor signals and pheromones, and egg laying (oviposition) sites having high levels of competition are avoided by deterring compounds. In addition, nectar-foraging insects, such as honeybees and moths, use olfactory cues emitted by flowers to find the food source. Consequently, insects have evolved considerably more advanced mechanisms with which to distinguish between the different constituents of the floral scent mixtures coming from diverse floral species. As alluded to above, the co-evolution between essential oil production in the flowers of plants and the highly specific sensing/detection systems in insects for these scent compounds has resulted in highly-successful and highly-specific pollination syndromes.

While the mechanisms behind detection, coding, and discrimination of single volatiles are fairly well investigated, odors are rarely encountered as single molecules under natural conditions. How insects are able to navigate the immensely complex world of scent and learn what specific flowers offer the best rewards largely remains a mystery. It has been well established that insects, such as honey-bees, learn their odor cues from visited flowers that have had good food rewards [73]. Presumably, the ability of pollinators to sense odor molecules combined with learning enables them to utilize resources more efficiently.

The major function of the olfactory organs is to provide the central nervous system with information about the identity and abundance of odor molecules in the environment. To accomplish this task, specific cells sense the presence of a chemical stimulus and transform it into changes in membrane potentials that can reliably send information to the target cells in the brain. In insects, olfactory receptors on the antennae and mouth parts bind to odor molecules, including floral scents and pheromones. Antennae are paired appendages connected to the front-most segments of arthropods (Figure 2).

The primary olfactory organs in insects are the antennae. On the third flagellum of most antennae are numerous cuticular formations, called sensilla, containing the sensory cells (Figure 3). Each sensillum normally houses two to five olfactory receptor neurons (ORNs), but rarely more than 100 [74]. The ORNs are bipolar cells connected directly to the brain. From the cell somata at the sensillar base, a dendritic end extends into an aqueous fluid, the sensillar lymph, which acts as the interface between neuron and environment. Odor molecules enter the sensilla through pores in the cuticular walls [75]. As most aroma compounds are lipophilic, the transfer from the pores to the receptor sites on the ORNs is believed to be facilitated by docking to "odorant binding proteins" (OBPs).

In contrast to other sensory systems, the olfactory system has to recognize and discriminate odor stimuli that are multidimensional with respect to physical properties. The solution as to how the olfactory sense deals with this problem came when the Nobel laureates Linda Buck and Richard Axel discovered the multigene family coding for odorant receptor proteins in rats [76]. Since then, such odorant receptor proteins have been found in other organisms, including insects [77-79].

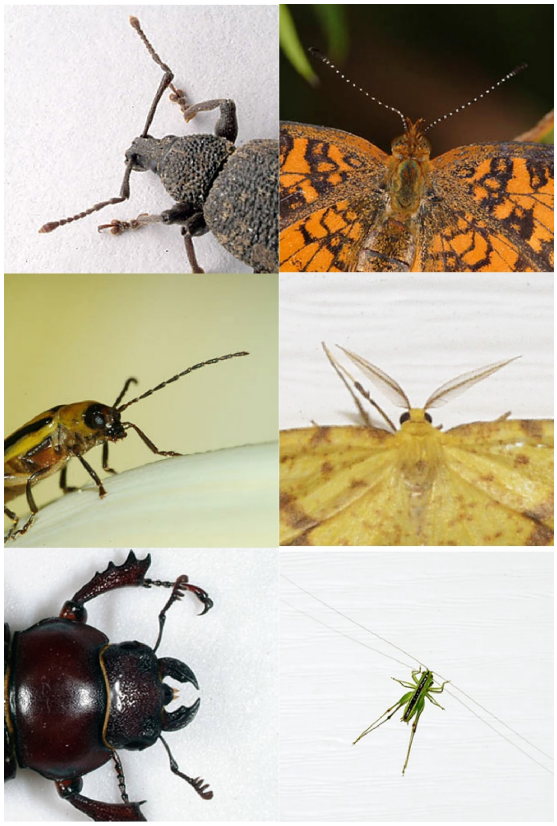

Figure 2: Some examples of the primary scent sensing organs of insects (paired antennae and mouth parts).

The size of the gene families coding for these receptors is remarkable and the number of different receptors expressed in olfactory tissues can be as large as 1300 in the mouse [80]. Even though the number is much lower in insects (~40 to 200), the gene family is still quite large [78, 81]. All odorant receptors identified so far are G-protein coupled 7-transmembrane proteins, but they show little homology between phylogenetically divergent groups of organisms [77, 82]. Most importantly, each insect ORN expresses only a single receptor type [77, 81, 83]. However, each type of receptor cell responds to several structurally similar compounds, and each of these compounds activates several types of receptor cells [84]. Any odorant will therefore excite several different types of receptor cells, and the pattern of cells excited by several odor compounds usually overlaps.

Binding of an odor molecule to a receptor protein triggers a second messenger cascade. The primary pathway in insects involves generation of inositol 1,4,5,-triphosphate (IP_3), which causes an influx of calcium ions into the dendrite [85]. The calcium then activates non-specific cation channels. The inflow of

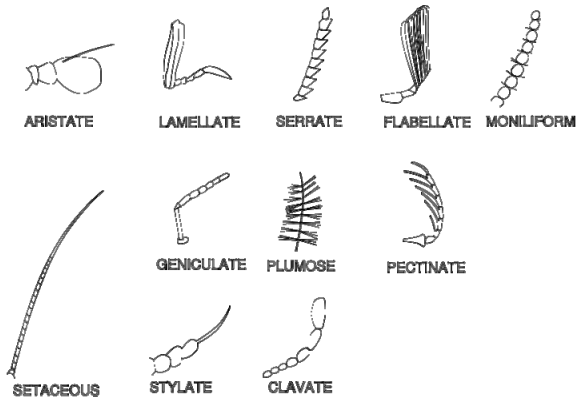

Figure 3: Examples of typical shapes of insect antennae.

cations through these channels changes the membrane potential, and (if the depolarization exceeds a certain threshold) an action potential is evoked at the initiation site near the soma. Action potentials carry information along the axons of the sensory cells into the primary olfactory center of the brain, the antennal lobe (AL). The AL is the locus of synaptic interactions with the brain interneurons, and the interneurons interconnect glomeruli, small cells in the olfactory bulb that form numerous synaptic connections with each other and with the output neurons [86,87]. The frequency of the evoking action potentials within a neuron is proportional to the concentration of the stimulus.

The molecular receptive range of ORNs that are tuned to specific floral aroma compounds has been covered by an extensive study performed by Shields and Hildebrand in the female hawkmoth (*Manduca sexta*) [84]. They used a large panel of volatiles (more than 100 different compounds) known to be emitted by flowers preferred by *M. sexta*. They found that some groups of ORNs are highly specific, while others have quite broad recognition. Since several different types of ORNs can be activated to a different degree by the same type of compound, the identity of the compound is likely contained in an "across-neuron" pattern. Likely, ORNs are tuned to a molecular feature shared by several different compounds, and each compound possesses several of these features, and thus activates different receptors. Since all ORNs expressing the same receptor protein converge on the same glomerulus in the AL, the identity of floral compounds is likely represented as unique combinations of activated glomeruli. These activity patterns depend on the odor identity, the odor abundance, and on previous experience. Such patterns can be quite complex and appear to explain how many types of compounds can be recognized by

the insect sensory system. As a comparison, it has been estimated that humans possess about 300 different functional receptor proteins [88]. Still, we can recognize more than 400,000 different odorous molecules [89].

VII. HOW DO POLLINATORS FINALLY DECIDE THAT THEY SHOULD COME TO A SPECIFIC FLOWER?

Activity patterns set up in the antennal lobe are made more complex when combined with the responses of other brain neuropils that represent reinforcing stimuli (such as color, shape, texture, taste). For example, honeybees have a cluster of cells located in the subesophageal ganglion that receive input from sucrose-sensitive taste hairs on the mouthparts [90]. They then send their outputs to the AL, where they influence the activity of most or all of the glomeruli. The anatomy and electrophysiological responses of one such cell cluster, called the VUMmx1, to odors and sucrose have been fairly well characterized [90]. The VUMmx1 may be an important linkage between odor and sucrose learning pathways in the insect brain. Likewise, two recent electrophysiological studies of ALs indicate that neural responses to odor are modified by reinforcement. In the honeybee, glomeruli that are activated by an odor show an increase in responsiveness to that odor after it has been associated with sucrose reinforcement [91]. Likewise, in the moth, individual units in the ALs show complex changes in response patterns when associated with reinforcement [92].

Similar reinforcement pathways have also been proposed by Raguso and Willis, where nectar-feeding insects use carbon dioxide (CO_2) as an additional indicator of nectar sources [93]. In fact, it was recently demonstrated that the CO_2 level was correlated with the secretion of nectar in the flower of *Datua wrightii* [94]. Thus, CO_2 may act as an additional indicator of food abundance to insects, but the unique structure of CO_2 suggested that its detection follows a different pathway. Indeed, ORNs tuned to CO_2 in moths are not located on the antenna, but in the labial palp pit organ (near the mouth parts) housing more than 2000 ORNs in *M. sexta* [94].

A foraging moth or bee visits from a few dozen to more than a hundred flowers on an average foraging trip, and it can make many such trips in a single day [95]. During these visits, it is able to associate floral stimuli, such as color, shape, texture, and odor, with nectar and pollen rewards produced by flowers [28, 96, 97, 98]. Based on these experiences, the insect's memory is continuously updated with current information about the nature and distribution of reward associated with a given species of flower. This memory influences ongoing decisions about staying or leaving a given food patch or whether to specialize on a particular species of flower [95].

Clearly odors do not work alone to attract floral pollinators. Instead, a combination of mechanisms and cues (e.g., visual cues, aroma compounds, CO_2) allow an insect to find important food sources. Highly selective ORNs are used to prepare the insect for especially important and predictable stimuli, while the broad and overlapping ORNs increase the coding capacity greatly and prepare the insect for an unpredictable and ever-changing odor world. In turn, the plants that provide the correct signals to potential pollinators benefit from the spread of genetic material to new generations.

(a) Case study: Production of mixtures of aromatic compounds by orchid flowers together with insect mimicry attracts highly species-specific insect pollinators: Orchids have evolved especially complex mechanisms for pollination. Orchid flowers are typically bisexual and consist of three sepals, three petals (two wing petals and the lip petal often adapted as a "landing platform"), a column of fused stamens and stigmas, and an ovary made up of three carpels. The lip petal of the flower encloses the column, resulting in the fusion of male and female parts. At the tip of this column is an anther cap with four masses of pollen called pollinia (pollen packets) tucked into two pocket-like structures. A pollinium has a sticky anther sac and a hooked caudicle. The remaining end of the column is formed by three fused fertile stigmas with the end of the stigma forming a sterile, sticky flap, the rostellum [1].

On many orchids, the lip (labellum) serves as a landing pad for flying insect pollinators. In some cases, the labellum is adapted to have a color, shape, and scent that attract particular male insects via mimicry of a receptive female insect. In fact, some orchids are completely reliant on this deception for pollination. For example, most species of the genus *Ophrys* ("eyebrow") imitate the female morphology of their specific pollinator, usually a bee, a wasp, or sometimes a large fly or beetle. This visual lure is enhanced by the production of pheromone compounds that mimic the female sex pheromones.

Ophrys has some species that look and smell so much like female bumblebees that male bees flying nearby are irresistibly drawn to the flower in an attempt to mate with the flower, such as with the Bumblebee Orchid (*Ophrys bombyliflora*). During this visit, the viscidium, and thus pollinia, stick to the head or the abdomen of the bumblebee, and upon "visiting" another orchid of the same species, the bumblebee ends up pollinating the sticky stigma with the pollinia. The filaments of the pollinia, during transport, take a position from which the waxy pollen is able to stick to the stigma in the second orchid, just below the rostellum; such is the refinement of the reproduction. If the filaments had not taken the new position on the bee, the pollinia could not have pollinated the original orchid.

Other species of *Ophrys* are mimics of different bees or wasps, and are also pollinated by males attempting to mate with the flowers. Many neotropical orchids are pollinated by male orchid bees, which visit the flowers to gather volatile chemicals that they require to synthesize pheromones used to attract mates. Each type of orchid places the pollinia on a different body part of a different species of bee, so as to enforce proper species-specific cross-pollination.

Orchids, such as Lady's Slipper (*Paphiopedilum*), have labella that are modified into a deep pocket that traps visiting insects, such as flies or bees that are lured into the pouch due to the bright colors of the flowers. In the process of climbing out of the pouch, the pollinator gets the flower's pollinium glued to its back. Pollination is then achieved when the same insect becomes trapped in other orchid of the same species, having to pass once again through the exit. Many other fascinating mechanisms of orchid pollination have evolved over time. Some of these include the following:

An underground orchid in Australia, *Rhizanthella slateri*, never sees the light of day, but depends on ants and other terrestrial insects to pollinate it. Many *Bulbophyllum* orchid species stink like rotting carcasses, and the flies they attract assist their reproduction.

Holcoglossum amesianum, native to China's Yunnan province, reproduces in a hermaphroditic manner, fertilizing itself by rotating its anther and inserting it into the flower's stigma cavity. This mode of pollination is likely due to the lack of wind and insects in the region where this species grows.

The bizarre *Catasetum* orchids produce either male or female flowers, depending on the individual. Male flowers have special triggers that literally flick away the pollinators they lure in the process of applying their pollinia. Darwin, himself, observed this spectacular process in *C. saccatum*, and was ridiculed by Thomas Huxley due to the event's alleged preposterousness.

The Star of Bethlehem orchid, *Angraecum sesquipedale*, of Madagascar, has an 18 inch long nectar-spur emanating from its labellum. Knowing that sphinxmoths pollinate all of its relatives, Darwin predicted that there was a sphinxmoth with an 18-inch long tongue that pollinates it. Over a hundred years after Darwin's death, the Madagascan sphinxmoth *Xanthopan morganii praedicta*, which has an 18 to 20 inch-long tongue, was discovered. Paradoxically, this particular sphinxmoth has never been observed feeding on the orchid in the wild.

(b) Case study: Changes in the production of the monoterpene, linalool, over evolutionary time controls the attraction of specific insect pollinators: Linalool is a naturally-occurring acyclic monoterpenoid alcohol found in the scent mixtures of many flowers and spice plants, and it has many commercial applications, the majority of which are based on its pleasant scent (floral, with a touch of spiciness). Like other monoterpenes, linalool is important in industry as a starting material in the production of perfumes and as a flavoring compound in food and drink [99, 100]. So, its study not only helps with the understanding of how plants communicate with insects, but may also benefit industry and agriculture, especially with the potential for the modification of scent production through transgenic plants or crop plants that are grown outside of their natural pollinator's living range and thus suffer from lower crop yields.

In addition to "linalool", this compound also has other names such as β-linalool, linalyl alcohol, linaloyl oxide, *p*-linalool, allo-ocimenol and 2,6-dimethyl-2,7-octadien-6-ol. In nature, over 200 species of plants produce linalool, mainly from the families Lamiaceae (mints, scented herbs), Lauraceae (laurels, cinnamon, rosewood) and Rutaceae (citrus fruits), but also, birch trees (*Betula* spp.) and other plants, from tropical to boreal climate zones [100-106]. Its chemical structure is shown in Figure 4.

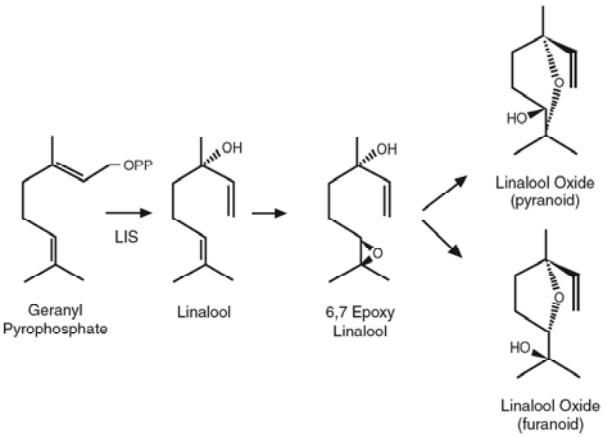

Figure 4: The linalool and linalool oxides pathway.

Linalool has a chiral center at C-3, and therefore, two stereoisomers: licareol is (*S*)-(+)-linalool (CAS No. 126–90–9) and coriandrol is (*R*)-(–)-linalool (CAS No. 126–91–0). Both enantiomeric forms are found in nature. *S*-linalool, for example, is found as a major constituent of the essential oils of coriander (*Coriandrum sativum* L., family Apiaceae) seed, palmarosa [*Cymbopogon martinii* var *martinii* (Roxb.) Wats., family Poaceae], and sweet orange (*Citrus sinensis* Osbeck, family Rutaceae) flowers.

R-linalool is present in lavender (*Lavandula officinalis* Chaix, family Lamiaceae), laurel (*Laurus nobilis*, family Lauraceae), and sweet basil (*Ocimum basilicum*, family Lamiaceae), among others. Interestingly, each enantiomer evokes different neural responses in humans, and therefore, are anthropophilically classified as possessing distinct scents. *S*-(+)-linalool is perceived as sweet, floral, petitgrain-like (odor threshold 7.4 ppb) and the 3*R*-form as more woody and lavender-like (odor threshold 0.8 ppb).

The enzyme resposible for linalool production is called linalool synthase (LIS), and it catalyzes the conversion of GPP directly to linalool (Figure 4). In *Clarkia breweri* plants (a small annual plant native to California and one of only a few species where LIS activity is characterized in detail), it is produced predominantly by the epidermal cells of the petals that are responsible for the majority of linalool emission from the flower [26]. Linalool also has its oxide forms that are produced through a suspected epoxide intermediate by an as-yet unidentified epoxidase (Figure 4). These oxides are produced predominantly in the transmitting tissue of the stigma and style of each flower where pollen tubes grow during pollination. The oxides, however, are a minor component of the floral scent mixture. Both linalool and its oxides are only produced when the flower is open, beginning as soon as the flower opens and ending just after the flower is pollinated. This timing has a distinct advantage for the plant since it avoids wasted energy by the production of compounds when they are not needed.

Interestingly, linalool is also known to be toxic to some insects, such as fleas. There is also some evidence through transgenic studies that linalool production can be toxic to young plant tissue. Thus, producing linalool only when a more mature tissue, such as a flower, has developed may avoid other toxic effects within the plant. In any case, the primary activity of linalool itself seems to be to attract a specific moth pollinator (a hawkmoth) that lives in the same regions as *C. breweri*. The oxides may also play a part in this role, but it seems likely from their expression patterns that linalool oxides have potential roles (1) in directing the visiting insect specifically to the stigma where it is most advantageous for the plant to have pollen placed or (2) in the inhibition of pollen tube growth of other species or the stimulation of pollen tube growth from the same species. The true function of the oxides, however, is not known.

Another interesting part of the *Clarkia* example deals with the general question of how the ability to produce linalool changes over evolutionary time [107]. As mentioned above, species that produce linalool are generally pollinated by moths, while species that do not produce linalool are pollinated predominantly by bees and butterflies. This part of the study focuses on the differences in the molecular genetics and biochemistry of scent production between *Clarkia* and *Oenothera* (evening primrose) species that determines the differences in primary pollinators.

Oenothera and *Clarkia* are in the same family (Onagraceae) and are thus very closely related. Most *Oenothera* species produce scent, including linalool; yet only two species within the *Clarkia* genus, *C. concinna* and *C. breweri*, produce any linalool at all [104, 105, 106]. Flowers of *C. concinna*, like those of all other *Clarkia* species, are odorless to the human nose. However, linalool and its pyranoid and furanoid oxides have been detected in *C. concinna* stigmata using gas chromatography/mass spectrometry (GC-MS), but at levels 1000-fold less than in *C. breweri*. Additionally, chromosomal, morphological, and genetic data suggest that

C. breweri has evolved relatively recently from *C. concinna* [102,106]. These observations raise at least two questions: (a) What is the function of the linalool pathway in non-scented plants such as *C. concinna*; and (b) what is the mechanism of evolution that allows the scent trait to be switched off and on over evolutionary time?

This evolution could occur through several mechanisms — enzymatic, morphological, or genetic — but research so far has narrowed the possibilities for differential scent production between *C. breweri* and *C. concinna* to control at the level of transcription [26,107]. It is generally accepted that *Oenothera* and *Clarkia* species share a common ancestor; yet, they show a surprising diversity in the ability to produce linalool. By characterizing the expression and regulation of genes that encode enzymes, such as linalool synthase, researchers are starting to uncover how scented species, such as *Oenothera*, evolve into non-scented species, such as most *Clarkia* species, and yet retain the ability to evolve into scented species again.

The case of the strongly scented *C. breweri* evolving from the more or less non-scented *C. concinna* is a clear example of gene level regulation of linalool synthase. As described above, the *LIS* gene of *C. breweri* has been shown to be highly expressed in stigmas and petals [26]. This *LIS* gene has also been isolated from *C. concinna* and has been shown to encode an identical protein [107]. However, in *C. concinna*, the gene is not expressed at all in the petals, but is expressed in the stigma at a drastically lower level than that of *C. breweri*. It is this difference in expression levels between the two species that draws hawkmoths as pollinators to *C. breweri*, but leaves *C. concinna* to be pollinated by more generalized insects, such as bees and butterflies.

VIII. HOW CAN BIOTECHNOLOGY OF ESSENTIAL OILS BENEFIT FLOWER POLLINATION?

Plants cultivated for their flowers, such as roses, have a major economic impact for countries around the world. Throughout history, people have harvested the flowers of particularly sweet smelling or otherwise distinctly scented plants for the shear enjoyment and subsequent profit of the smell. This is especially true for essential oil extracts from flowers. In fact, the original perfume industry arose from the observation that floral volatile compounds could be isolated and concentrated into essential oils and used as perfumes. On the other hand, while many essential oils are still collected, the bulk of perfumes are now produced from synthetic reactions.

Today, many of our commercially available flowers have been bred, using either inbreeding techniques or genetic transformation protocols, in order to produce plant cultivars having a greater diversity of colors (e.g., blue roses with genes for blue anthocyanin pigment biosynthesis being obtained from *Petunia hybrida*), larger or smaller flower sizes, and/or abnormal flower shapes (e.g., flowers with supernumerary petals resulting in so-called "double" flowers). Unfortunately, these recent commercial plant breeding programs in the "cut flower" industry have resulted in many new cultivars of formerly scented species that have substantial reductions in their floral scents. The reasons for this are not well understood, although it is likely that this resulted from the selection process being more focused on visual attractiveness and shelf life rather than the scent of the flowers [108]. The exact genetic mechanisms for such losses are not clear. However, an alteration in gene expression leading to the production of scent is likely.

For example, the scent of *Rosa chinensis* is rich in 1,3,5-trimethoxybenzene, but most modern roses, which are believed to be hybrids obtained by crossing *R. chinensis* with other rose species, do not emit this compound. The methyltransferase enzymes responsible for the last steps in its synthesis are present in modern roses [109]. However, it is hypothesized that hybrid roses lack the ability to synthesize 1,3,5-trihydroxytoluene, the substrate of the methyltransferases [110]. Still, the exact cause has not yet been determined.

A current initiative of plant breeders is to restore and/or alter floral scent, especially because of public demand, commercial potential, and the need to restore attraction of diverse kinds of pollinators to improve the productivity of various crop plants. One relatively new field devoted to controlling how flowers smell is called "scent engineering" [111]. Many groups of investigators are now beginning to focus on "scent genes" with an aim to understand how the expression of these genes can be manipulated in order to manipulate floral scent and essential oil production. The metabolic pathways and the genes that regulate the synthesis of the enzymes in these pathways are mainly those that produce

terpenes, phenylpropanoids, or fatty acid derivatives, as these are the largest and best understood of the scent compound categories.

Still, the complexity of the pathways can be mind-boggling with many interconnecting branch-points and chemical modifications, each of which is controlled by the expression of different genes. Recent attempts to re-engineer terpenoid production to enhance scent compounds in flowers of transgenic plants point to the importance of substrate availability for the enzymes that catalyze the reactions throughout the pathways. In many cases, the nature of the product and the efficiency of its formation are determined by the availability of substrates for the final reaction. This is especially true when the final reaction is catalyzed by an enzyme with broad substrate specificity, such as some methyltransferases and acyltransferases, as in the case for roses described above [23,112,113].

The role of substrate in the regulation of the biosynthesis of volatile compounds was recently confirmed by metabolic engineering, as denoted in the two examples below:

Example 1: When the *LIS* gene was introduced under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter into transgenic Petunia (*Petunia hybrida*) [114] and carnation (*Dianthis caryophyllus*) [115] flowers and leaves, the organ-specific differences in the amount of synthesized linalool or its glycoside depended more on the availability of the GPP substrate within each tissue than on the level of expression of the *LIS* gene [114]. These plants normally do not emit linalool from either their leaves or flowers.

Example 2: Introduction of three lemon (*Citrus* × *limon*) terpenoid synthases in tobacco (*Nicotiana tabacum*) flowers and leaves, again using the constitutive 35S promoter, resulted in the emission of native terpenoids, which are present in the non-transgenic plants, as well as new terpenoids that included ß-pinene, limonene, and γ-terpinene [116]. Subsequently, mint (*Mentha* spp.) limonene-3-hydroxylase genes were introduced into these transgenic tobacco plants, resulting in consequent production of (+)-*trans*-isotranspipiterinol from (+)-limonene via hydroxylation [116, see [111] for more examples]. However, the directions that the branched pathways appear to take depend again on the abundance of the substrates for these reactions and to a lesser degree the expression of the transgene.

The above two cases represent examples of *de novo* scent production in transgenic plants. However, scent restoration in plants that have lost their scent via inbreeding has not yet been achieved. In contrast to *de novo* scent production, the elimination of some of the floral scent volatile constituents produced in the phenylpropanoid/benzenoid pathways has been achieved in *P. hybrida* using gene silencing RNAi technology [117-120]. Thus, the use of new technology (including gene silencing) allows such studies to ask the question: What would be the effect of reduced scent volatile diversity on the numbers and kinds of insect pollinators that visit such flowers? In the near future, the answers to such questions will likely lead to some exciting new directions for (1) the productivity of crop plants, (2) the resurrection of and manipulation of floral scent, and (3) the importance of essential oil compounds in our modern society.

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