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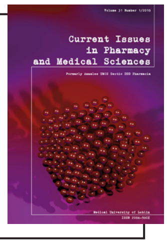

Chemical composition, toxicity, and acaricidal activity of *Eucalyptus globulus* essential oil from Algeria

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ABSTRACT

The study was aimed at determining the chemical composition, toxicity effect and field varroacidal efficacy of the essential oil distilled from the leaf of *Eucalyptus globulus* (Eg) grown in Algeria. Brine shrimp lethality (BSL) assay and bee hives infected by *Varroa destructor* were used to assess the toxicity and acaricidal effect, respectively. Steam distillation of leaves yielded 0.93 % (v/w) of essential oil (EO). GC/MS Analysis revealed 39 compounds, essentially oxygenated monoterpenes (86.01%). The main constituents of the oil were 1,8-cineole (78.45 %), o-cymene (2.18 %), isopinocarveole (1.74 %), α-pinene (1.69 %), pinocarvone (1.34%) and veridiflorol (1.31%). The BSL assay revealed a highly toxic value of LC50 (67.55 µg/mL). Furthermore, field efficacy testing on bee hives infected with *Varroa destructor* has confirmed the effectiveness of *Eucalyptus globulus* essential oil (EgEO) or thymol as varroacidal agents. Moreover, a EgEO + thymol association was more effective than EgEO or thymol alone. Finally, the use of EgEO + thymol may constitute a viable alternative to the thymol-based commercial treatment.

INTRODUCTION

Varroa destructor is an ectoparasite that contributes to the collapse of bee colonies, resulting in economic losses and ecological problems related to the role of bees as the most important pollinators on Earth [1]. Varroatosis has been treated by methods that include special apiculture practices (mesh floors, smoke, selection of resistant bees etc.) biological and chemical approaches [2-4]. Synthetic pesticides have been widely used throughout the world, however, they have often proved to be harmful to the environment and pose potential risks of contamination of honey and other hive products with chemical residues [5]. There is also clear evidence for the evolution of resistance in *Varroa* mite populations to conventional pesticides [6]. Given this situation, where the number of infected bee colonies is increasing steadily, there is an urgent need for an effective and environmentally-friendly means of control available to beekeepers. This problem has led to the appearance of several programs of prospecting natural products with anti-varroa potential [7-10]. Commercially available botanical products include formulations based on natural molecules such as camphor, thymol, and eucalyptol. Thymol-based formulations (e.g. Apiguard®, Thymovar®) are particularly effective, with a percentage that exceeds 90% [11]. Although thymol treatment has been shown to be effective in neutralizing mites, volatility problems have arisen [12], and treatment based solely on thymol may generate the potential resistance of *V. destructor* [13]. A special practice of local beekeeping in the forest regions of Skikda (city by the sea, 471 km from Algiers) is to combine conventional treatments with aromatic plants for better efficiency and lasting effect, thus, Eucalyptus leaves are used as fumigation in nearby hives. The efficacy of this traditional practice is plausible given the many scientific studies supporting the insecticidal properties of *Eucalyptus* essential oils [10-14].

A large number of *Eucalyptus* species have been introduced in Algeria, notably by the French settlers from 1864 to 1876 [15]. *Eucalyptus globulus* of the Myrtaceae family,

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also known as Tasmanian blue gum or blue gum, is one of the species originated from Australia which has well acclimated in Algeria, especially in the northern part where it found a favorable climate for its development. *Eucalyptus* leaves are largely used in the Algerian folk medicine for a range of therapy conditions. For instance, vapor from a hot water extract of the dried leaves is inhaled to retrieve symptoms associated with respiratory infections, such as cold, flue and sinus congestion [16].

The chemical composition of EgEO has revealed more than twenty compounds with 1,8-cineole, α-pinene and δ-limonene as major constituents [17,18]. EgEO is also widely used in modern cosmetics, food and pharmaceutical industries [19]. EgEO was shown to possess anti-inflammatory, analgesic, antiviral, antimicrobial, antioxidant and insecticidal activities [20,21]. Moreover, its has exhibited a varroacidal activity against *V. destructor* [1,2,9,10]. Essential oils components offer an attractive alternative to synthetic acaricides for the control of *V. destructor*. They are generally inexpensive and most pose few health risks. It is on this basis that we have studied the chemical composition, the acaricidal and toxic effects of the essential oil extracted from the leaves of *Eucalyptus globulus*.

This study is initiated on the basis that thymol is a product whose efficacy in the control of mites is established and that this effect could be potentiated by combining it with local *Eucalyptus* essential oils. The ultimate goal is to develop new products which are more efficient, but, above all, will overcome the problem of increasing pest resistance to treatments.

MATERIALS AND METHODS

Plant material and distillation

Leaves were collected in April 2014 at the herbarium of "Draa Naga" Djbel El Ouahch, located at 15 km east of Constantine, Algeria. The study area (Draa Naga herbarium) is situated between the longitude X1: 6°42'5", X2: 6°42'30" and the latitude Y1: 36°20'45", Y2: 36°22'15". *Eucalyptus globulus* was identified by a taxonomist (Dr Bouzid Mosbah) and the voucher specimen (Eg006501) was deposited for future reference at the herbarium of the Constantine Forestry Conservation. Essential oil was extracted from fresh leaves (1400 g) by steam distillation using a Clevenger apparatus for 4 hours. Distilled oil was immediately dried over anhydrous sodium sulfate and stored in screw-capped dark glass vials at 4°C until further testing.

GC/MS analysis

Essential oil extracted from the leaves of *Eucalyptus globulus* was analysed by a gas chromatograph coupled with mass spectrometer "GC/MS" (Agilent System HP-5MS.) as described below: Capillary chromatographic column of 30 m (length), 0.25 mm (diameter), and 25 µm (film thickness)], with apolar stationary phase of 5% phenyl and 95% dimethyl polysiloxane. Column compartment temperature was programmed from 50 to 200°C for 10°C/min; GC/MS interface was maintained at 230°C and the ionization source at 150°C; Helium was used as gas vector with a flow of 0.5 ml/min; Injection volume was 0.5 µl; MS ionisation energy was 70ev with scan band of 45-400 u. The essential components were tentatively identified by comparison with mass spectra data (MS) obtained from NIST-Wiley-MS library and confirmed by comparison with Kovats index on HP-5MS column.

BIOACTIVITY

Brine Shrimp lethality assay

The brine shrimp lethality (BSL) assay was used to predict the toxicity of the essential oil, as previously described [22]. Different concentrations (1000, 100, 10, 1 ppm) of EgEO were prepared using dimethyl sulfoxide (DMSO 1%). After 48 h, a drop of DMSO and 4 ml of sea water were added to each of the sample bottles containing the oil sample; Ten brine shrimp larvae of *Artemia salina* were carefully counted into each of the sample bottles and the volume of the sea water was made up to 5 ml. Tests for each concentration was done four times, and a control experiment containing 5 ml of sea water, a drop of DMSO and ten brine shrimp larvae was set along side. The experiment was maintained at room temperature for 24 hrs, the number of surviving larvae were counted and recorded, and the data obtained were subjected to Finney's probit analysis to determine the "LC50" of the oil.

ACARICIDAL ACTIVITY

Experimental apiary

This Experiment was conducted in an apiary located in nearby Azzaba (36°45'41.1"N, 7°03'50.3"E). Langstroth type hives of bees (*Apis mellifera*) whose colonies were infected with *V. destructor* were randomly assigned into four batches: batch 1 to 3 was treated with (1mL/hive/week) of each of EgEO, thymol, and EgEO + thymol (v/v) [2]. Batch 4 was used as a control (untreated hives).

Collection, counting and analysis

The method followed is the biological method deemed "raised diapers" or "covers background" [2,3]. This method is designed to track and count the fallen mites. Vaseline greased diapers are first placed in the hive, then removed and carefully examined with a hand lens to detect the dead *Varroa*. This method lasts 21 days, during which the counting is done every two days. After each count, the diapers are thoroughly cleaned and then replaced. The essential oil is deposited on a cardboard tab of 1 mm thickness to a width of 4 cm and a length of 20 cm, the deposited volume is 1 ml [2]. The tab is inserted through the main entrance of the hive; the treatment is repeated at the 7th day and then continues to the 14th day. The results are expressed in means of mortality ± standard deviation. The temperature during the experiment varied between 20°C and 22°C.

RESULTS AND DISCUSSION

Yield of essential oil

The essential oil extracted from the Eg leaves was yellow colored and had a camphor-like smell and pleasant odor, as previously described [17,23]. The extraction yield of essential oil was 0.96 %, a value within the range of those previously reported [24-27]. Of note, disparity of yields in the same species can be linked to genetic and environmental factors, time of collection and extraction method [28].

GC/MS analysis of essentials oils

The results of the GC/MS analysis revealed 39 compounds (Table 1), essentially oxygenated monoterpenes (86.01%), monoterpenes (5.74%), monoterpenes alcohols (4.05%) and sesquiterpenes alcohols (2.74%) (Table 2). The main constituents of the oil were 1,8-cineole (78.45 %), o-cymene (2.18 %), isopinocarveole (1.74 %),α-pinene (1.69 %), pinocarvone (1.34%) and veridiflorol (1.31%).

Table 1. Chemical composition of leaf essential oil of *E.globulus*

Compounds	KI	% composition
1,8-cineole	1030	78.45
o-cymene	1026	2.18
Isopinocarveole	1226	1.74
alpha-pinene	939	1.69
alpha-terpineol	1189	1.36
Pinocarvone	1165	1.34
Veridiflorol	1593	1.31
(+) spathulenol	1578	1.05
trans-p-mentha-1 (7), 8-dien-2-ol	1185	0.79
methyl benzene	773	0.77
Camphene	954	0.7
cis-p-mentha-1 (7),8-dien-2-ol	1235	0.51
4-terpineol	1177	0.5
1-phellandrene	1003	0.35
thymol	1290	0.28
Ledol	1569	0.28
trans-carveol	1217	0.22
butylester	2388	0.17
D-carvone	1243	0.16
alpha-selinene	1498	0.14
beta-myrcene	991	0.13
Myrtenol	1327	0.13
2-beta-pinene	979	0.12
Valencene	1496	0.11
gamma-terpinene	1060	0.1
Isospathulenol	1644	0.1

KI: compounds were tentatively identified by comparison with mass spectra data (MS) obtained from NIST-Wiley library and confirmed by comparison with Kovats index on HP-5MS column. **(%) composition**: percentage of concentrations based on peak area integration

Table 2. The percentage of particular groups of *E. globulus* essential oil components

Groups	Percentage (%)
Monoterpenes	5.47
Oxygenated monoterpenes	78.45
Monoterpenes alcohols	4.05
Monoterpenes ketones	3.41
Monoterpenes aldehydes	0.1
Sesquiterpenes	0.65
Sesquiterpenes alcohols	2.74
Aromatic hydrocarbons	0.87
Aldehydes	0.16
Esters	0.27

Previous investigations of EgEO have revealed various chromatographic profiles with different percentages of 1,8-cineole [29]. For example, EgEO samples from Brazil and Australia showed a high amount of 1,8-cineole, with an average of 85 and 90 %, respectively [30,31]. In contrast, an analysis of a *Eucalyptus* oil sample from Argentina displayed a moderate percentage of 60% [32], while those of Morocco and Kenya gave a low percentage - with an average of 22.4 and 17.22%, respectively [26,33]. Studies from Algerian EgEO collected from different sites revealed a variability in the composition from 47.05 to 53.3% [23,34]. These variations in Algeria EgEO and those of elsewhere denote the existence of several chemotypes, as previously reported [29].

BIOACTIVITY

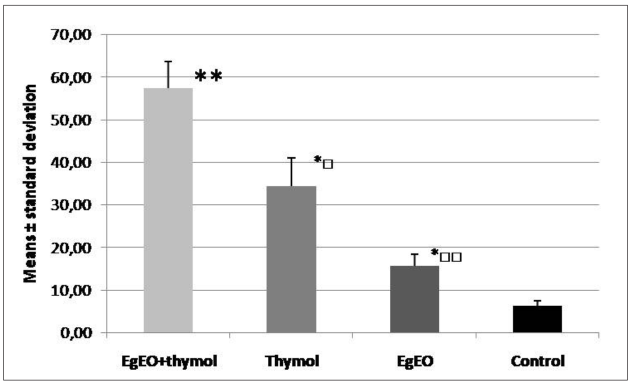
Toxicity and acaricidal activity

The result of the brine shrimp lethality assay of EgEO is shown in (Table 3). The (BSL) test revealed a value of "LC50" of 67.55 µg/mL. According to the toxicity scale established previously by Clarkson *et al*., the value of "LC50" obtained for EgEO is considered as highly toxic [35]. It is worth noting that the toxicity of our EO is lower than that obtained from a Nigerian sample which showed on the same assay an "LC50" value of 9.59 µg/mL [24]. The Nigerian EO has a different composition, with terpinen-4-ol at 23.46% and δ-terpinene at 17.01% as predominant components, while 1,8-cineole was present with only a 2.52%. This low value of 1,8-cineole suggest that other constituents of the Nigerian EgEO might contribute to its toxicity.

Table 3. Brine shrimp lethality assay of leaf extracts of *E. globulus* essential oil

Plant extract	Dose (µg/mL)	Nbre of tested shrimps	Nbre of survivors	"LC50" (µg/mL)
E. globulus	1	40	13	67.55
	10	40	20	
	100	40	25	
	1000	40	32	

The results of the field acaricidal testing of EgEO are shown in (Figure 1). All treated infected bee hives have showed significant fall of *Varroa destructor* compared to the control. The EgEO + thymol-treated batch showed as being the most efficient in removing the ectoparasite compared to the thymol (p = 0.05) and EgEO (p = 0.006) batches.



Value of fallen Varroa expressed as means ± SD (n=3);
(** p<0.01, * p<0.05) value vs control;
(□□ p<0.01, □ p<0.05) value vs (EgEO ± thymol) treatment

Figure 1. Number of dead *Varroa* expressed as means ± SD

However, no significant difference was observed between the thymol and the EgEO treated batches. Despite the situation that EgEO alone has a lower value of fallen *Varroa* (15.60±2.70) than that of thymol (34.37±6.56), the difference is not significant (p=0.07).

Several studies have been conducted world-wide, to understand the effectiveness of essential oils against *Varroa destructor* [29]. Aromatic species such as *Ferula assa-foetida. Allium sativum. Sizygium aromaticum, Piper aduncum, Thymus* spp.*, Eucalyptus* spp. has been tested successfully against the ectoparasite [29]. The acaricidal activity of EOs from *E. camaldulensis* on *V. destructor* mite was also investigated, and a LD50 of 1.74 µL/L was found [8]. A previous study using *Eucalyptus globulus* EO at a level of 10 µL, has showed an acaricidal effect of 45.75% of dead *Varroa* [10]. Our result revealed a slightly lower toxic effect toward the ectoparasite than the last study. This difference may be justified by the more important number of repeated treatments used by this study [10]. Finally, all these studies have outlined the usefulness of an EgEO treatment against *Varroa destructor*.

Despite the fact that the thymol treated group has shown a higher percentage of fallen *Varroa* than that of EgEO, no statistically difference between the two groups was observed. One of the reasons might be linked to the high variability detected in the apiary. To overcome this issue, the number of hives and apiaries that form such a study must be raised to obtain a better understanding of the effects of the applied treatments. It should be underlined that the most important fall of the ectoparasite was recorded with the association of EgEO + thymol. This result is significant and raises other crucial issues. Among the raised questions is whether the observed effect of EgEO is attributable or not to 1,8-cineole?, if this is the case, it will be interesting to investigate the association of thymol + 1,8-cineole as a potential acaricide.

CONCLUSION

The chemical analysis of EgEO revealed a composition dominated by oxygenated monoterpènes in the main, represented by 1,8-cineole. Our study has confirmed that an EgEO + thymol association was more effective in reducing the ectoparasite populations than was EgEO or thymol alone. This result is interesting in that the use of EgEO+thymol may constitute a viable alternative to thymol-based commercial treatment. Finally, the use of a natural pesticide is of immense significance in view of the environmental and toxicological implications of the indiscriminate use of synthetic pesticides and in view of the need to overcome the problem of the increasing pesticide resistance of *V. destructor.*

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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