Contribution to the Study of the Antimicrobial Properties of Eucalyptus pauciflora Essential Oil and Evaluation of its Acaricidal Effect on Varroa destructor ofBees

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Abstract

The antibacterial, antifungal, and acaricidal properties of *Eucalyptus pauciflora* (Ep) essential oil (EO) from the Constantine region of northeastern Algeria are investigated in this study. The plant's dry leaves yielded 0.73%. The GC/MS analysis identified 39 compounds, with 1,8-cineole (54.45%) being the most abundant. The *E.pauciflora*'s antibacterial activity was tested using the Muller Hinton agar diffusion method on *Escherichia coli (E.coli*), *Staphylococcus aureus (S.aureus), Klebsiella pneumoniae (K. pneumonia)* and *Pseudomonas aeruginosa (P.aeruginosa*) at various oil concentrations with DMSO. The diameters of the inhibition zones ranged from 6 mm to 20 mm. The plant's antifungal power was tested against the tomato fungus *Fusarium oxysporum (F.oxysporum)* by incorporating the product into the Potato Dextrose Agar (PDA) agar medium. The action of EpEO at different concentrations on mycelial growth was compared to determine inhibition rates. The rates of inhibition ranged from 39.27–84.48%. The oil's acaricidal activity was tested on beehives infested with *Varroa destructor*. The biological "swaddling" or "cover crop" method was used. The oil has a statistically significant effect ($p < 0.05$). The *E.pauciflora* EO recorded antimicrobial and acaricidal results, indicating that this plant could be used in integrated pest management against the bacteria and fungus tested and the *Varroa destructor* mite.

INTRODUCTION

Eucalyptus originated in Australia and is now found almost everywhere on the planet, with over 900 species Brooke and Kleinig (2004). It has spread throughout the world, including Algeria. Eucalyptus is primarily grown for its wood, paper pulp, and essential oils, which have medicinal and therapeutic properties. It is an important source of essential oils in treating infectious diseases and traditional medicine. Eucalyptus essential oil has numerous benefits, including being a respiratory tract antiseptic, expectorant, and analgesic Duraffourd and Lapraz (2000), and having anti-inflammatory, antimicrobial, anti-dermatophyte, and anti-parasitic properties (Vratnica et al. 2011; Filomeno et al. 2016). *Varroa destructor* is a parasitic mite of adult bees, larvae, and pupae that causes significant economic losses in beekeeping and causes bee population loss in Algeria and around the world (Brodschneider et al.2010; Vanengels Dorp et al.2010). Using conventional phytosanitary products harms soil microflora, microfauna, and beneficial insects such as the honeybee *Apis mellifera*. The situation is constantly deteriorating, and the search for alternatives to chemical products is becoming increasingly important. Alternative treatments containing EOs of Eucalyptus are effective against mites while remaining safe for bees (Atmani-Merabet et al. 2018; Habbi-Cherifi 2014). To identify the active compounds responsible for these various biological activities, we tested antibacterial power of *E.pauciflora* species against *E. coli*, *S.aureus*, *K.pneumoniae*, and *P. aeruginosa*, its antifungal power against *Fusarium oxysporum*, and its acaricidal effect against *Varroa destructor* of bees.

MATERIAL AND METHODS

Plant material

Leaves of *E.pauciflora* were collected in March 2020 at the Draa Naga Arboretum in the Djebel El Ouahch forest of the Wilaya of Constantine in northeastern Algeria. The arboretum is situated between longitudes [X1: 6° 42’ 5”, X2: 6° 42’ 30”) and latitudes (Y1: 36° 20’ 45”, Y2: 36° 22’ 15”), and the reference specimen of species Ep (Ep006504) has been deposited with the Constantine Forest Conservation Service, which manages the arboretum. The essential oil was extracted from dry leaves (700 g) using Clevenger hydrodistillation for 4 h, then dried over anhydrous sodium sulphate and stored at 4°C in an opaque glass bottle.

Yield calculation

The yield Rd is determined by the ratio of the weight of essential oil to the weight of dry plant matter used for extraction, multiplied by 100 (Fallehand al.2008).

$Rd = m_1 / m_0 \times 100$ (1)

Where: $\mathbf{m_1}$ is the mass in grams of the essential oil and $\mathbf{m_0}$ is the mass of the dry plant matter.

GC/MS analysis

The essential oil extracted from fresh leaves of *E.pauciflora* was analyzed using a gas chromatograph coupled to a GC/MS mass spectrometer (Agilent System HP-5MS.) as follows: Capillary chromatographic column of 30 m (length), 0.25 mm (diameter), and 25 m (film thickness)], with an apolar stationary phase of 5% phenyl and 95% dimethyl polysiloxane. The column compartment temperature was programmed to rise at a rate of 10°C/min from 50 to 200°C; the GC/MS interface was kept at 230°C, and the ionization source was kept at 150°C; helium was used as the gaseous carrier with a flow rate of 0.5 ml/min; the injection volume was 0.5 µl; and the MS ionization energy was 70ev with a scan band of 45–400 u. By comparing mass spectral (MS) data from the NIST-Wiley-MS library, essential components were tentatively identified and confirmed using the Kovats index on the HP-5MS column.

Antimicrobial activity

Because of the frequency with which certain species contaminate foods and the pathogenicity of some of them, we chose four bacterial strains (*S. aureus, Escherichia coli, K. pneumoniae,and P. aeruginosa*) and one fungal strain (*Fusarium oxysporum*) from Algeria’s Constantine Biotechnology Research Center (CRBT).

Antibacterial activity

The disk diffusion method (Rios et al. 2005) was used to test the antibacterial activity of four human pathogenic bacteria. Gram-positive strains include *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and a Gram-negative strain, *E. coli*. Bacteria were grown for 18 to 24 h on a Mueller Hinton (MH) plate at 30°C before being inoculated onto nutrient agar. A sterile Pasteur pipette was used to extract 3 to 5 perfectly identical colonies. The Pasteur pipette was then inserted into a 5 ml sterile (MH) medium (which can be replaced with physiological water). The optical density of this suspension was adjusted to 0.13–0.19 at 600 nm,

and this inoculum was used to inoculate sterile (liquid Mueller Hinton) agar plates poured into Petri dishes with a swab soaked in the suspension in tight ridges. Various EO concentrations in DMSO (dimethyl sulfoxide) were prepared (5%, 10%, 50%, and 100%), and 6 mm diameter Whatman paper discs were soaked in EO at various concentrations and deposited on the surface of Petri dishes. The procedure was repeated four times for each can and dilution. Each strain was tested on MH agar without extract. The plates were kept at 4°C for 2 h to allow the EO to diffuse, and the diameters of the inhibition zones were measured after 24 h of incubation at 37°C. The results are expressed as mean value ± standard deviation and interpreted using the (Poncé et al. 2003) scale, which includes the following categories: **non-sensitive (-)**: less than 8 mm; **sensitive (+)**: 8 mm to 14 mm; **very sensitive (++)**: 15 mm to 19 mm; and **extremely sensitive (+++)**: more than 20 mm.

Antifungalactivity

The *E.pauciflora*'s antifungal activity against the fungus *F.oxysporum* was tested using the direct contact method on PDA agar medium. The technique involves adding oil to the still-liquid culture medium (PDA) at various concentrations in DMSO (0.2%, 0.1%, and 0.05%). For each concentration, 5 mm diameter mycelial disks are deposited on the surface of the agar medium in the center of Petri dishes after the culture medium has solidified. The procedure was repeated three times for each concentration, with a control of agar (PDA) without extract. *F.oxysporum* was incubated for 96 h in an oven set to 37°C. Two methods were used to evaluate antifungal activity of the plant.

Mycelial growth

After 96 h of incubation, mycelial growth was measured by measuring the diameters corresponding to each concentration without considering the diameter of the disc. This value is always compared to control cultures that were started on the same day and under the same conditions (Benouaer 2016).

Inhibition rate

After 96 h of incubation, mycelial growth was measured by measuring the diameters corresponding to each concentration without considering the diameter of the disc. This value is always compared

to control cultures that were started on the same day and under the same conditions (Benouaer 2016).

I% = C - T / C x 100

Where: **I%** is the rate of growth inhibition, **C** is the diameter of the control colony, and **T** is the diameter of the colony in the experiment. (Abd-Ellatif et al. 2011) interpreted the results obtained regarding the inhibition rate of diametral growth of tillers. Notably, **30 to 40%** indicates low activity, **50 to 60%** indicates moderate activity, **60 to 70%** indicates good activity, and **> 70%** indicates excellent activity.

Acaricidal activity

Study area

The experiment was conducted in an apiary in the commune of Azzaba (36°45’41.1 “N, 7°03’50.3 “E) in Skikda. Langstroth-type bee hives (*Apis mellifera*) infested with *Varroa destructor* were selected and divided into four batches of three hives each: batches 1 to 3 were treated with (1mL/hive/week) of (EpEO + thymol), EpEO and thymol (v/v) respectively (Labeste 2013). Batch 4 is the untreated control batch, in which the *V. destructor* died naturally after falling.

Collection, countingand analysis

The biological “swaddling” or “covering” method was used (Labeste2013; Ghomari et al. 2014). This method is intended for tracking and counting fallen mites. Each hive has a grid tray on which Vaseline-greased diapers are placed, then removed and carefully examined with a hand lens for dead varroa mites. The 1 mL treatment was applied to a cardboard strip that was 1 mm thick, 4 cm wide, and 20 cm long and was placed on the tray grid (Labeste2013). The study lasted 21 days, with dead varroa counted every two days. The layers were meticulously cleaned and replaced after each count. The treatment was repeated on days 7 and 14, and the results were expressed as mean mortality ± standard deviation. During the experiment, the temperature ranged between 20°C and 22°C.

RESULTS AND DISCUSSION

Essential oil yield

The *E.pauciflora* essential oil was light yellow and had a fresh, heady aroma. The essential oil yield was 0.73%, which was relatively high when compared to the Brazilian species (0.60%) (Filomeno et al. 2016) and the Tunisian species (0.23%) (Ghazghazi et al. 2021). Indeed, EO content is influenced primarily by environmental and genetic factors (Piccaglia et al. 2001) and extraction methods and conditions (Bagheri et al. 2014).

Chemical composition

E.pauciflora essential oil’s GC/MS analysis revealed 39 compounds (Table 1) (Figure1). 1,8-cineole (57.45%), β-cymene (5.44%), spathulenol (5.38%), trans verbenol (4.31%), α-pinene (3.11%), α-terpineol (2.84%), and globulol (2.76%) were the main constituents of the oil. The chemical composition of EpEO is consistent with the findings of studies that have revealed the presence of numerous bioactive compounds, including 1,8-cineole, which has been identified as a specific marker for essential oils of this genus globally (Atmani-Merabet et al. 2018; Atmani-Merabet 2018; Filomeno et al. 2016). However, analysis of Brazilian oil (Filomeno et al. 2016) revealed a difference in composition, with 'the major compounds being spathulenol (22.6%), p-cymene (19.4%), and globulol (10.4%), which could translate into different biological and pharmacological properties (Bruneton1999).

Table1. Result of GC-Ms analysis of *E.pauciflora* leaf essential oil

Compounds	% composition	KI
α-thujene	0,14	930
α-pinene	**3,11**	939
β-pinene	0,12	979
β-myrcene	0,35	991
1-phellandrene	0,33	1003
p-cymene	0,14	1025
m-cymene	**5,44**	1031
1,8-cineole	**57,45**	1030
δ-terpinene	0,14	1047
Durol	0,14	1124
3-methyl Butylester	0,43	2388
D-fenchylalcool	0,35	1139
α-campholenal	0,12	1126
***Trans*-verbenol**	4,31	1144
Pinocarvone	1,02	1165
Borneole	0,55	1166
4-terpineol	**2,84**	1177
Trans-p-mentha-1(7),8-dien-2-ol	1,04	1185
α-terpineol	1,92	1189
Myrtenol	0,22	1327
Phellandreneepoxyde	0,16	1190
Cis-piperitol	0,12	1208
Carvéol	0,23	1217
Cis-p-mentha-1(7),8-dien-2-ol	0,74	1235
2-methyl-3-phenyl propanal	0,29	1244
Piperitone	0,14	1259
Phellandral	0,16	1273
Carvacrol	0,35	1317

Camphene	**1,98**	966
Tetradecene	0,14	1400
2,4-di-ter-butylphenol	0,39	1513
Epiglobulol	0,18	1569
Aromadendrene	0,39	1441
Spathulenol	**5,38**	1578
Globulol	**2,76**	1578
Viridiflorol	0,82	1593
Isospathulenol	0,29	1644
Octadecene	0,16	1795

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

Antibacterial activity

To estimate the plant's antibacterial activity, we used the (Poncé et al. 2003) scale, and the values of the zones of inhibition are expressed as the mean of four tests ± standard deviation, with diameters ranging from 6 mm to 20 mm (Table 2, Fig. 2).

Table2. Diameters of *Eucalyptus pauciflora* inhibition zones

Bacteria	**Dilution 5 µl**	**Dilution 10 µl**	**Dilution 50 µl**	**Dilution 100 µl**	**DMSO**
Diameters (mm)					
E. coli	6 ± 0	13 ± 1,41	17,5 ± 0,54	20 ± 0	0 ,0
G -	(-)	(+)	(++)	(++)	
S.aureus	7 ± 1,41	14,5 ± 0,71	16,5 ± 0,71	20 ± 0	0,0
G+	(-)	(-)	(+)	(++)	
K.pneumoniae	7,5 ± 1,12	15 ± 0	17,5 ± 0,54	20 ± 0	0,0
G-	(-)	(++)	(++)	(++)	
P.aeruginosa	9 ± 0	14 ± 0	16 ± 0	17 ± 0	0,0
G-	(+)	(++)	(++)	(++)	

G+: Gram+, G- : Gram –, (+++): Extremely sensitive, (++): very sensitive, (+): Sensitive, (-): non sensitive

Our findings show that the oil's inhibitory power increases with concentration, which can be attributed to an increase in the bioactive molecules responsible for its antibacterial effect (Kheyar et al. 2014).The findings also show that the EO of the species studied had a higher activity on *E. coli,K. pneumoniae* and *P. aeruginosa* than *S. aureus*. Several studies testing the inhibitory activity of EOs found the same result, confirming that Gram (+) bacteria are less sensitive to EOs than Gram (-) bacteria. The complexity of their cell envelope, which contains a double membrane, is linked to their resistance (Filomeno et al. 2016).

Antifungal activity

The susceptibility of the fungus (*F.oxysporum*) to the essential oil of *E.pauciflora* was tested using the direct contact method, and the antifungal activity of the EO was evaluated in vitro using two approaches:

Mycelial growth

The findings indicate that the mycelial growth of *F.oxysporum* f. sp. Lycopersici (FOL) decreased as EO concentration increased (Fig.3 and Fig.4). Because the concentration was low and did not significantly inhibit FOL filament divergence, the 0.05% concentration produced the largest diameter of mycelial growth (4.6 mm). The diameter is 2.725 mm for 0.1% and 1.175 mm for 0.2% concentration. After 96 h of incubation, the + control had a diameter of 7.575.

Inhibition rate

For each concentration, the rate of inhibition of *F.oxysporum* mycelial growth is

I% (0.2%) = 84.48%, I% (0.1%) = 64.02%, and I% (0.05%) = 39.27%. (Abd-Ellatif et al. 2011) interpreted the results based on the inhibition rate of diametral growth of tillers. As a result, we can conclude that the species studied in this study has excellent in vitro antifungal activity against *F.oxysporum* at concentrations of 0.2% and 0.1% but is weak at 0.05% (Table 3, Fig. 5).

Table 3. Antifungal activity of *Eucalyptus pauciflora*

Dilutions	0.2%	0.1%	0.05%
Inhibition rate	84,48%	64,6%	39,27%
(I %)	(+++)	(++)	(+)

(+++): Excellent activity(++) : Good activity **(+)** : Low activity

The findings indicate that the antifungal activity of the EO is proportional to its concentration. The diameter of the FOL decreases as we increase the concentration of EO. Furthermore, these findings suggest that the control's mycelial growth is significant, with a Diameter that varies depending on the

incubation time. Our findings support the findings of (Dahou2017) and (Mehani 2015), who discovered an inverse relationship between essential oil concentration and mycelial growth.

Acaricidal activity

Figure 6 depicts the results of *E.pauciflora*'s acaricide field tests. Compared to the control, all infested hives treated showed a significant decrease in *Varroa destructor*. The batch treated with EpEO+ thymol was the most effective in eliminating the ectoparasite compared to those treated with thymol ($p = 0.04$) and EpEO ($p = 0.003$). However, there was no significant difference between batches treated with thymol and EpEO. Although EpEO had a lower fallen varroa value (15.41 ± 2.74) than thymol (34.37 ± 6.56), the difference was not statistically significant ($p=0.06$). The high variability detected in the apiary could be one reason for this. Other studies (Atmani-Merabet et al. 2018; Atmani-Merabet et al. 2022; Atmani-Merabet 2018; Labeste 2013; Abu Bakar et al. 2019; Charpentier 2013) have found that essential oils of Eucalyptus species have a significant acaricidal potential against *Varroa destructor*.

While our findings agreed with previous authors, we discovered that the combination (EpEO+ thymol) was more effective than thymol treatment in reducing parasite populations within the apiary. The same outcome was observed in four Algerian species: *E.robusta, E.sideroxylon, E.globulus, and E.amygdalina* (Atmani-Merabet et al. 2018; Atmani-Merabet et al. 2022; Atmani-Merabet 2018). Traditional thymol-only treatments are currently encountering resistance from *V. destructor* (Charpentier2013; Bruneau 2015), so the approach proposed in our study is based on a combination (EO+thymol) to overcome this resistance problem. On the one hand, the essential oil's complex composition and remarkable structural diversity will be a difficult barrier for the parasite to overcome in order for it to develop resistance. On the other hand, because most anti-varroa drugs used in Algeria contain a single active ingredient, essential oils with their complex structure may help reduce varroa resistance.

CONCLUSION

The *E.pauciflora*'s essential oil contains a high concentration of 1,8-cineole and demonstrated a significant toxicity threshold in antibacterial, antifungal, and acaricidal tests. It can therefore be considered a promising antimicrobial agent for the pharmaceutical industry in various pharmaceutical forms. In addition, our results confirmed that the EO + thymol combination was more effective than EO or thymol alone in reducing ectoparasite populations. Finally, the use of *E.pauciflora* oil as a bio-control method against *Varroa destructor* may help to combat varroa resistance to current treatments by alternating their use with that of essential oils alone or in combination with other compounds such as thymol and other essential oils.

Abbreviations

E:eucalyptus, **Ep:** *Eucalyptus pauciflora*, **EOs**: essential oils, **EpEO**: *Eucalyptus pauciflora* essential oil, ***E.coli***: *Escherichia coli*, ***S.aureus***: *Staphylococcus aureus,* ***K. pneumonia***: *Klebsiella pneumonia,* ***P.aeruginosa***: *Pseudomonas aeruginosa,* **MH**: Muller Hinton, **DMSO**: dimethyl sulphoxide, **PDA**: Potato Dextrose Agar, ***F.oxysporum***: *Fusarium oxysporum,* **FOL:** *F.oxysporum* f. sp. Lycopersici, ***V.destructor:*** *Varroa destructor.*

Declarations

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants performed by any of the authors.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Figures

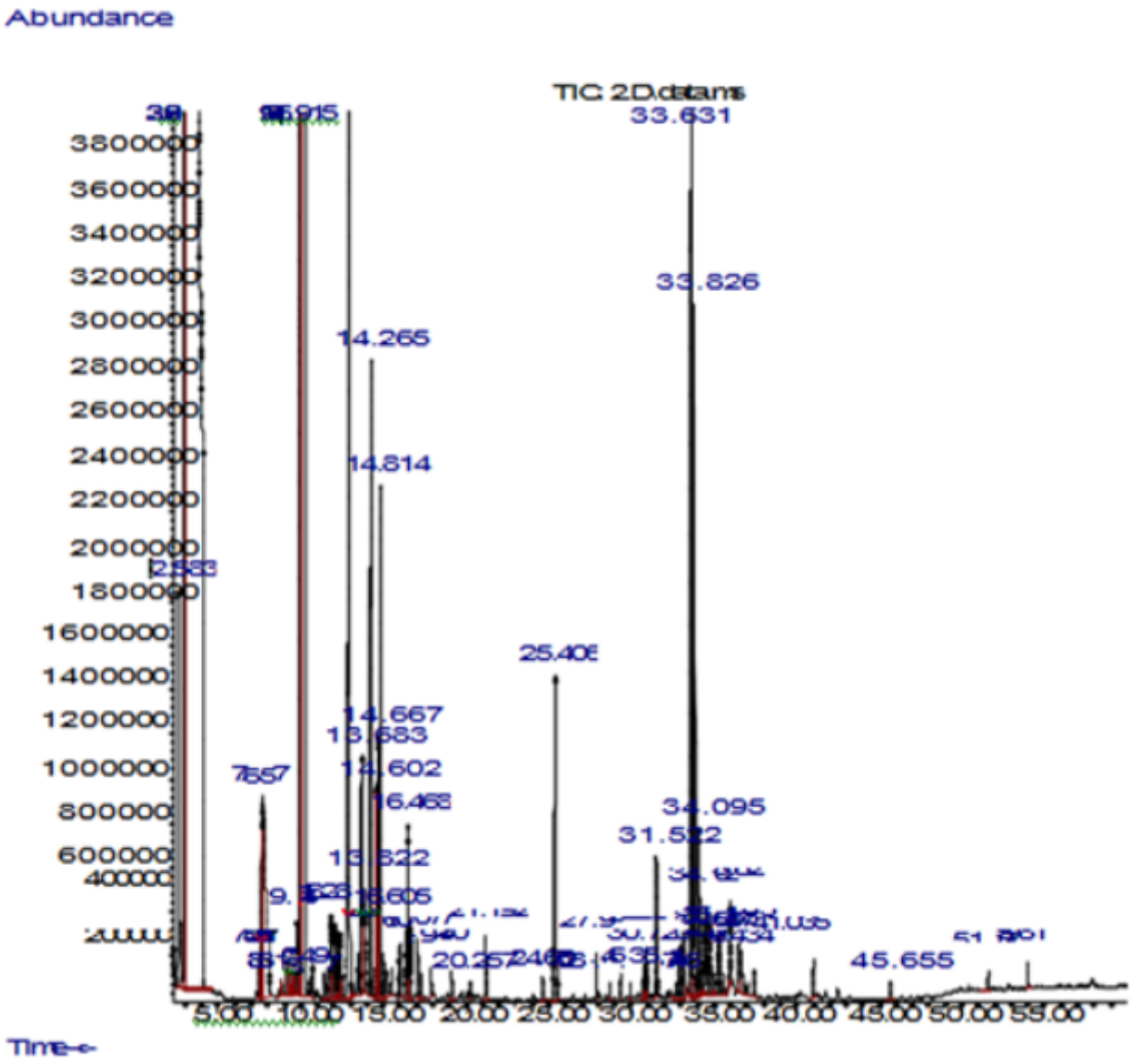


Figure 1

Chromatogram of E. pauciflora leaf essential oil

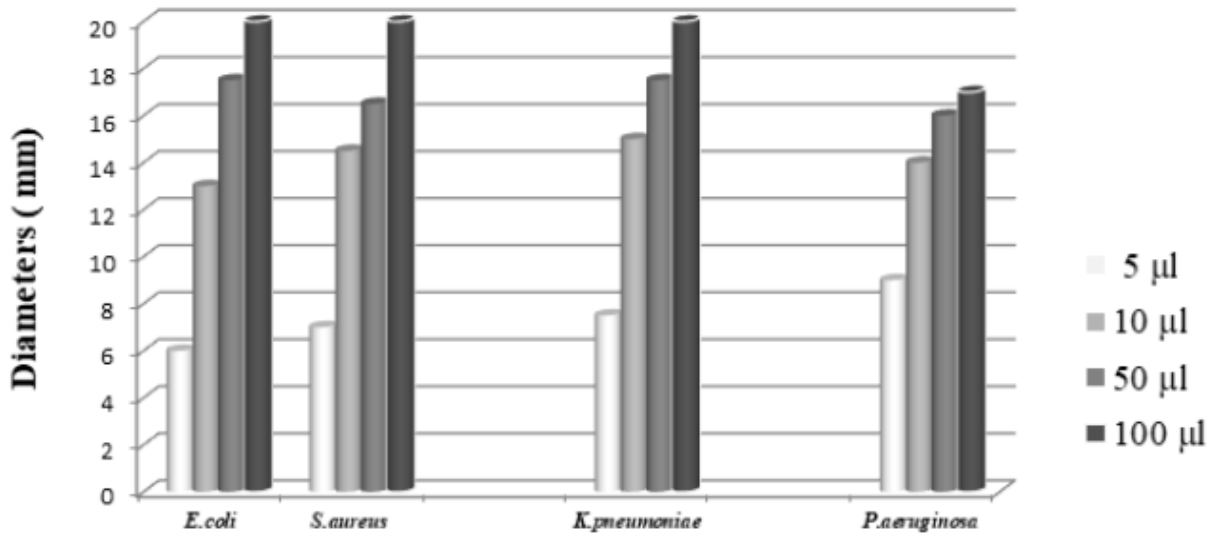


Figure 2

Antibacterial activity of E.pauciflora essential oil

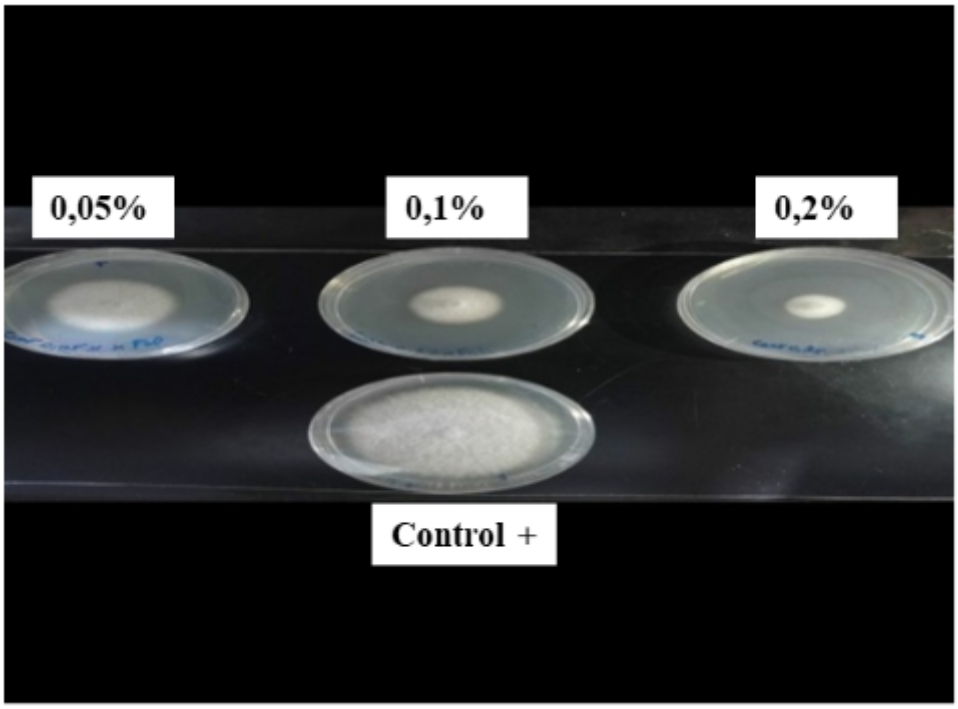


Figure 3

Morphology of FOL mycelial growth after 4 days of incubation

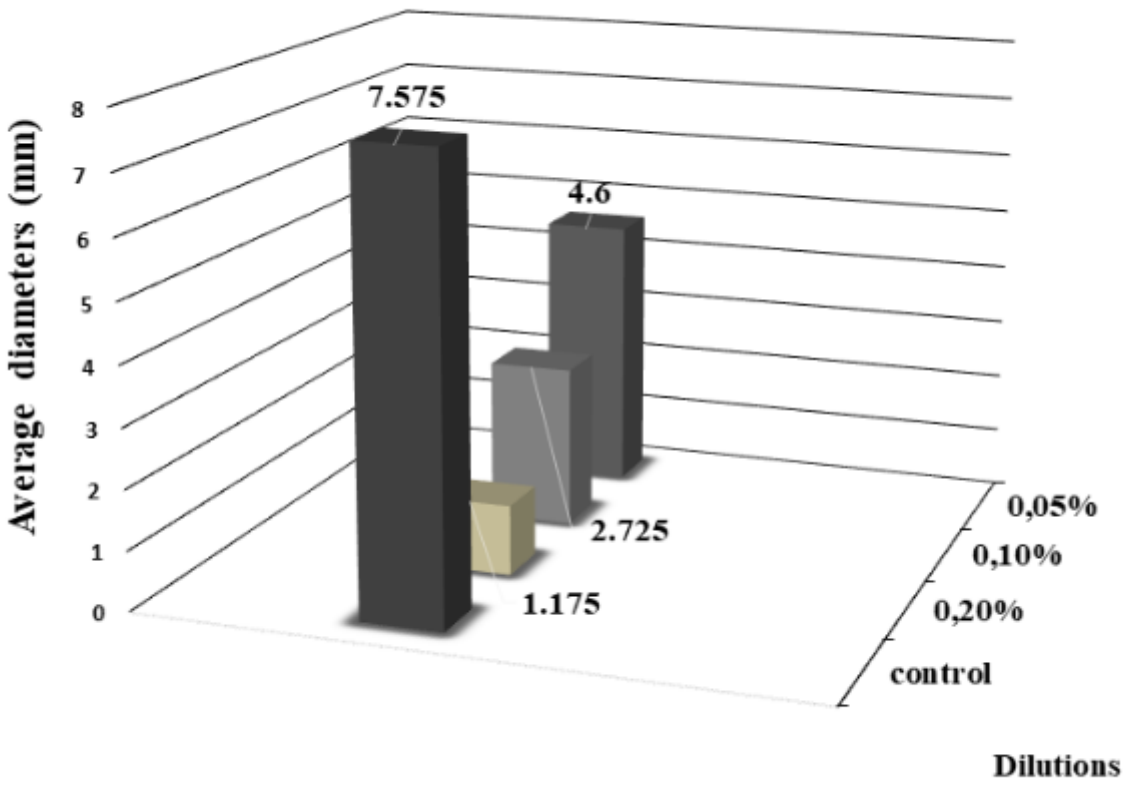


Figure 4

Diameters of the F.oxysporum mycelial growth at different concentration

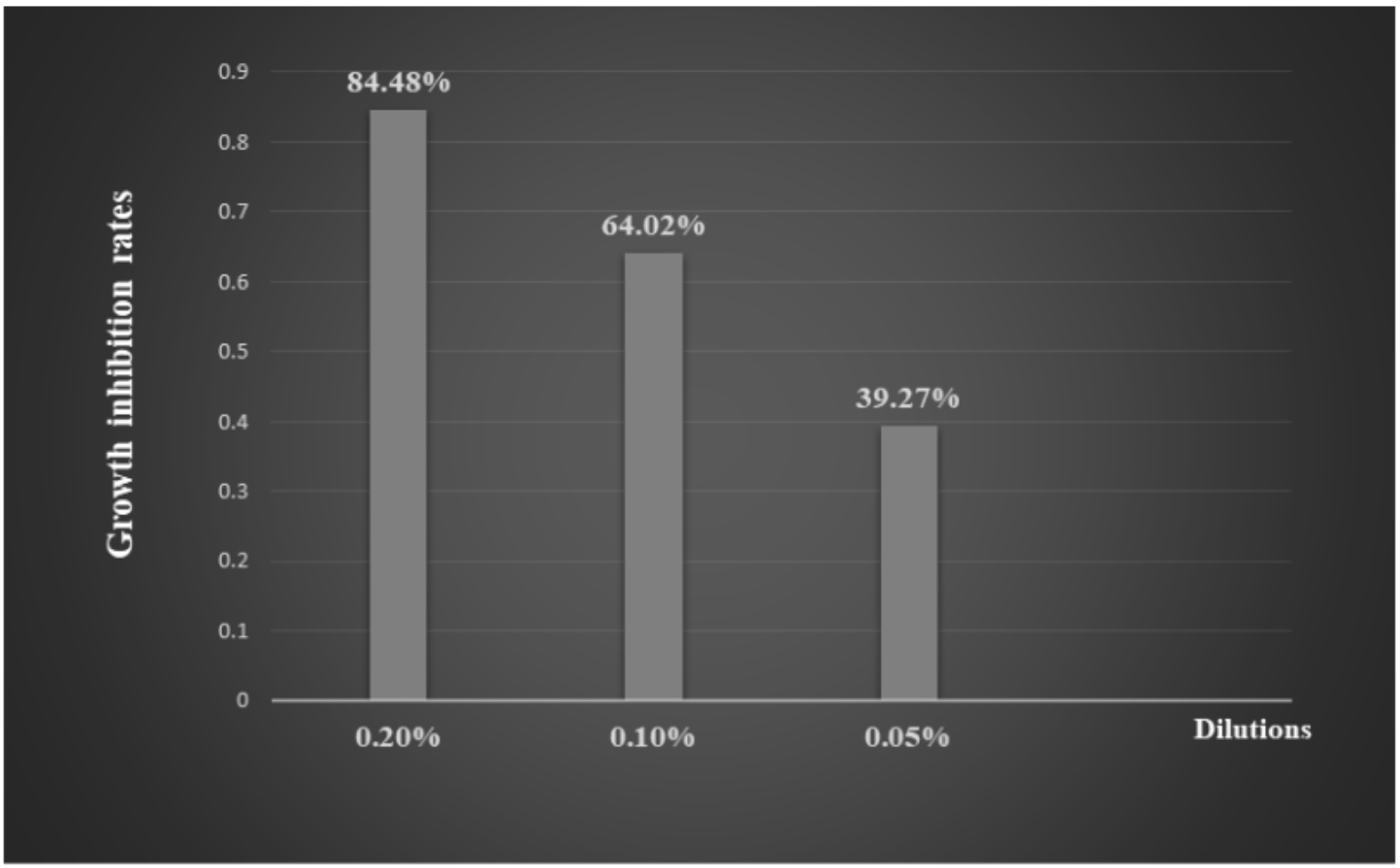
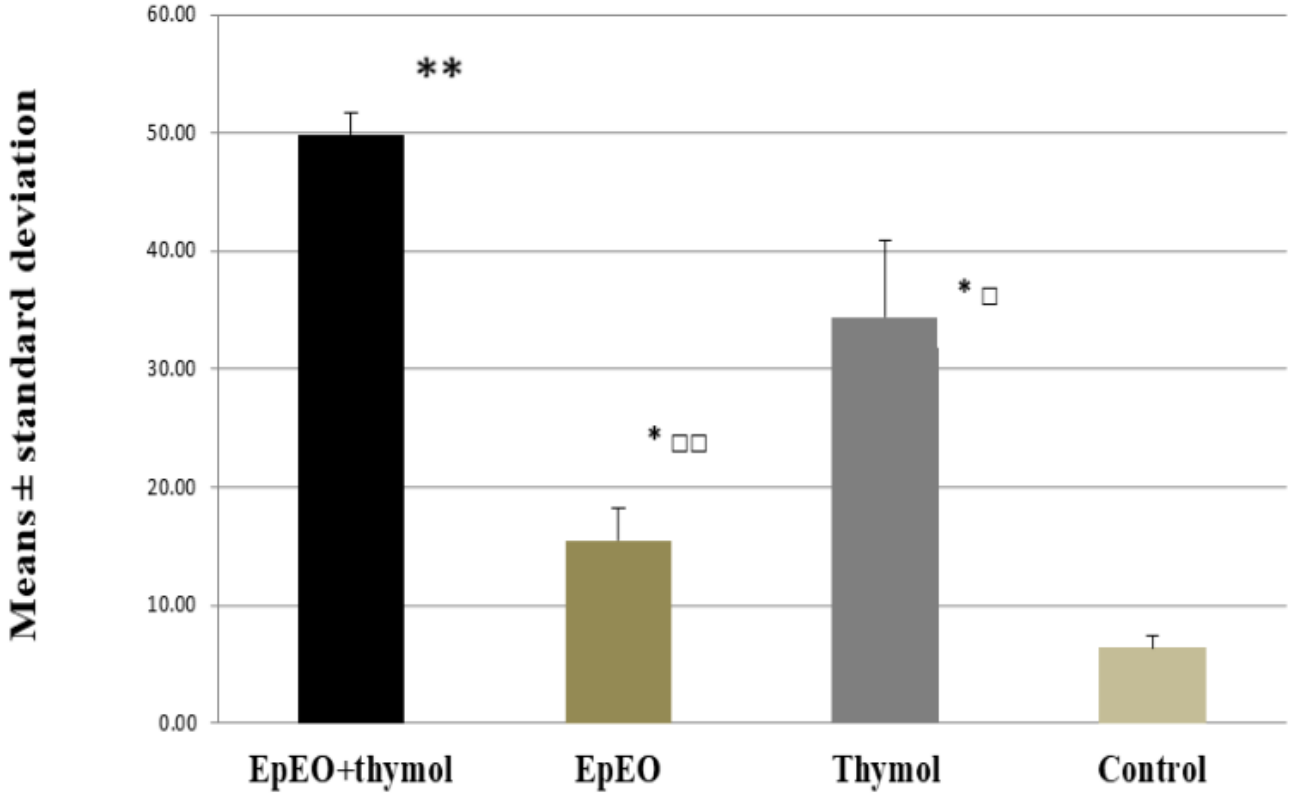


Figure 5

The effect of E. pauciflora oil on the growth inhibition rate of Fusarium oxysporum



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* (Ep EO+ thymol) treatment

Figure 6

Acaricidal effect of EpEO against V.destructor