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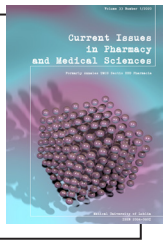

Comparative study of two *Eucalyptus* species from Algeria: chemical composition, toxicity and acaricidal effect on *Varroa destructor*

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ABSTRACT

Varroa destructor is an external parasitic mite that is a serious pest of honeybees and has caused severe losses of colonies worldwide. One of the feasible alternative treatments being used for their control is the application of essential oils, which are generally inexpensive and most pose few health risks. The investigation was designed to determine the chemical composition, toxicity and acaricidal effects of *Eucalyptus amygdalina* leaf essential oil (EaEO) grown in Algeria and to compare its activity on *Varroa destructor* with that of *Eucalyptus globulus* from the same region. Fresh leaves of *E. amygdalina* (Ea) by steam distillation yielded 0.77% (v/w), and investigation of the oil on GC/MS resulted in the identification of 35 compounds, with 1.8-cineole (35.78%) as most abundant constituent. Other notable compounds include spathulenol (12.58%), camphene (7.73%), α-pinene (4.38%), valencene (2.64%), while 2-carene and ledol (1.45%) were also among the constituents identified. The acaricidal features of the essential oil was evaluated using bee hives infected by *Varroa destructor*, and a significant effect of oil application was observed ($p < 0.05$). Cytotoxic effect was assayed using the brine shrimp lethality assay, Probit's analysis of the result revealed an LC50 value of 116.06 µg/mL. Essential oil of *E. amygdalina* (EaEO) has potential acaricidal effect on *Varroa destructor*, but this effect is less important than the one recorded by *E. globulus*. Further studies are needed to determine the active component responsible for this effect.

INTRODUCTION

Eucalyptus species, belonging to Myrtaceae family, are native to Australia. There are more than 800 species, divided in 13 subgenera, and hybrids [1,2]. *Eucalyptus* was introduced in Algeria in 1854 by Ramel [3], the species being *E. globulus*. Since then thanks to their adaptability, the species *E. globulus*, *E. camaldulensis*, and *E .gomphocephala* have become widespread in the Mediterranean region [4]. Other species, however, are planted and listed in the herbariums– including the species of our study, *E. amygdalina*. The oil of *Eucalyptus* possesses a wide spectrum of biological activity, including, analgesic, anti-inflammatory [5], antioxidant, antibacterial [6,7], antifungal [8], antiviral and insecticidal [9]. It is one of the best remedies against the chronic inflammation of gastric mucosa and intestinal mucosa and it is a powerful antiseptic of the respiratory tract. It is used in treating bronchitis, influenza, pulmonary tuberculosis and asthma [10].

Several varieties of the genus *Eucalyptus* are of particular use in Algerian folk medicine for their therapeutic properties. Hot water extracts of dried leaves of *Eucalyptus globulus* are traditionally applied as analgesic, anti-inflammatory and antipyretic remedies for the symptoms of respiratory infections, such as cold and sinus congestion. Studies with essential oils of various species of this genus report the presence of the compound 1,8-cineole, a monoterpene already in use in the pharmaceutical industry for its antimicrobial activity in the treatment of respiratory diseases. The compound also has applicability as an acaricide [11,12] and has displayed

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insecticidal activity against *V. destructor*, 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 [13-16].

A specific practice of local beekeeping in Algeria is to combine conventional treatments with aromatic plant fumigation for greater efficiency and lasting effect. Herein, *Eucalyptus* and *Thyme* leaves are used as fumigation tools of hives. The effectiveness of this traditional practice is plausible given the many scientific studies supporting the insecticidal properties of *Eucalyptus* essential oils [16,17]. Synthetic pesticides have been widely used and pose potential risks of contamination of honey and other hive products with chemical residues [18]. There is also clear evidence for the evolution of resistance in *Varroa* mite populations to conventional pesticides [19]. Essential oils and essential oil components offer an attractive alternative to synthetic acaricides for the control of *Varroa destructor*. They are generally inexpensive and most pose few health risks.

It is on this b*a*sis that we have studied the chemical composition, the acaricidal and toxic effects of the essential oil extracted from the leaves of *E. amygdalina* and compared them with those of *E .globulus* [16]. Thus, we hope we can reduce the spread of *Varroa* in our country by helping beekeepers with natural products such as plant essential oils. These products are inexpensive and safe for the environment and the evolution of bees. It is worth noting that what is most worrying is the resistance of *Varroa* to the different acaricides currently used in Algeria and all over the world. Therefore, alternating an acaricidal treatment with a treatment with essential oils that are characterized by a high chemical diversity will help reduce this phenomenon of resistance.

MATERIALS AND METHODS

Plant material and distillation

Leaves were collected in April 2014 at the herbarium of "Draa Naga" Djebel El Ouahch, located 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 amygdalina* (Ea)was identified by a taxonomist (Dr Bouzid Mosbah) and the voucher specimen (Ea006505) was deposited for future reference at the herbarium of Constantine Forestry Conservation. Essential oil was extracted from fresh leaves (1450 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. The same protocol was followed for species *E. globulus* [16].

GC/MS analysis

Essential oil extracted of the leaves of *Eucalyptus amygdalina* (henceforth Ea) was analysed by gas chromatograph coupled with mass spectrometer "GC/MS" (Agilent System HP-5MS.) as described below: Capillary chromatographic column [30 m (length), 0.25 mm (diameter), and 25 µm (film thickness)], with apolar stationary phase [5% phenyl, 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 70 ev with scan band of 45-400 u. The essential components were tentatively identified by comparison with mass spectra data (MS) obtained from the NIST-Wiley-MS library and confirmed by comparison with Kovats index on a HP-5MS column. The same protocol was followed for species *E. globulus* [16].

BIOACTIVITY

Brine Shrimp lethality assay

The brine shrimp lethality (BSL) assay was used to predict the toxicity of the essential oil, as previously described [20]. Different concentrations (1000, 100, 10, 1 ppm) of EaEO were prepared using dimethyl sulfoxide (DMSO 1%). After 48 h, a drop of DMSO and 4 ml of seawater 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 24hrs, the number of dead larvae were counted and recorded, and the data obtained were subjected to Finney's probit analysis to determine the"LC50" of the oil. The same protocol was followed for species *E. globulus* [16].

ACARICIDAL ACTIVITY

Experimental apiary

The experiment was conducted in an apiary located nearby Azzaba (36°45'41.1"N 7°03'50.3"E). Langstroth type hives of bees (*Apis mellifera*) colonies infected with *V. destructor* were randomly assigned into four batches: batch 1 to 3 was treated with (1 mL/hive/week) of each of EaEO, thymol, and EaEO+thymol (v/v) [2]. Batch 4 was used as a control (untreated hives).The same protocol was followed for species *E. globulus* [16].

Collection, counting and analysis

The method followed is the biological method "raises diapers" or "covers background" [2,3].This method is designed to track and count the fallen mites. Vaseline greased diapers are 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, 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 7th day and then goes back to 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 and composition of *E. amygdalina* essential oil

The essential oil extracted from Ea leaves was light yellow and had a very fresh smell, characteristics that was close to the essential oil of *E. globulus* previously described [16].The extraction yield of essential oil was 0.77%, a value lower than that of *E. globulus* (0.93%) [16], and relatively higher than yields of *E. camaldulensis* from Nigeria (0.38%) [22] and *E. saligna* from Kenya (0.38%) [23].

The results of the GC/MS analysis revealed 35 compounds (Table 1, Figure 1). The main constituents of the oil were 1,8-cineole (35.78%), spathulenol (12.58%), camphene (7.73%), α-pinene (4.38%), valencene (2.64%),2-carene and ledol (1.45%). This composition remained less rich in 1,8-cineole and richer in α-pinene compared to that of the *E. globulus* species of the same herbarium with the percentages of (78.45%) for 1,8-cineole and (1.69%) for the pinene[16]. It is worth noting that the 1,8-cineole is the specific marker of the genus [24].

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

Compounds	% composition	KI
α-thujene	0.58	930
α-pinene	4.38	939
β-myrcene	0.23	979
p-cymene	0.23	1025
1,8-cineole	35.78	1030
D-fenchylalcool	0.22	1139
Pinocarvone	0.76	1165
Endo-borneole	0.44	1152
Cis-p-mentha-1(7),8-dien-2-ol	0.92	1235
α-terpineol	1.31	1189
Valencene	2.64	1484
Allospathulenol	0.71	1575
Isospathulenol	1.69	1644
Ledol	1.45	1569
Muurolol	0.72	1640
β- Eudesmol	0.69	1645
Oplopenone	0.22	1600
Phellandral	0.27	1273
Carveole	0.45	1225
Camphene	7.73	966
Spathulenol	12.58	1578
Epiglobulol	0.23	1573
α-terpinolene	0.30	1017
3-méthyl butylester	0.34	1105
Trans-sabinene hydrate	0.49	1075
2-Carene	1.58	1001
Thymol	0.95	1290
Cis-piperitol	0.21	1204
Cuminic aldehyde	0.69	1226
Bicyclogermacrene	0.71	1500
Alloaromadendrene	0.56	1457
3,3,4-trimethyl-2-cyclohexenone	1.48	1647
Carvacrole	0.62	1293
Linalool	0.77	1106
Total (%)		83.48

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-5MScolumn. **(%) composition**: percentage of concentrations based on peak area integration

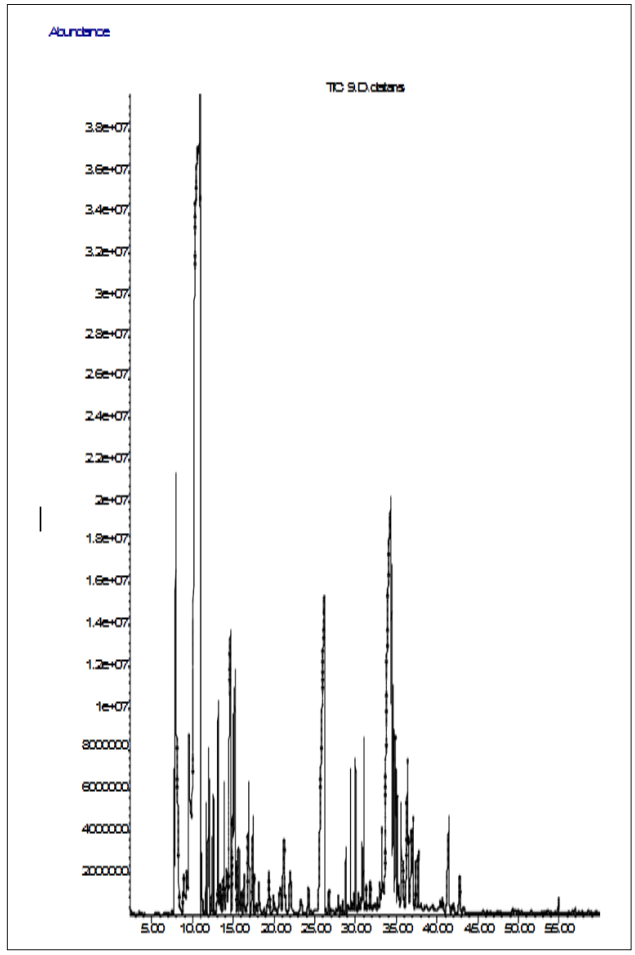


Figure 1. Chromatogram of leaf essential oil of *E. amygdalina*

The medicinal properties of the oil are specified by minimum quantity of constituents. This is defined in the British pharmacopoeia [25], and requires *Eucalyptus* oil to contain not less than 70% 1.8-cineole and be practically free of phellandrene. Therefore, the oil extracted from *E. amygdalina* containing (0.14%) of phellandrene, and (35.78%) of 1,8-cineole content, may not be suitable for medicinal purposes, but can be used in industry as insecticidal agents.

BIOACTIVITY

Toxicity and acaricidal activity

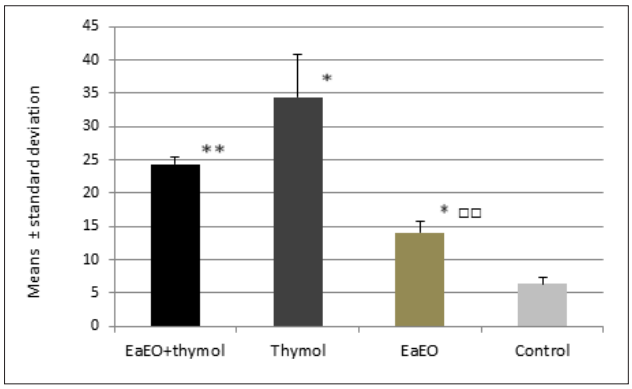
The result of BSL assay of EaEO revealed an LC50 value of 116,06 µg/mL (Table 2) indicating moderate toxic potentials [26]. The results ascertained documented use of the plant extracts as repellant and insecticidal agents [27]. The toxicity against *Artemia salina* nauplii of our oil was moderate compared to thetoxicity of *E. globulus* (65.5 µg/ml), *E. robusta* (9.42 µg/ml), *E .sideroxylon* (34.80 µg/mL) collected in the same herbarium from Algeria [28] and that of *E. globulus* from Nigeria which revealed an LC50 value

of (9.59 µg/ml) [29]. However, the toxicity of the EaEO was higher compared to that of *Eucalyptus baileyana* and Eucalyptus major from Australia against *Artemia franciscana* nauplii, which have LC50 (µg/mL) values of 216 and 762, respectively [30]. This difference is due to the chemical composition of each species.

Table 2. Brine shrimp lethality assay of leaf extracts of *E. amygdalina* essential oil

Plant extract	Dose (µg/mL)	Nbre of tested shrimps	Nbre of dead shrimps	LC50 (µg /mL)
E. Amygdalina	1 10 100 1000	40 40 40 40	9 14 17 19	116.06

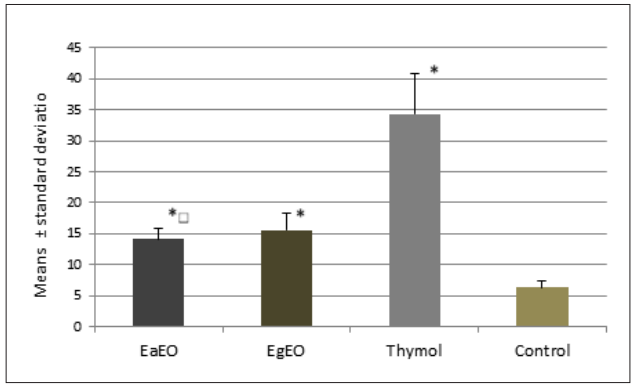
The results of the field acaricidal testing of EaEO are shown in (Fig. 2). All treated infected bee hives have showed significant fall of *Varroa destructor* compared to the control. A significant difference was observed between EaEO and thymol (p=0.02) batches and between batches treated with (EaEO+thymol) and EaEO (p=0.001). However, no significant difference was observed between thymol and (EaEO+thymol) (p=0.09) treated batches, which implied that the essential oil of *E. amygdalina* has a moderate acaricidal effect on *V .destructor*, and that this effect has been potentiated by the association of the oil with the thymol. This result has been also verified by the value of fallen *Varroa* of EaEO (14.11±1.67) which was lower than those of thymol (34.11±6.51).



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

Figure 2. Number of dead Varroa expressed as means ± SD

In comparison, no significant difference was observed between *E. globulus* essential oil and thymol (p=0.07) [16] and between EaEO and EgEO (p=0.58) (Figure 3), which indicate that the acaricidal effect of *E. globulus* is more important on *Varroa destructor.* It is worth noting that this effect is related to the chemical composition of the oil. The 1,8-cineole compound is present in both species *E. Amygdalina* and *E. globulus* with respective percentages of 35.78% and 78.45% and the high level of 1.8-cineole in *E. globulus* suggest that it is probably at the origin of its potential mite effect [16].Our study, although it joins the conclusions of the previous authors which showed a significant acaricidal potential of *Eucalyptus* species essential oils against *V. destructor* [16,21,28,31], showed that the association(EEO + thymol) gives a better efficiency than the treatment with thymol alone to reduce the population of the parasite inside the apiary. It should be known that the conventional treatments based on thymol alone currently encounter a resistance phenomenon on the part of *V. destructor* [32,33], and the track proposed by our study is based on an association (EO+thymol) to overcome this problem of resistance.



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* Thymol treatment

Figure 3. Number of dead *Varroa* expressed as means ± SD

Finally, the species *E amygdalina* is less toxic and has a moderate acaricidal effect compared to that of the *E. globulus* species (Table 3), this is due to the chemical composition, which is different as to the percentages of the majority compounds. The same result was found in the species *E. robusta* and *E. sideroxylon* which exhibited a strong acaricidal effect on *Varroa* and a chemical composition rich in 1,8-cineole, with respective percentages of 65.79% and 40.24% [28].

Table 3. Main constituents, toxicity and acaricidal activity of *E. amygdalina* and *E. globulus*

Eucalyptus ssp.	Components of EOs	EOs yields (%)	Toxicity (Artemia salina)	Acaricidal activity (Varroa destructor)
E. amygdalina	1,8-cineole (35.78%), spathulenol (12.58%) camphene (7.73%) α-pinene (4.38%) valencene (2.64%),	0.77	Moderate (LC50=116.06 µg/mL)	Moderate effect
E. globulus	1,8-cineole (78.45%) o-cymene (2.18%) isopinocarveole (1.74%) α-pinene (1.69%) pinocarvone (1.34%)	0.96	Toxic (CL50=65.5 µg/mL)	High effect

CONCLUSION

This study revealed that *Eucalyptus amygdalina* essential oil is moderately toxic and has moderate acaricidal effect on *Varroa destructor* compared to *E. globulus* essential oil. The purpose of the test of essential oils was to demonstrate that *Eucalyptus* could be used as a natural fighting means against *Varroa* without danger to the environment and bees. Finally, 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 that are more efficient, but above all useful in overcoming the problem of increasing pest resistance to treatments.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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