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In vitro evaluation of the effects of some plant essential oils on *Paenibacillus larvae*, the causative agent of American foulbrood

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ABSTRACT

Paenibacillus larvae is one of the major bacterial pathogens of honey bee broods and the causative agent of American foulbrood disease. The factors responsible for the pathogenesis of American foulbrood disease are still not fully understood, and the increasing resistance of *P. larvae* to commonly used antibiotics necessitates a search for new agents to control this disease. The *in vitro* antibacterial activities of 28 plant essential oils against *P. larvae* ATCC 9545 were evaluated. Out of the 28 plant essential oils tested, 20 were found to be effective in killing *P. larvae*. Based on their minimum bactericidal concentration (MBC) values, the effective oils were grouped into three categories: highly effective, moderately effective and minimally effective. Jamaica pepper oil, mountain pepper oil, ajwain oil, corn mint oil, spearmint oil, star anise oil, nutmeg oil and camphor oil were highly effective, with MBC values between 162.0 and 375.0 μg/mL. Jamaica pepper oil was the most effective essential oil, with an MBC value of 162.0 μg/mL. The results of the time-response effect assays showed that no viable *P. larvae* cells were observed after 24 h of treatment with Jamaica pepper oil (162.0 μg/mL), 36 h of treatment with mountain pepper oil (186.0 μg/mL), 48 h of treatment with ajwain oil (224.8 μg/mL) or 48 h of treatment with oxytetracycline (5.89 μg/mL). The tested essential oils exhibited significant antimicrobial activities against *P. larvae*, and they may contain compounds that could play an important role in the treatment or prevention of American foulbrood disease.

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Essential oils; antibacterial activity; antibiotics; *Paenibacillus larvae*; American foulbrood; biological control

Introduction

Honey bee health is directly associated with sustainable agriculture and various non-agricultural ecosystems. Honey bees are affected by various pathogens. Among the brood diseases, caused by these pathogens, American foulbrood disease (AFB) is by far the most contagious and destructive brood disease among honey bees. AFB is caused by the Gram-positive and spore-forming bacterium *Paenibacillus larvae*. The spores of *P. larvae* are the only form of the bacterium that is infectious to honey bees. These spores are resistant to hot, cold, humid and drought conditions, and they may remain infectious for more than 35 years.[1–3] AFB infection is a challenging problem for apiculture worldwide. In the year of 2000, AFB infection in the United States resulted in an annual economic loss of approximately 5 million USD.[4] AFB is a transmissible destructive disease that affects the larval and pupal stages of honey bees. The disease is spread by the exchange of materials between honey bee colonies, the presence of numerous hives in a confined area and the trade of bee packages and bee products.[5] The burning of contaminated colonies and hive materials is considered to be the only effective control strategy for AFB.

Some antibiotics, such as oxytetracycline (OTC) hydrochloride and sulfathiazole, are commonly used for the treatment of infected colonies, but in most European countries, the use of these antibiotics is banned. Common problems associated with antibiotic use include the ineffectiveness of these agents against the infectious spores, adverse effects on the vitality of the brood and the longevity of the bees,[6] and an increased rate of resistance of various *P. larvae* strains. Furthermore, antibiotic residues present in honey represent a major human health hazard.[7–10] The discovery of new antibiotics to control AFB could lead to the emergence of additional resistant *P. larvae* strains. Therefore, there is great interest in the investigation of alternative and

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efficient AFB-controlling substances, such as plant essential oils (EOs), which contain natural antibacterial substances.[5]

EOs from plants, herbs and spices exhibit antimicrobial activity against a wide spectrum of Gram-positive and Gram-negative bacteria and some fungal pathogens.[11] This antimicrobial activity is mainly due to the presence of phenolic and terpenoid compounds, which have a well-known antimicrobial activity.[12] EOs and aqueous herbal extracts have been used as remedies for infections since ancient times.[13,14] Several reports have been published, describing the role of EOs in controlling honey bee diseases [15−18] and several attempts have been made to investigate the *in vitro* antibacterial activity of plant EOs against *P. larvae*.[18−21] The EOs from *Carapa guianensis* and *Copaifera officinalis* exhibit considerable antibacterial activity against *P. larvae*, with high minimum inhibitory concentration (MIC) of 1.56% and 25%, respectively.[18]

The aim of this study was to evaluate the *in vitro* antimicrobial activity of EOs from 28 plant species against *P. larvae* by using a standard agar diffusion assay. The MIC and the minimum bactericidal concentration (MBC) values of these oils were determined using broth microdilution assay and broth macrodilution assay to screen for highly effective, moderately effective, minimally effective and ineffective plant oils. Some of the plant oils tested in this study exhibit antimicrobial activity against various bacterial and fungal pathogens [22,23]; however, little information about their antimicrobial activity against *P. larvae* has been published. Therefore, this study investigated the activity of 28 different plant EOs against *P. larvae*.

Materials and methods

Microorganisms and culture conditions

The reference strain of *P. larvae* from American Type Culture Collection (ATCC 9545) was used in this study. This strain was obtained from the Department of Biotechnology at the Indian Institute of Technology, Roorkee, India. The bacterial stock was stored and conserved by lyophilization (Lyodel Freeze Dryer, Delvac Pumps Pvt LTD Chennai, India) until further use. *P. larvae* was cultured in MYT medium (Mueller-Hinton broth, yeast extract and thiamine) (0.2% Mueller−Hinton broth (Oxoid) and 1.5% yeast extract (Oxoid) supplemented with 0.1 μg/mL of thiamine) and incubated at 36 °C ± 0.5 °C for 48 h with agitation (120 rpm) and cultured in MYPGP (Mueller-Hinton broth, yeast extract, potassium phosphate, glucose, and pyruvate) agar (10 g Mueller−Hinton broth (Difco), 15 g yeast extract, 2 g glucose, 3 g K_2HPO_4, 1 g sodium pyruvate, 20 g agar and water with a final volume of 1000 mL).[16] Following incubation, cells were collected by centrifugation (Eppendorf 5810R Centrifuge, Fisher Scientific, UK) at 5000 × *g* for 10 min and 4 °C, washed twice with phosphate-buffered saline (PBS) (pH 7.2) and finally re-suspended in PBS. The inoculum was prepared and adjusted to an inoculation level of 1.5×10^8 colony-forming units (CFU)/mL.

Chemicals and plant oils

The EOs used in this study are summarized in Table 1, together with their main chemical constituents. Almond (*Prunus glandulosa* L.), cedar wood (*Juniperus virginia* L.) and neem seed (*Azadirachta indica* A. Juss) oils were purchased from Himedia chemicals (Himedia, India), and the oils from cardamom (*Elettaria cardamomum* (L.) Maton), curry leaf (*Murraya koenigii* (L.) Sprengel.), ginger (*Zingiber officinale* Rosc), khus (*Vetiveria zizanoides* (L.) Nash), carrot seed (*Daucus carota* L.) and tulsi (*Ocimum tenuiflorum* L.) were purchased from Imperial Extracts (Ernakulam, India). Ajwain (*Trachyspermum ammi* L.), bay leaf (*Laurus nobilis* L.), bergamot (*Citrus bergamia* Risso and Poit), cajuput (*Melaleuca leucadendron* L.), camphor (*Cinnamomum camphora* (L.) J. Presl.), Jamaica pepper (*Pimenta dioica* (L.) Merr.), mountain pepper (*Litsea cubeba* Pers.), nutmeg (*Myristica fragrans* Gronov.), rosewood (*Aniba rosaeodora* Duke), spearmint (*Mentha spicata* L.) and star anise (*Illicium verum* Hook.f.) oils were purchased from Mother Herbs P (Ltd), New Delhi, India. Linseed (*Linum usitatissimum* L.), babuna (*Matricaria chamomilla* L.), corn mint (*Mentha arvensis* L.), dill (*Anethum graveolens* L.), geranium rose (*Pelargonium graveolens* L.), jojoba (*Simmondsia chinensis* (Link) C. K. Schneid.), sesame (*Sesamum indicum* L.) and wheat germ (*Triticum vulgare* L.) oils were obtained by using the steam distillation method.[24] OTC hydrochloride (Sigma-Aldrich, India) was used as a standard negative control.

Screening of essential oils for antibacterial activity

The antibacterial activity of the EOs from the 28 plants were tested using a disk diffusion assay.[25] MYPGP agar plates were prepared, and 50 μL of 1.5×10^8 CFU/mL *P. larvae* was aseptically spread over each plate. Sterile paper discs (6 mm in diameter) saturated with plant EOs (10 μL) were placed on the agar surface of the seeded plates, and the plates were incubated at 36 °C ± 0.5 °C for 48 h under microaerobic conditions. After 48 h of incubation, the diameter of the zone of growth inhibition was measured in millimetres with a calliper and was considered as the zone of inhibition (ZOI). All of the experiments were carried out in triplicate.

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Table 1. Essential oils and their main chemical constituents.

Plant essential oils	Botanical name	Family	Main constituents (over 10%)
Ajwain oil	*Trachyspermum ammi* L.	Apiaceae	Thymol (43.7%)
Almond oil	*Prunus glandulosa* L.	Rosaceae	α-Tocopherol (24.2%)
Babuna oil	*Matricaria chamomilla* L.	Asteraceae	α-Bisabolol (56.9%)
Bay leaf oil	*Laurus nobilis* L.	Lauraceae	1,8-Cineole (60%)
Bergamot oil	*Citrus bergamia* Risso and Poit	Rutaceae	Limonene (35.9%), Linalyl acetate (31.9%), Linalool (14.6%)
Cajuput oil	*Melaleuca leucadendron* L.	Myrtaceae	1,8-Cineole (55.8%), α-Terpineol (13.56%)
Camphor oil	*Cinnamomum camphora* (L.) J. Presl.	Lauraceae	Camphor (68%)
Cardamom oil	*Elettaria cardamomum* (L.) Maton	Zingiberaceae	α-Terpinyl acetate (46.0%), 1,8-Cineole (27.7%)
Cedar wood oil	*Juniperus virginiana* L.	Cupressaceae	Cedrol (26%), α-Cedrene (23.6%), Thujopsene (18.2%)
Corn mint oil	*Mentha arvensis* L.	Lamiaceae	Menthol (45.7%), Menthone (20.4%)
Carrot seed oil	*Daucus carota* L.	Apiaceae	Carotol (67.53%)
Curry leaf oil	*Murraya koenigii* (L.) Sprengel.	Rutaceae	α-Pinene (55.7%), β-Pinene (10.6%)
Dill oil	*Anethum graveolens* L.	Apiaceae	R-(-)-Carvone (38.89%), Apiol (30.81%), Limonene (15.93%)
Geranium rose oil	*Pelargonium graveolens* L.	Geraniaceae	Citronellol (30.4%), Geraniol (17.8%)
Ginger oil	*Zingiber officinale* Rosc	Zingiberaceae	Geranial (25.9%), α-Zingiberene (14.5%)
Jamaica pepper oil	*Pimenta dioica* (L.) Merr.	Myrtaceae	Eugenol (62.1%), Methyl eugenol (22.9%)
Jojoba oil	*Simmondsia chinensis* (Link) C. K. Schneid.	Simmondsiaceae	9-Octadecen-1-ol (41.35%), 1,21-Docosadiene (20.65%)
Khus oil	*Vetiveria zizanoides* (L.) Nash	Poaceae	Sesquiterpenols (30%−42%), Sesquiterpenones (14%−22%)
Linseed oil	*Linum usitatissimum* L.	Linaceae	Methyl linolenate (11.9%−33.9%)
Mountain pepper oil	*Litsea cubeba* Pers.	Lauraceae	Citral (72%)
Neem oil	*Azadirachta indica* A. Juss	Meliaceae	β-Caryophyllene (12.73%), Limonene, (10.17%)
Nutmeg oil	*Myristica fragrans* Gronov.	Myristicaceae	β-Pinene (11.69%), α-Pinene (10.06%), Sabinene (41.7%)
Rosewood oil	*Aniba rosaeodora* Duke	Lauraceae	Linalool (77.56%)
Sesame oil	*Sesamum indicum* L.	Pedaliaceae	γ-Tocopherols (52.5%)
Spearmint oil	*Mentha spicata* L.	Lamiaceae	Carvone (65.10%), d-Limonene (16.11%)
Star anise oil	*Illicium verum* Hook.f.	Schisandraceae	*trans*-Anethole (89.5%)
Tulsi oil	*Ocimum tenuiflorum* L.	Lamiaceae	Eugenol (75.30%)
Wheat germ oil	*Triticum vulgare* L.	Poaceae	1-Eicosene (13.77%), Hexadecyl acetate (12.19%)

Determination of the MICs of plant oils

The MICs (μg/mL) of the plant EOs were measured using a microbroth dilution assay in 96-well microtiter plates (Jet Biofil, China). To perform the microbroth dilution assay, 100 μL of each EO from stock solutions (3200 μg/mL) emulsified with propylene glycol (8 mg/mL) (Sigma-Aldrich Germany), was suspended in 100 μL of autoclaved MYT broth and placed in each well of the 96-well microtiter plate. Twofold serial dilutions of each emulsified sterile EO ranging from 3200 to 0.78 μg/mL were added to the remaining wells of the microtiter plates to make a final volume of 200 μL with sterile broth. Different concentrations (25 to 1.25 μg/mL in MYT broth) of OTC hydrochloride were used to determine the MIC value of the standard negative control. One well (MYT broth + propylene glycol) was used as a control, and another well (MYT broth + propylene glycol + test plant oil) was used as a sterility control. All wells except the sterility control well were inoculated with *P. larvae* suspension (10 μL) at a concentration of 1.5×10^8 CFU/mL, prepared as described above. This assay was performed for all of the EOs. Each microtiter plate was incubated at 37 °C under microaerobic conditions. The MICs were recorded after 48 h of incubation. Wells that contained white 'pellets' on the bottom were considered positive for bacterial growth. The extent of bacterial growth was determined based on the turbidity, which was measured spectrophotometrically using a GloMax®-Multi Microplate Multimode Reader (Promega, China) at 600 nm. The MIC was determined to be the lowest concentration of plant oil that resulted in the inhibition of the visible growth of *P. larvae* after an overnight incubation, compared with the growth control.[26] On the basis of effectiveness of EOs against *P. larvae*, we categorized the EOs as highly effective, moderately effective, minimally effective and ineffective. All samples were tested in triplicate.

Determination of the MBCs of plant oils

The MYPGP solid agar medium was inoculated with 20 μL inoculum from each of the wells with no visible bacterial growth. The Petri dishes were incubated at 36 °C ± 0.5 °C for 48 h under microaerobic conditions to determine the MBC (μg/mL) value. The MBC was determined to be the lowest concentration of plant oils that was required to kill 99.9% of the original inoculum of *P. larvae*.[27]

Time-kill analysis

To explore the bactericidal effect of the most effective plant oils, along with OTC, survivor (time-kill) curves were plotted. The bactericidal effects of the oils against *P. larvae* were studied using the MBCs of the most effective oils. At different time intervals after incubation (0, 3,

6, 9, 12, 24, 36 and 48 h), a 50-μL inoculum was removed from the control (MYT broth + propylene glycol) and plant oil suspensions, serially diluted in a physiological saline solution, and plated on an MYPGP agar plate for colony counting. All of the tests were performed in triplicate, and the averages were plotted as the log CFU/mL versus time (h) for each time point.

Statistical analysis

All experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation. Statistical analysis of the differences between the mean values obtained for the experimental groups was performed using Student's *t*-test. A *P*-value of 0.05 or less was considered significant.

Results and discussion

Since ancient times, plant EOs have been used for domestic and therapeutic purposes; these oils possess broad-spectrum antimicrobial properties. Plant EOs have been selectively used to treat various microbial infections.[28−30] The antimicrobial properties of EOs suggest that these substances could be used to control *P. larvae*. Recently, a few studies have demonstrated the activity of plant EOs against honeybee pathogens.[16,18−20] Natural antibiotics based on plant EOs may represent alternatives to chemically synthesized antibiotics. It is important to control honeybee diseases with natural antibiotics because most honeybee by-products, such as honey, must be free from contaminants. OTC and sulfamethoxazole continue to be the drugs of choice for the treatment of AFB infections, but the emergence of resistance of *P. larvae* to these antibiotics has been documented in some countries.[7]

We studied the *in vitro* efficacy of 28 plant EOs against *P. larvae* (Table 1). Out of the 28 selected plant EOs, 20 exhibited antibacterial activity against *P. larvae*, with zones of inhibition ranging from 1 to 20 mm (Table 2). The zones of inhibition produced by Jamaica pepper oil, mountain pepper oil and ajwain oil were 19.6, 19.2 and 16.5 mm, respectively. The zones of inhibition produced by corn mint oil, spearmint oil, star anise oil, nutmeg oil and camphor oil ranged from 11.6 to 15 mm, whereas those produced by tulsi, carrot seed, ginger and

Table 2. Classification and antibacterial activity of selected plant oils against *P. larvae*.

Group	Plant oils	Botanical name	ZOI (mm)	MIC (μg/mL)	MBC (μg/mL)
Highly effective	Jamaica pepper oil	*Pimenta dioica* (L.) Merr. (Myrtaceae)	19.6 ± 1.6	78.0 ± 8.2	162.0 ± 18.2
	Mountain pepper oil	*Litsea cubeba* Pers. (Lauraceae)	19.2 ± 1.2	85.0 ± 7.9	186.0 ± 21.2
	Ajwain oil	*Trachyspermum ammi* L. (Apiaceae)	16.5 ± 0.9	137.0 ± 12.2	224.8 ± 25.6
	Corn mint oil	*Mentha arvensis* L. (Lamiaceae)	15.0 ± 0	144.7 ± 17.2	248.0 ± 23.4
	Spearmint oil	*Mentha spicata* L. (Lamiaceae)	14.8 ± 0.5	145.6 ± 15.4	256.0 ± 26.5
	Star anise oil	*Illicium verum* Hook.f. (Schisandraceae)	12.7 ± 0.8	278.6 ± 21.2	365.0 ± 32.1
	Nutmeg oil	*Myristica fragrans* Gronov. (Myristicaceae)	11.9 ± 0	285.8 ± 29.2	371.3 ± 29.0
	Camphor oil	*Cinnamomum camphora* (L.) J. Presl. (Lauraceae)	11.6 ± 0.6	286.2 ± 27.9	375.0 ± 34.8
Moderately effective	Tulsi oil	*Ocimum tenuiflorum* L. (Lamiaceae)	7.9 ± 1.0	412.8 ± 26.0	589.6 ± 48.2
	Carrot seed oil	*Daucus carota* L. (Apiaceae)	6.8 ± 0	482.0 ± 36.5	612. 6 ± 52.0
	Ginger oil	*Zingiber officinale* Rosc (Zingiberaceae)	5.2 ± 0	488.0 ± 28.2	618.2 ± 63.0
	Geranium rose oil	*Pelargonium graveolens* L. (Geraniaceae)	4.6 ± 0.2	495.4 ± 32.9	690.5 ± 75.0
Minimally effective	Cajuput oil	*Melaleuca leucadendron* L. (Myrtaceae)	2.7 ± 0	1067 ± 35.8	1190.8 ± 68.9
	Bay leaf oil	*Laurus nobilis* L. (lauraceae)	2.2 ± 0	1287.9 ± 32.0	1355.9 ± 85.9
	Cardamom oil	*Elettaria cardamomum* (L.) Maton (Zingiberaceae)	1.9 ± 0	1322.0 ± 68.2	1488.2 ± 42.9
	Bergamot oil	*Citrus bergamia* Risso and Poit (Rutaceae)	1.6 ± 0	1550.9 ± 65.2	2110.8 ± 32.2
	Curry leaf oil	*Murraya koenigii* (L.) Sprengel. (Rutaceae)	1.5 ± 0	1685.5 ± 54.8	2234.7 ± 105.9
	Dill oil	*Anethum graveolens* L. (Apiaceae)	1.4 ± 0	1890.5 ± 48.7	2110.8 ± 63.5
	Cedar wood oil	*Juniperus virginiana* L. (Cupressaceae)	1.2 ± 0	1897.2 ± 52.2	2310.3 ± 56.8
	Rosewood oil	*Aniba rosaeodora* Duke (Lauraceae)	1.2 ± 0	1910.3 ± 32.2	2290.2 ± 49.9
Ineffective	Khus oil	*Vetiveria zizanoides* (L.) Nash (Poaceae)	0 ± 0	–	–
	Sesame oil	*Sesamum indicum* L. (Pedaliaceae)	0 ± 0	–	–
	Babuna oil	*Matricaria chamomilla* L. (Asteraceae)	0 ± 0	–	–
	Wheat germ oil	*Triticum vulgare* L. (Poaceae)	0 ± 0	–	–
	Almond oil	*Prunus glandulosa* L. (Rosaceae)	0 ± 0	–	–
	Jojoba oil	*Simmondsia chinensis* (Link) C. K. Schneid. (Simmondsiaceae)	0 ± 0	–	–
	Linseed oil	*Linum usitatissimum* L. (Linaceae)	0 ± 0	–	–
	Neem oil	*Azadirachta indica* A. Juss (Meliaceae)	0 ± 0	–	–
	Oxytetracycline*		21.0 ± 1.6	4.2 ± 1.20	5.89 ± 1.37
	Growth control (MYT broth + propylene glycol)		0 ± 0	–	–
	Sterility control (MYT broth + propylene glycol+ test plant oil)		0 ± 0	–	–

Note: Zone of inhibition (ZOI); minimum inhibitory concentration (MIC); minimum bactericidal concentration (MBC); standard negative control (*). Results were obtained as mean values ± SD.

geranium rose oils ranged from 4.6 to 7.9 mm. The zones of inhibition produced by eight other oils (cajuput, bay leaf, cardamom, bergamot, curry leaf, dill, cedar wood and rosewood) ranged from 1.2 to 2.7 mm. The other tested oils – from khus, sesame, babuna, wheat germ, almond, jojoba, linseed and neem – were found to be ineffective. The ZOI produced by the OTC hydrochloride was 21.0 mm, which was almost equal to the ZOI produced by Jamaica pepper oil. The largest ZOI was obtained using Jamaica pepper oil (19.6 ± 1.6 mm) and the lowest one was obtained using Rosewood oil (1.2 ± 0 mm). Therefore, Jamaica pepper oil has high antibacterial activity against *P. larvae*. Our findings demonstrated that these plant oils might not only have the potential to control *P. larvae* infections but may also represent possible alternatives to the use of OTC to control AFB. Our data (Table 2) clearly demonstrated that most of the used EOs inhibited the growth of *P. larvae*, which is in contrast to the results of earlier studies on EOs.[16,17,19]

The MICs of the 20 effective plant oils and OTC against *P. larvae* were determined using a microbroth dilution assay. The plant oils showed concentration-dependent growth inhibition of the *P. larvae*. OTC (4.2 μg/mL) completely inhibited the growth of *P. larvae*. Jamaica pepper oil was the most effective, with complete inhibition occurring at 78.0 ± 8.2 μg/mL. Eight plant oils completely inhibited the growth of *P. larvae* at concentrations ranging from 78 to 286.2 μg/mL. Four plant oils were inhibitory at concentrations between 412.8 and 495.4 μg/mL, and the other eight plant oils were inhibitory at concentrations ranging from 1067 to 1910.3 μg/mL. The MIC of OTC was 4.2 ± 1.20 μg/mL (Table 2).

The MBC values for the tested plant oils were greater than their respective MIC values. Based on the MBC values, effective plant oils were placed into three categories: plant oils having MBC values of 162−375 μg/mL were considered to be highly effective, plant oils with MBC values ranging from 589 to 700 μg/mL were considered to be moderately effective, and plant oils with an MBC > 1000 μg/mL were considered to be minimally effective. OTC's MBC was 5.89 ± 1.37 μg/mL. Jamaica pepper, mountain pepper, ajwain, corn mint, spearmint, star anise, nutmeg and camphor oils were highly effective, with bactericidal values ranging from 162.0 to 375.0 μg/mL. Four oils (tulsi, carrot seed, ginger and geranium rose) were moderately effective, with MBC values ranging from 589.6 to 690.5 μg/mL. Eight oils were less effective, with MBC values from 1190.8 to 2290.2 μg/mL. The other eight plant oils were not effective, even at a concentration of 3400 μg/mL (Table 2).

To determine the time-response effect of the most effective oils (Jamaica pepper oil, mountain pepper oil and ajwain oil) and that of OTC on *P. larvae*, bacteria were exposed to the oils for 48 h. After 24 h of treatment with Jamaica pepper oil (162.0 μg/mL), 36 h of treatment with mountain pepper oil (186.0 μg/mL) and 48 h of treatment with ajwain oil (224.8 μg/mL) or OTC (5.89 μg/mL), no viable cells of *P. larvae* ATCC 9545 were observed (Figure 1). Furthermore, a steep decline in the CFU/mL was observed after treatment with Jamaica pepper oil, mountain pepper oil, ajwain oil and OTC for 12 h and 24 h.

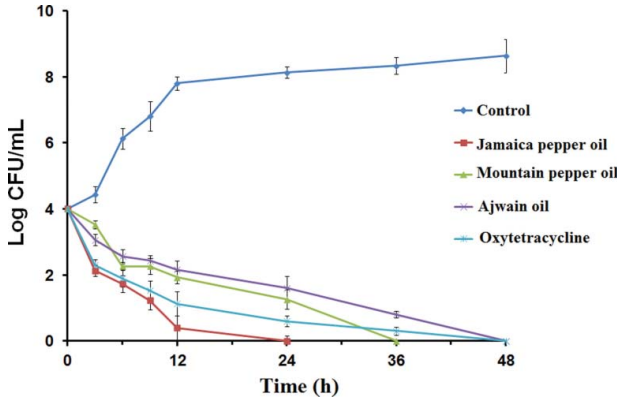

Figure 1. Time-course effect on *P. larvae* ATCC 9545 of Jamaica pepper oil, mountain pepper oil, ajwain oil and oxytetracycline.

Jamaica pepper oil has a medicinal value and has been used in the treatment of various bacterial infections. It has been reported that cinnamon oil possesses *in vitro* antibacterial activity against *P. larvae* and *Staphylococcus aureus* strains.[16,31,32] The antibacterial activity of cinnamon oil is due to the presence of eugenol.[16] This study supports our results, as Jamaica pepper oil showed a maximum antimicrobial activity (ZOI: 19.6 ± 1.6 mm) with eugenol, as a major constituent. In addition, lemongrass is recognized as an aromatic and medicinal plant. Lemongrass oil is commonly used to control some fungal diseases and AFB. The antimicrobial activity of lemongrass oil is due to the presence of a citral compound.[33] This is also supported by our findings, as mountain pepper oil showed antimicrobial activity (ZOI: 19.2 ± 1.2 mm) and its major constituent is citral compound. Thyme oil, which is rich in thymol, also has strong effects against some pathogens.[34] The antagonistic effects of Thymol against *Listeria monocytogenes* and *Bacillus subtilis* have been reported.[35] Thymol is present in some commercial products used to control the mite *Varroa destructor*.[36] In this study, we found that the majority of the plant oils tested were effective and showed anti-*P. larvae* activity at low concentrations. The *P. larvae* strain was found to be highly susceptible to cinnamon oil (*Cinnamomum zeylanicum*), lemongrass oil (*Cymbopogon citratus*) and thyme oil (*Thymus vulgaris*).[16,37] Our findings are supported by the findings of

Gurgulova et al.,[38] who reported the high antimicrobial activity of EOs from thyme. Roussenova [17] has also reported the use of cinnamon oil against *P. larvae*. OTC is known to be effective against *P. larvae* and is the drug of choice to treat AFB infection, despite the emerging resistance of *P. larvae* to this antibiotic.[39] Little information is available about the mode of action of natural substances that inhibit the *P. larvae* growth.

Conclusions

In conclusion, the results presented in this paper demonstrated that most of the selected plant EOs have potential antibacterial activities. Jamaica pepper, mountain pepper and ajwain oils have a bacteriostatic effect against *P. larvae*. The use of non-toxic natural compounds could represent a natural alternative to the use of synthetic antibiotics in the control of AFB. This should, therefore, reduce the antibiotic resistance and the levels of antibiotic residues. Further research must be conducted on these essential plant oils, in order to isolate the active ingredients that kill *P. larvae*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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