

Full Length Research Paper

The interaction between the gall wasp *Leptocybe invasa* and *Eucalyptus camaldulensis* leaves: A study of phyto-volatile metabolites

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A comparative study of the volatile terpene fraction isolated from the leaves of *Eucalyptus camaldulensis* attacked by the gall wasp (*Leptocybe invasa*) and from the healthy leaves of the plant was carried out using gas chromatography-mass spectrometry. A total of 59 components representing 89.13 and 88.6%, respectively, of their total volatile fraction contents were analyzed. Of these volatiles, 26 compounds with concentrations greater than (0.1± 0.02%) have been used for statistical comparison. A number of 21 of these compounds were identified, in different concentrations, in the leaves volatile fraction of both healthy plants and plants attacked by the wasps. The other 5 volatiles: p-mentha-2-4(8) diene, δ-elemene, β-elemene, E-caryophyllene and bicyclogermacrene, were exclusively found in the volatile fraction of the attacked leaves. The newly produced compounds in the attacked leaves or the change in concentration of those commonly found in that fraction, could be part of the plant defense mechanisms, or/and an element of the plant allelopathic and communication mechanisms. Identifying the components of the gall wasp-damaged leaves can help in their recycling for different physiological, pharmacological and medicinal uses.

Key words: *Eucalyptus*, gall wasp, plant defense mechanisms, plant recycling, terpene fraction.

INTRODUCTION

Eucalyptus camaldulensis (Myrtaceae) (Figure 1) is one of the best known species of the genus *Eucalyptus* in the Middle East, its importance rely on the high production of its unique volatiles composition that exhibits medicinal and pharmacological activity. The volatile constituents in *Eucalyptus* have been extensively studied (Zrira et al., 2004; Barra et al., 2010; Elaiissi et al., 2011, 2012;

Akolade et al., 2012). All these investigations revealed the major constituents to be qualitatively more or less the same; however, quantitatively they are very different. The minor constituents show qualitative and quantitative variation depending on the soil, climate and degree of maturity (Giamakis et al., 2001; Ben Marzoug et al., 2010). Generally, 1,8-cineole is one of the most

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abundant and unique volatile constituent in *Eucalyptus*. However, in *E. camaldulensis*, the aromatic cyclic monoterpene p-cymene is more abundant than the 1,8-cineole (Begum et al., 2000; Barra et al., 2010). *E. camaldulensis* offers a broad spectrum of physiological, pharmacological and medicinal properties, which are ascribed to its volatile constituents that enable the use of *E. camaldulensis* oil as antimicrobial and antifungal (Sartorelli et al., 2007; Barra et al., 2010; Gilles et al., 2010; Panahi et al., 2011), antioxidant (Akolade et al., 2012), antispasmodic (Begum et al., 2000), insecticide, larvicide, pesticide and antimalarial (Batish et al., 2008; Cheng et al., 2009; Alzogaray et al., 2011) agent.

The gall forming wasp (*Leptocybe invasa* Fisher and La Salle, Hymenoptera: Eulophidae), commonly referred to as the blue gum chalcid, has recently come into sight as the main pest attacking *E. camaldulensis* and causing severe injury. It was first identified in Northern and Eastern Africa, the Middle East and Mediterranean countries, then the pest has expanded to all over the world (Mendel et al., 2004). The wasp lays its eggs in the petiole and midrib of leaves and stems of young shoots, which leads to gall formation. Gall formation by *L. invasa* damages the growing shoot and leaves of *Eucalyptus*, resulting in the abscission of leaves and drying up of shoots (Karunaratne et al., 2010), as shown in Figure 1. Because *L. invasa* completes its development within the gall, chemical control is not applicable. Possible control measures would include breeding of less susceptible *Eucalyptus* species as well as biological control represented by the natural enemies of the wasp (Kelly et al., 2012).

Plant defense mechanisms protect the plant against any biotic or abiotic attacks. One of the most common plant defense mechanism is the production of specific secondary metabolites. These chemicals can either have an anti-microbial, antifungal or insecticidal activity that can affect the attacking organism, or allelochemical characteristics, thus spreading and allowing communication mechanisms with other parts of the plant or with the adjacent plants to warn against the wasp attack. Most of these allelopathic chemicals belongs to mono-or-sesqui-terpene type of secondary metabolites and are located in the volatile components of the plant metabolome (Henery et al., 2008; Troncoso et al., 2011). These compounds are made either constitutively in low concentration (constitutive defense mechanisms), or as a response to an attack on the plant (induced defense mechanism). In constitutive defense, secondary metabolites are produced and their biosynthesis is increased in case of attack. On the other hand, in induced defenses, a set of new metabolites are produced as a consequence of the attack.

The presented study is an attempt to investigate the effect of the gall wasp (*L. invasa*) attack on the volatile constituents of *E. camaldulensis*. This could help in the control of this wasp through stimulating plant defense or

production of new allelochemicals or insecticides. Studying and comparing the volatiles fraction of *Eucalyptus* prior to and post plant attack by the wasp can help the re-use and recycle of the infected *Eucalyptus* trees for new pharmacological and medicinal activities.

MATERIALS AND METHODS

Plant

The fresh gall wasp (*L. invasa*)-attacked and healthy leaves of *E. camaldulensis* (Myrtaceae) were collected from the Medicinal Plants Experimental Garden of the Faculty of Pharmacy, Zagazig University, Egypt in March 2014. The identification of the plants was confirmed by Dr. A. H. Abdel-Baset, plant taxonomist, Egyptian Agricultural Museum, Agriculture Research Centre, Cairo, Egypt. Identification of the insect species and wasp gall disease was done in the Ornamental, Medicinal and Aromatic Plants Diseases Department, Plant Pathology Research Institute, Agricultural Research Centre, Cairo, Egypt.

Isolation of the volatile terpene fraction

The fresh gall wasp-attacked and healthy leaves (100 g each) were cut and immediately subjected to hydrodistillation using Clevenger-type apparatus for 3 h (Leicach et al., 2010). The volatile fraction was recovered by decantation and dried over anhydrous sodium sulphate and stored at -20°C until analysis. The *E. camaldulensis* leaves attacked by the gall wasp *L. invasa* terpene fraction (ALTF) and the healthy leaves of the same plant terpene fraction (HLTF) gave a yield of 1.2 and 1.3% (v/fresh weight), respectively. Terpene fraction samples were kept in brown vials in the refrigerator at 4°C until further analyses. Six ALTF and HLTF samples from different trees were prepared as mentioned earlier. 20 µl of terpene fractions were diluted with n-hexane (HPLC-grade, Fisher chemicals) to 500 µl and used for the gas chromatography analysis.

Gas chromatography analysis

Gas chromatography/flame ionization (GC-FID) analysis was done using GC-2010 Plus, Shimadzu Corporation, Kyoto, Japan, gas chromatograph equipped with FID-2010 Plus detector. The following conditions were applied; column, RTX-5MS[®] fused silica capillary (30 m × 0.25 mm i.d and 0.25 µm film thickness); carrier gas He (2 ml/min); detector temperature 300°C, injection temperature 250°C; oven temperature program: initial temperature 45°C, 2 min isothermal, 300°C, 4°C/1 min, then 20 min isothermal; split ratio was 1:15, injection volume was 5 µl. Gas chromatography/mass spectrometry (GC-MS) data were recorded on GCMS-QP2010 Plus, Shimadzu corporation, Kyoto, Japan. The ionization energy for the mass spectrometer was 70 eV. Split ratio was 1:30; full scan mode was used, other conditions were identical to those mentioned for GC-FID. GC-MS was used to identify the compounds, which were then quantified using GC-FID.

Components identification and percentage area calculation

Kovat's retention indices (RI) were calculated with respect to a set of co-injected standard hydrocarbons (C10-C28). The analytes were identified by comparing their spectral data and retention indices with Wiley Registry of Mass Spectral Data 10th edition (April 2013), NIST 11 Mass Spectral Library and by comparing with



Figure 1. *Eucalyptus camaldulensis* leaves. Photos on the left represent the healthy *E. camaldulensis* leaves and photos on the right represent *E. camaldulensis* attacked by the gall wasp *Leptocybe invasa*. The samples were taken from the Medicinal Plants Experimental Garden, Faculty of Pharmacy, Zagazig University, Egypt.

published data (Adams, 2007). Calculations of peak percentage areas, based on FID response, are as follow:

Percentage area of peak= (The FID peak area / The sum of all the FID peaks areas) × 100

Most of non-identified components are present as traces with relative abundances of less than 0.1%. The most important constituents identified in the volatiles fractions analysed are listed in Table 1.

The percentage area ratio was calculated for each component and displayed in Table 1. This ratio indicates that this component increased in concentration due to the gall wasp attack (the ratio will become than 1) or decreased due to the attack (the ratio will be less than 1). The percentage area ratio was calculated using the following formula:

Percentage area ratio for a compound= (Percentage area of this compound in ALTF/ Percentage area of this compound in HLTF)

Statistical analysis

Six samples were used for both ALTF and HLTF (n=6), respectively and each sample was injected in triplicate. Quantitative values are expressed as mean ± standard error of mean (SEM) of percentage areas and significance difference was determined using unpaired student-sample-t-test performed using SPSS statistical package

version (SPSS for Windows, Version 11.5, SPSS Inc., Chicago, IL). $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Volatile terpene fraction components

The gas chromatographic analysis of the volatile terpene fraction produced by *E. camaldulensis* leaves attacked by the gall wasp *L. invasa* (ALTF, Figure 2B) and the healthy leaves of the same plant (HLTF, Figure 2A) allowed the separation and identification of 59 components representing 89.13 and 88.60% of the total fraction contents respectively. The results of HLTF samples were very much in agreement of published data of the same species elsewhere (El-Ghorab et al., 2003; Cheng et al., 2009) with little qualitative and quantitative variation in minor constituents. This variation could be attributed to the stage of maturity or other ecological factors. Twenty-six major compounds at an average concentration greater than $0.1 \pm 0.02\%$ have been retained for the statistical comparison (Table 1). From these main components, twenty-one were found in both HLTF and ALTF, in different percentages, whilst five components were found exclusively in ALTF. These specific compounds were the open chain mono terpene p-mentha-2-4(8) diene and the sesquiterpenes δ -elemene, β -elemene, E-caryophyllene and bicyclogermacrene. These compounds existed in different concentration in ALTF samples varying from 0.69% for p-mentha-2-4(8) diene to 2.10% in case of bicyclogermacrene with a total of 5.58% of the total fraction components' percentage (Table 1 and Figure 2C). The percentage areas of other volatile components showed an increase or decrease in concentration between the ALTF and HLTF samples. Some compounds dramatically increased in ALTF samples such as myrecene, α -phellandrene, sabinene and β -phellandrene (increased by 10.56, 7.91, 3.37 and 5.45 folds, respectively). Interestingly, the main component in HLTF, p-cymene, decreased in ALTF samples to reach 0.61 of its percentage area in HLTF samples (12.8% of the total components' percentage). Moreover, all oxygenated monoterpenes component in ALTF, such as 1,8-cineole, cryptone and 1-terpeniol decreased to 0.55, 0.60 and 0.14 of their percentage area values in HLTF samples, respectively (Table 1).

The sesquiterpene fraction increased from 8% in HLTF to reach 11% of the total percentage area of components in ALTF samples and nearly all the newly produced components were found in this fraction. The amount of spathulenol decreased by gall wasp attack to reach 0.70 of its percentage area in HLTF samples.

Biosynthesis of volatile components

The attack of gall wasp induced the production of

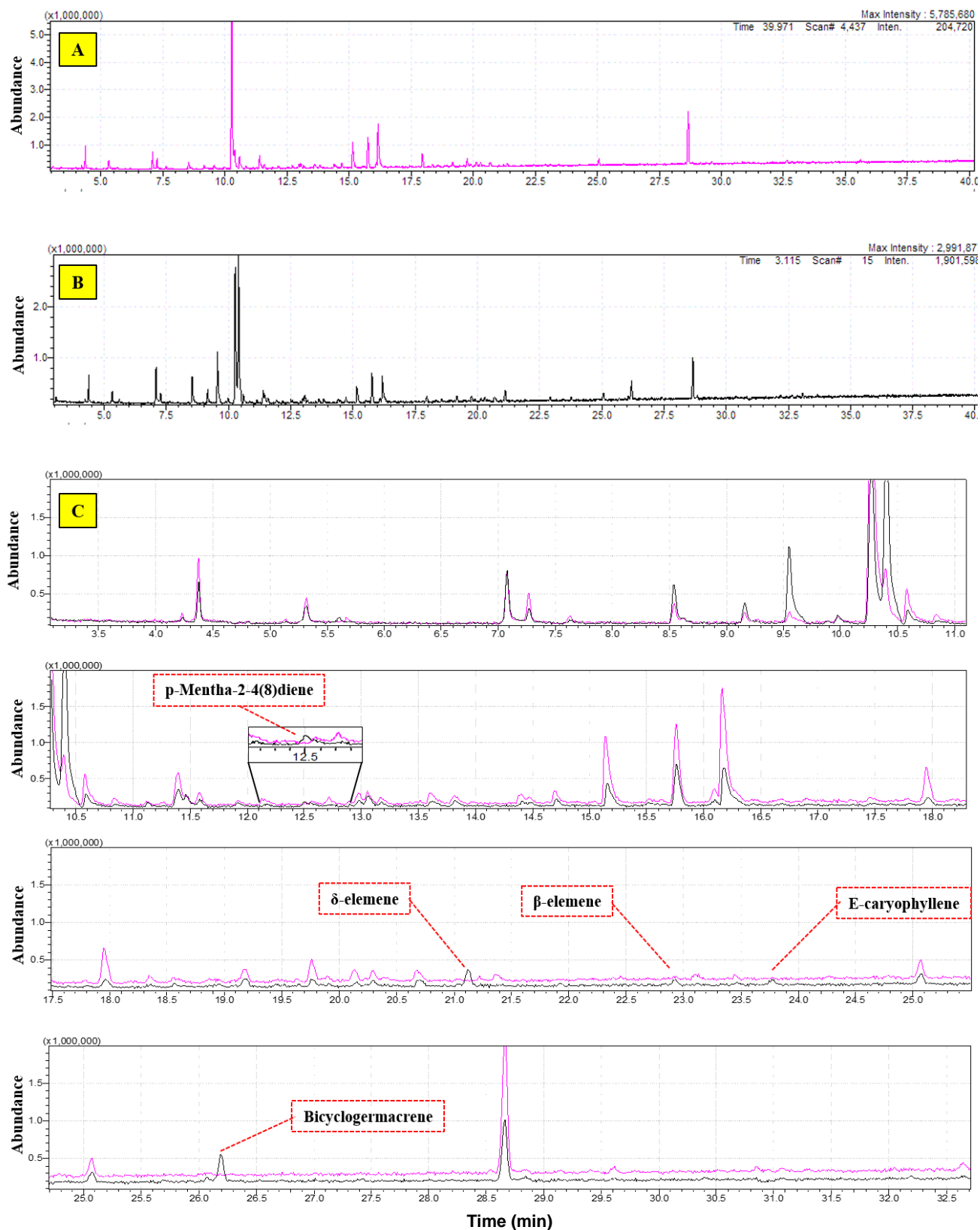


Figure 2. GC-MS chromatograms of the volatiles fraction extracted from *Eucalyptus camaldulensis* healthy leaves (HLTF) (A) and leaves attacked by the gall wasp (ALTF) (B). (C) Expanded and aligned GC-MS chromatograms of healthy (HLTF, upper) and gall wasp-attacked (ALTF, lower) volatile terpene fraction of *Eucalyptus* leaves. The signals of the five compounds produced only in ALTF samples are labeled in red dotted boxes.

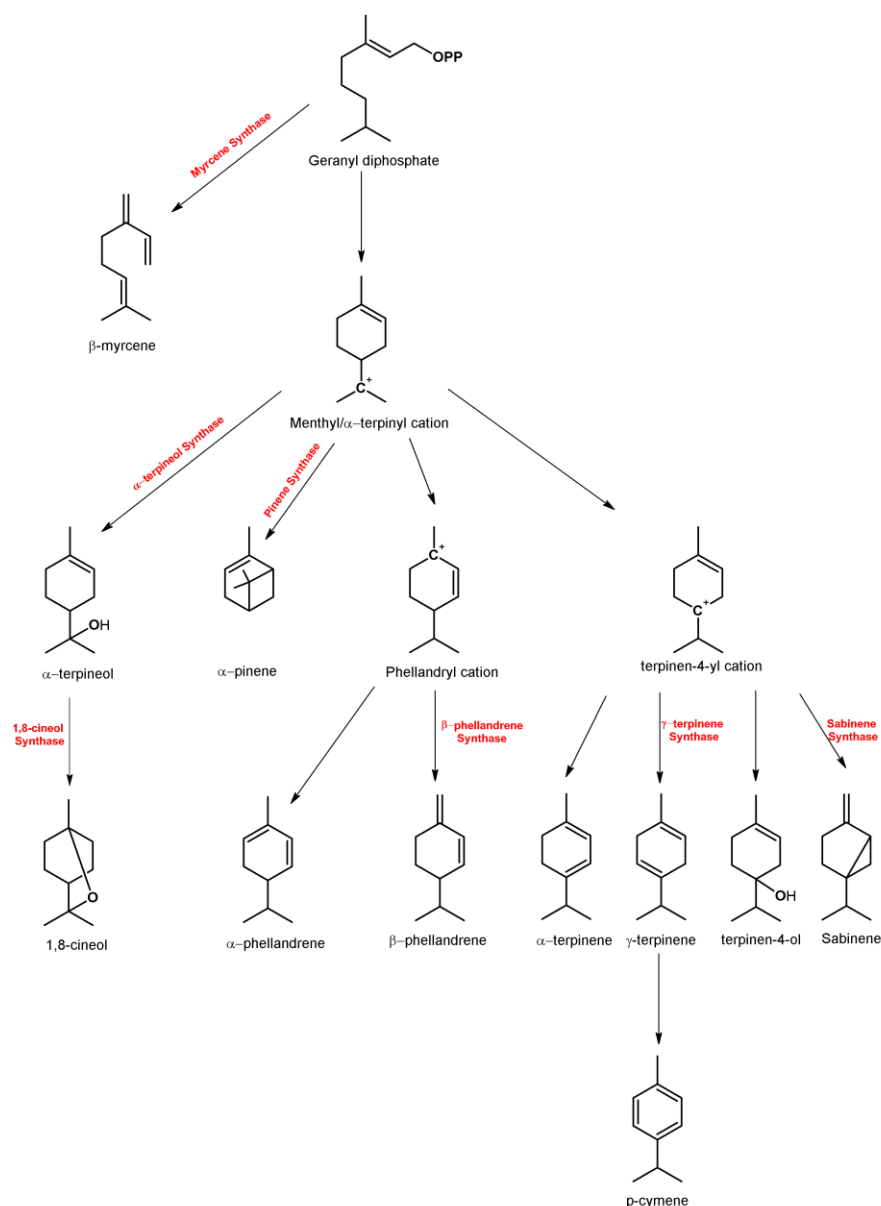


Figure 3. Biosynthesis cascade of some monoterpenes found in *Eucalyptus* volatile fraction.

aliphatic non-oxygenated monoterpenes as in the case of myrcene and phellandrenes. Oxygenation process was generally inhibited and thus the production of compounds such as 1,8-cineol, cryptone and terpinen-4-ol was reduced. Furthermore, production of aromatic compounds such as p-cymene was also diminished; consequently, the production of p-cymene-7-ol and carvacrol was affected (Table 1). The biosynthesis of monoterpenes in *Eucalyptus* could go through four different metabolic pathways as shown in Figure 3. These pathways produce α -terpineol, pinenes, phellandrenes and terpinen-4-yl cation. The attack with gall wasp might have induced enzymes in the pinene and phellandrene

pathways and some enzymes in the terpinen-4-yl cation pathway; however, it could have inhibited enzymes in the α -terpineol pathway (Figure 3). These induction processes might have resulted in the increase of phellandrenes on the expense of oxygenated and aromatic monoterpenes such as 1,8 cineol and p-cymene. The gall wasp attack could have induced the myrcene synthase enzyme resulting in the 10-fold increase in the open chain monoterpene, myrcene. The biosynthesis of p-mentha-2-4(8)diene could be achieved through the phellandryl cation by satisfaction of carbon 1 cation from carbon 4 and formation of a double bond between carbons 4 and 8, however, this pathway needs

Table 1. Comparison between the volatile constituents of gall wasp attacked- and healthy *Eucalyptus camaldulensis* leaves. Twenty six components of the volatile terpene fraction with concentrations greater than (0.1±0.02%) were compared. The ratio of attacked (ALTF) to healthy (HLTF) fraction of the same compound are represented in the ALTF/HLTF ratio column. The values of components' percentage area are expressed as mean ± SEM of six independent samples (n=6).

Compound name	R _t (min)	RI	Percentage area		Significance difference*	ALTF/HLTF Ratio [#]
			HLTF	ALTF		
α-Pinene	7.081	917	1.96±0.090	3.22±0.101	Yes	1.642
Tricyclene	7.268	924	1.32±0.070	0.92±0.036	Yes	0.698
Sabinene	8.538	967	0.82±0.018	2.76±0.037	Yes	3.374
β-myrcene	9.159	988	0.15±0.012	1.63±0.025	Yes	10.562
α-Phellandrene	9.553	1001	0.71±0.024	5.59±0.120	Yes	7.910
α-Terpinene	9.978	1012	0.21±0.010	0.61±0.006	Yes	2.842
p-Cymene	10.266	1020	20.6±0.157	12.8±0.106	Yes	0.618
β-Phellandrene	10.401	1024	2.6±0.132	14.6±0.113	Yes	5.458
1,8-Cineole	10.59	1029	1.9±0.073	1.05±0.081	Yes	0.556
γ-Terpinene	11.463	1052	0.24±0.010	0.95±0.046	Yes	3.891
p-Mentha-2-4(8)diene	12.514	1080	0.00±0.000	0.69±0.033	Yes	Present in ALTF only
Trans-sabinene hydrate	13.064	1095	0.72±0.023	0.94±0.046	Yes	1.306
1-Terpineol	14.399	1131	0.61±0.016	0.09±0.009	Yes	0.142
Terpinen-4-ol	15.761	1167	4.08±0.047	3.12±0.087	Yes	0.765
p-Cymen-8-ol	16.101	1176	0.75±0.011	0.64±0.019	Yes	0.860
Cryptone	16.178	1178	5.91±0.034	3.57±0.088	Yes	0.605
Cumin aldehyde	17.969	1228	1.81±0.032	0.87±0.049	Yes	0.478
p-Cymen-7-ol	19.772	1280	1.07±0.025	0.68±0.029	Yes	0.637
Carvacrol	20.294	1295	0.83±0.016	0.75±0.024	Yes	0.899
Veloutone	20.679	1306	0.91±0.024	0.77±0.022	Yes	0.841
δ-Elemene	21.121	1319	0.00±0.000	1.31±0.047	Yes	Present in ALTF only
β-Elemene	22.926	1370	0.00±0.000	0.74±0.024	Yes	Present in ALTF only
E-Caryophyllene	23.789	1395	0.00±0.000	0.74±0.018	Yes	Present in ALTF only
Aromadendrene	25.071	1435	1.07±0.023	1.11±0.063	No	1.034
Bicyclogermacrene	26.195	1471	0.00±0.000	2.10±0.086	Yes	Present in ALTF only
Spathulenol	28.666	1549	7.12±0.105	4.99±0.158	Yes	0.702
Total fraction percentage area			55.5	67.2	-	-

Rt: Retention time, RI: Retention index. *Significance difference between HLTF and ALTF percentage area for each component, determined using unpaired student-sample-t-test ($p < 0.05$). [#]The (ALTF/HLTF) ratio indicate if this component increased in concentration due to the gall wasp attack (the ratio is more than 1) or decreased due to the attack (the percentage is less than 1).

more investigation.

Similar scenarios can be implemented in sesquiterpenes biosynthesis; however, the situation is more interesting. The humulyl cation pathway might have been induced after the gall wasp attack, resulting in the production of E-caryophyllene. Furthermore, germacrene A and C beside bicyclogermacrene were produced after the attack and during GC analysis and due to high temperature, germacrene A and C undergo cope rearrangement to produce β- and δ-elemene, so the latter compounds are considered as artefacts of germacrene A and C (Adio, 2009). Both β- and δ-elemene were produced in concentrations of 0.7 and 1.3% of the total components' percentage areas, respectively. Bicyclogermacrene was produced, which is the precursor of guaiyl derivatives such as aromadendrene and

spathulenol (Mulyaningsih et al., 2010) (Figure 4). Therefore, it is better to say that bicyclogermacrene has accumulated due to inhibition in the production of the oxygenated sesquiterpene spathulenol, however, no trace for bicyclogermacrene can be found in HLTF samples. These compounds present only in ALTF samples, were not reported in previous studies of *Eucalyptus* volatiles, except for the analysis of the oil produced from immature *Eucalyptus* flowers (Giamakis et al., 2001). This study reports on the mechanisms for the production of such compounds in *Eucalyptus*, although they are not produced unless needed. In the case of immature flowers, these compounds are needed for the protection of flowers in this sensitive stage of plant growth. Similarly, the wasp attack induces in the plant, the production of such defense compounds.

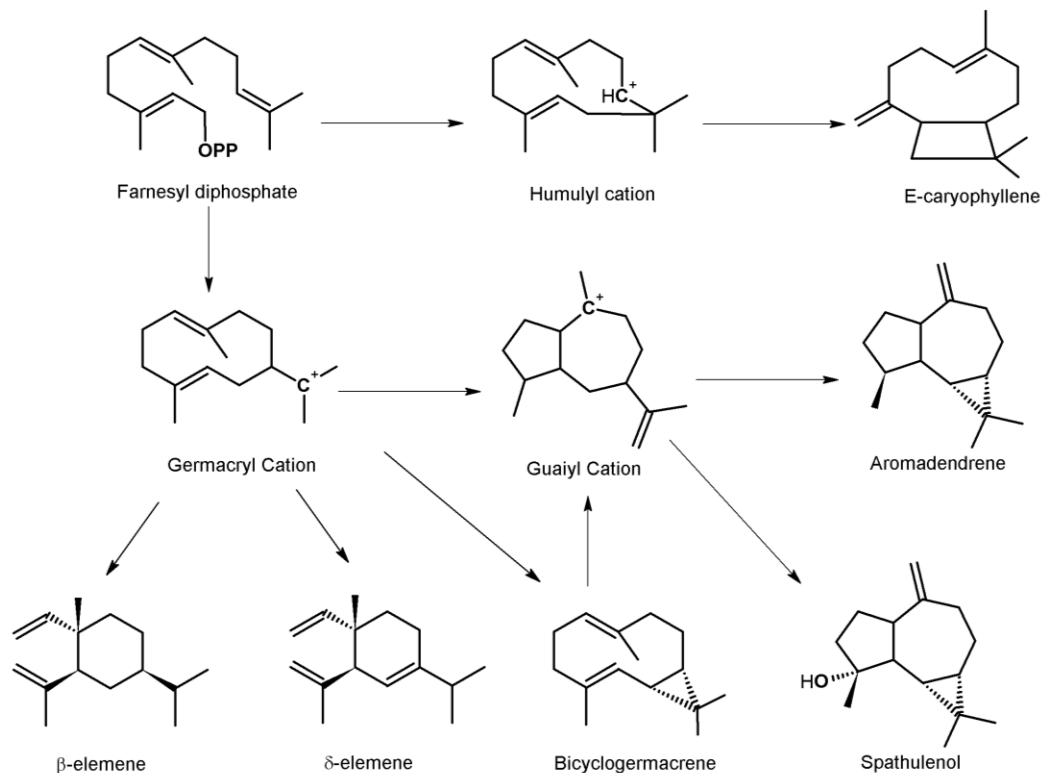


Figure 4. Biosynthesis cascade of some Sesquiterpenes found in *Eucalyptus* volatile fraction.

Plant defense and allelopathy

The present study reveals that, due to the wasp attack, the concentrations in the *Eucalyptus* leaves were increased for several volatile components, such as myrcene, α-phellandrene, sabinene and β-phellandrene. These components are assumed to represent the plant constitutive defense mechanisms or could be a part of the allelopathic mechanisms. *Eucalyptus* volatile components are known for their insecticidal and larvicidal activities (Batish et al., 2008; Cheng et al., 2009; Alzogaray et al., 2011; Gomes and Favero, 2013). Unfortunately, almost all these studies concentrate on the effect of total fraction, not the individual components. Cheng et al. (2009) demonstrated that α-phellandrene, γ-terpinene and α-terpinene exhibited good larvicidal activity against the mosquitoes' larvae, *A. aegypti* and *A. albopictus* (Cheng et al., 2009). These compounds' concentrations increased due to the gall wasp's attack on *Eucalyptus* leaves in the study hereby. The newly produced sesquiterpenes can also be a part of the plant-induced defense mechanism or they can be involved in allelopathy. Germacrene are known for their wide spectrum of biological activities, including antibacterial, antifungal, insect pheromones and phytotoxic activities (Adio, 2009; Vandermoten et al., 2011). The allelopathic effect of *Eucalyptus* is well known (Nishimura et al., 1984;

Inderjit et al., 1999; Verdeguer et al., 2009; Zhang and Fu, 2009), however, these published studies do not identify the compounds that determine this effect of *Eucalyptus*.

Reuse and recycle of attacked *Eucalyptus*

The gall wasp attacks thousands of *Eucalyptus* trees every year and the insect is not easy to be controlled. The leaves of the plants affected by this condition are inappropriate for medicinal use. The present study identifies the components of volatile fraction after the wasp attack. Many of these have increased concentration, such as myrcene. Myrcene has a good effect in gastric ulcers (Bonamin et al., 2014), so the elevation of this compound concentration in ALTF samples (to 1.6% of the total components percentage areas, a 10 fold) could make this fraction more suitable to treat ulcers. Germacrene are known for their cytotoxic and anti-cancer, anti-parasites and anti-inflammatory activities (Adio, 2009; Vandermoten et al., 2011). Phellandrenes are recognized for their antimicrobial, pain-inhibition and promotion of immune responses (Iskan et al., 2012; Lima et al., 2012; Lin et al., 2013). The presence of these new compounds or the change of other volatiles' concentration could add more medicinal and pharmacological properties

for *Eucalyptus*. Comparing the major terpene constituents of *Eucalyptus* attacked by the gall wasp and of the healthy plant leaves, might help in finding new uses and medicinal applications of the attacked plants.

Conclusion

The volatile terpene fraction of *E. camaldulensis* leaves has been analysed after the attack of the gall wasp (*L. invasa*) and compared to the same fraction obtained from healthy leaves of the same plant. The insect attack has triggered changes in the production of many phyto-volatile constituents, and also induced the synthesis of five new components: one open chain monoterpene and four sesquiterpenes. The insect switched on some metabolic pathways, and inhibited others, especially those for the production of the oxygenated and aromatic compounds. This regulation could be attributed to the plant induced or constitutive defense mechanisms against the insect attack, or to the plant communication to other plants or insects through allelochemicals production. A way to reuse and recycle the attacked *Eucalyptus* plants is to get benefit from their volatile constituents. This study reported some metabolic changes in the terpenes concentrations, plus the production of new compounds in the affected plants, thus suggesting that the medicinal uses and value of *Eucalyptus* can be further diversified.

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Conflict of interests

The author has not declared any conflict of interests.

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