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Antibacterial Activity of Potential Essential Oil Extracts against the American Foulbrood, *Paenibacillus larvae larvae*

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ABSTRACT

The present study was conducted to investigate the antibacterial activity of potential essential oils at five concentrations (100, 50, 25, 12.5 and 6.25 %) against *Paenibacillus larvae larvae* (the causative agent of AFB). Different parts of ten plants belong to four families, Rutaceae, Myrtaceae, Anacardiaceae and Lamiaceae were used in present experiment to extract fifteen essential oils. The efficiencies, in inhibition the growth of *P. l. larvae* bacteria, of these crude oils and four mixtures of some of them beside their diluents were evaluated using disk diffusion assay. Obtained results indicated that the efficiencies, in inhibiting *P. l. larvae* growth, of *Citrus limon* essential oil from leaves, peels and mixture of them (at tested concentrations) varied from weak to very high. The most effective one was the crude oil of leaves with inhibition zone of 34.2 mm with 213.5 % relative efficiency (R.E.) of Tylosine. Considering the four plants belonging to Family Lamiaceae, results emphasized the high efficiency of the crude essential oil from *Thymus vulgaris* whole plant, beside its tested diluents, in inhibiting the growth of *P. l. larvae*. It was most the effective treatment achieving inhibitory activity (inhibition zone of 47.5 mm) with 245.5 % R.E. Moreover, the same trend was observed when the concentration decreased to be 50, 25 or 12.5 %, which still exhibiting high activities (inhibition zones of 36.3, 33.5 and 30.5 mm with 198.4, 178.3 and 163.4 % R.E., respectively). In general, the antibacterial activities of the evaluated essential oils were concentration-dependent.

Key words: *Honey bees; foulbrood; Paenibacillus larvae; essential oils*

1. INTRODUCTION

Honeybees, *Apis mellifera* L., are the most important beneficial organisms in many agro-ecosystems throughout the world, because of their vital role in pollination, and values of their products: honey, pollen, beeswax, royal jelly, propolis and venom. Recently, increasing reports about the global phenomenon called colony collapse disorder (CCD) caused by many factors drew the attention to this serious problem to beekeeping industry. Various pathogens and beekeeper-applied antibiotics are some of these factors. American foulbrood (AFB) is one of the most destructive bacterial diseases of the honeybee causing great economic losses in beekeeping industry worldwide; decrease of bee population, colony productions and pollination (Eid *et al*., 2019).

The spore forming bacterium, *Paenibacillus larvae*, subspecies *larvae* cause the severe brood infection of AFB. These endospore Gram-positive bacteria are the most virulent

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brood pathogen infecting honeybees. The *P. l. larvae* spores germinate in the midgut of bee larvae to be the vegetative cells, which invade the tissue and exhaust the nutrients of host until form the spores. Therefore, inhibiting spore germination or impeding early vegetative growth is considered the most effective strategy for limiting the impact of AFB (Lamei *et al*., 2019). Oxytetracycline (OTC), Tylosine and Sulfathiazole are common antibiotics used reportedly in beekeeping to control AFB (Johnson and Jadon, 2010). However, resistance in these bacteria and contamination of bee products related to the use of antibiotics became great problems resulting in banning the use of these antibiotics in most European countries (Alonso-Salces *et al*., 2017). Therefore, searching for safe alternative strategies for disease control, based on the application of plant essential oils (EOs) became very crucial. Many researchers (González and Marioli 2010, Santos *et al*., 2012, Pimentel-Betancurt *et al.*, 2021 and Morshdy *et al.*, 2023) evaluated the antimicrobial activities of EOs extracted from different parts of aromatic and medicinal plants against various pathogens including *P. l. larvae* (Awad, 2011; Eid *et al*., 2019 and Paletti-Rovey *et al.*, 2022).

However, few studies were done in this area, so this research was carried out focusing on bioassay of safe and effective natural substances to control AFB. Therefore, this study was launched bearing in mind the following objectives:

1. Extracting essential oils or extracts from various plant parts (leaves, seeds and peels) of 10 plants belonging to four families.

2. Determining the *in vitro* activity of essential oils extracted from tested plants against local strain of *P. l. larvae* compared with Doxycycline, Tylosin/Doxycycline and Tylosine antibiotics.

2. MATERIALS AND METHODS

2.1. *Paenibacillus larvae* strain

The strain was obtained from Plant Protection Research Institute (PPRI), Apiculture Research Center (ARC), Cairo, Egypt. The bacteria were cultured to obtain the vegetative cells. The laboratory tests were performed, and then healthy colonies were artificially infected before the sensitivity tests.

2.1.1. Culturing of *P. l. larave*

Brain Heart Infusion (BHI) medium was sterilized using the autoclave (at 10 lb/ sq. in for 20 min at 116 °C). Then, it was fortified with 0.1 mg thiamine hydrochloride per liter of medium and adjusted to pH 6.6 with HCl. After that, suspension of the obtained strain of *P. larave* was spread over petri dishes containing the medium, and the plates were incubated at 37.5°C for 72 hours.

2.1.2. Artificial infection of healthy colonies

Suspension of the cultured pathogen was added to sugar syrup (1:1 w/v), and then sprayed on brood combs of three healthy colonies, left untreated for three months, to be artificially infected. AFB symptoms were routinely examined, after two weeks, based on biological and morphological characters.

2.1.3. Rapid diagnostic tests

The Holst milk test using smear of bacteria in a test tube, and the microscopic examination using Gram and Carbol fuchsin staining of bacteria according to Thai Agricultural Standard (TAS) (2007) were performed to rapidly diagnose the pathogen.

2.1.4. Confirmation tests

Culture techniques as bacteriological methods using growing on medium of Brain Heart Infusion with Thiamin (BHIT) (Baily and Lee, 1962), and biochemical tests such as Catalase production and growth on nutrient agar medium were conducted to confirm the pathogen.

2.2. Extraction of essential oils

2.2.1. Sampling

Different parts of ten plants belonging to four families **(Table 1)** sampled from different places from which the essential oils were extracted.

2.2.2. Extraction method

The essential oils were extracted by hydro distillation method using the modified Clevenger (flask of 2000 ml) (Cole *et al.*, 2014). Different parts from sampled fresh plants were separately ground in a blender to achieve uniform particle size. Then, the ground sample was put in a flask with 1,000 ml of distilled water and left until start of reflux. All extracted essential oils were stored under 4 °C until tested.

Table (1): Different essential oils extracted from different parts of ten plants belonging to four families using hydro distillation method.

Family	Botanical name of plant	Extract of part
Rutaceae	*Citrus limon*	Leaves, Peels, Leaves and peels
	Citrus reticulate	Leaves, Peels Leaves and peels
	Citrus sinensis	Leaves
Myrtaceae	*Syzygium aromaticum* (L.)	Flower petals
	Eucalyptus camaldulensis	Leaves, Seeds, Leaves and seeds
Anacardiaceae	*Schinus terebinthifolius*	Leaves, Seeds, Leaves and seeds
Lamiaceae	*Ocimum bacilicum*	Leaves, Seeds
	Mentha piperita	Leaves plus stem
	Mentha pulegium	Leaves plus stem
	Thymus vulgaris	Whole plants

2.3. Estimating the antibacterial activity of diluted essential oils on *P. l. larvae*

The efficiencies of fifteen essential oils extracted from different parts of ten plants belonging to four families, and four mixtures on the growth of *P. l. larvae* bacteria were evaluated using disk diffusion assay. Five concentrations (100, 50, 25, 12.5 and 6.25%) of each tested essential oil were prepared using emulsifier solution (Triton X100 at rate of 0.5 ml / 100 ml sterile water).

Three filter paper discs (0.6 cm in diameter) of each concentration, three discs of antibiotics (Tylosine, Doxycycline and Tylosin/Doxycycline) at concentration of 200 µg/ml as positive controls, and two discs of sterile water and Triton X100 as negative controls were placed onto the BHIT plates (10 ml/plate). Each concentration of each essential oil was evaluated in three replicates. All plates were incubated for 72 hrs at 37.5° and inhibition zones were measured and recorded. Tylosine was used as a reference to calculate the relative efficiency percentage (R.E.) using the following equation:

$$\text{Relative efficiency}\ (\%) = \frac{\text{Measured inhibition zone from treatment disk}}{\text{Measured inhibition zone from Tylosin disk on the same plate}} X100$$

Local isolate of *Paenibacillus larvae* subsp. *larvae* was obtained, cultured to artificially infect healthy honeybee colonies and the symptoms of the disease began to appear after two weeks. Also, the laboratory tests confirmed the pathogen. After that, it was tested for their sensitivity to a wide range of crude extracts and their diluents. Fifteen plant extracts and four mixtures from different parts of ten plants belonging to four families were subjected to sensitivity tests.

2.4. Statistical analysis

Data were subjected to analysis of variance using a one-way factorial design, with a random effect for block (position of Petri plates in incubator), in Glmmix procedure in SAS 9.4 (SAS Inc., Cary, NC, USA). Significant effects and interactions were determined at significance level of 5% (P≤0.05) and compared using to Tukey's test.

3. RESULTS

3.1. Antibacterial activity of five concentrations of *Citrus limon* essential oil against *P. l. larvae*

The antibacterial activities by the disc diffusion method (inhibition zone, mm) of these essential oils or mixtures (at five concentrations), compared with that of three antibiotics, against *P. l. larvae* were studied. The relative efficiencies (R.E.) of them expressed as percentages of that of reference antibiotic (Tylosin) are shown. Generally, in this research, the tested extracts varied in their efficiency in inhibiting the growth of pathogen. Concerning the three plants belonging to family Rutaceae, data in Table (2) reveal that the essential oil of *Citrus limon* leaves (at concentrations of 100 and 50 %) had antibacterial activities (with R.E. of 213.5 and

Table (2): Antibacterial activity (inhibition zone, mm) of five concentrations of *Citrus limon* essential oil against *P. l. larvae* by the disc diffusion method and their relative efficiency (R.E.) percentages.

Plant parts / Antibiotics	Concentrations %	I. Z (mm) ± S. D.*	R. E (%). #
Leaves	100	34.2 ± 0.2	213.5 a
	50	22.3 ± 1.2	125.2 c
	25	18.0 ± 0.4	101.0 de
	12.5	15.3 ± 0.5	89.4 e
	6.25	12.2 ± 0.2	71.6 f
Peels	100	16.2 ± 0.5	101.0 de
	50	12.7 ± 0.2	71.0 f
	25	10.0 ± 0.0	56.1 f-h
	12.5	9.0 ± 0.0	52.4 gh
	6.25	7.0 ± 0.0	41.2 h
Leaves and peels	100	26.7 ± 0.6	166.7 b
	50	19.7 ± 0.6	110.3 cd
	25	12.3 ± 0.2	69.3 fg
	12.5	10.0 ± 0.0	58.3 f-h
	6.25	8.3 ± 0.2	49.0 h
Tylosin	0.2 g $	17.0 ± 0.7	100.0 de
Doxycycline	0.2 g	20.5 ± 0.7	121.3 c
Tylo-Doxcine	0.2 g	20.3 ± 1.2	119.2 c

* : I. Z (mm) ±S.D: Average of inhibition zone ± Standard deviation.
#: R. E (%): Average of relative efficiency, significance letters apply within column only.
$: Recommended field application rate of Tylosin.

125.2 %, respectively) which are significantly higher than that of Tylosin. Meanwhile, the crude essential oil of peels had R.E. of 101.1% with no significant difference, compared with that of Tylosin, and when it diluted to be at concentrations of 50, 25, 12.5 and 6.25%, the antibacterial activities decreased to be with R.E. of 71.0, 56.1, 52.4and 41.2%, respectively. Mixing the two extracts of *C. limon* exhibited additive effect recording 166.7% R.E. that is significantly higher than that of Tylosin, while the diluted mixture (at 50 % concentration) exceeded the reference antibiotic, but with no significant difference. Data in Table (3) show that the essential oil of *Citrus reticulata* leaves (at concentrations of 100 %) had antibacterial activity (with R.E. of 121.6 %) which was not significantly higher than that of Tylosin, while lower concentrations exhibited low effect on the growth of pathogen. On the other hand, the essential oil of peels (at any tested concentration) significantly lower antibacterial activity than that of Tylosin. Mixing leaves and peels essential oils of *C. reticulata* exhibited additive effect and all concentrations of the mixture revealed significantly lower levels of antibacterial activity against the pathogen when compared with Tylosin. As shown in Table (4), the essential oil of *Citrus sinensis* leaves (at concentration of 100 %) had antibacterial activity (with R.E. of 116.0 %) which is significantly higher than that of Tylosin. Meanwhile, the diluted essential oil of peels (at concentration of 50 %) had R.E. of 91.3% with no significant difference, compared with that of Tylosin, and when it diluted to be at concentrations of 25, 12.5 and 6.25%, the antibacterial activities of them decreased to be with R.E. of 58.3, 53.3 and 49.0 %, respectively.

Table (3): Antibacterial activity (inhibition zone, mm) of five concentrations of *Citrus reticulata* essential oil against *P. l. larvae* by the disc diffusion method and their relative efficiency (R.E.) percentages.

Parts / Antibiotics	Concentrations %	I. Z (mm) ± S. D.*	R.E (%). #
Leaves	100	20.2 ± 0.5	121.6 a
	50	10.0 ± 1.6	61.3 cd
	25	8.2 ± 0.2	48.5 cd
	12.5	7.3 ± 0.2	44.9 cd
	6.25	7.3 ± 0.2	43.6 cd
Peels	100	10.8 ± 1.2	65.1 cd
	50	7.7 ± 0.9	47.1 cd
	25	7.3 ± 0.2	43.6 cd
	12.5	7.0 ± 0.0	42.9 d
	6.25	7.0 ± 0.0	41.6 d
Leaves and peels	100	12.7 ± 0.2	76.1 bc
	50	9.7 ± 0.5	59.2 cd
	25	7.7 ± 0.5	45.6 cd
	12.5	7.2 ± 0.2	43.9 cd
	6.25	7.0 ± 0.0	41.6 d
Tylosin	0.2 g $	16.6 ± 0.5	100.0 a
Doxycycline	0.2 g	18.1 ± 1.8	109.2 a
Tylo-Doxcine	0.2 g	18.3 ± 1.7	110.2 a

* : I. Z (mm) ±S.D: Average of inhibition zone ± Standard deviation.
#: R. E (%): Average of relative efficiency, significance letters apply within column only.
$: Recommended field application rate of Tylosin.

Table (4): Antibacterial activity (inhibition zone, mm) of five concentrations of *Citrus sinensis* **essential oil against** *P. l. larvae* **by the disc diffusion method and their relative efficiency (R.E.) percentages.**

Parts / Antibiotics	Concentrations %	I. Z (mm) ± S. D.*	#R. E (%).
Leaves	100	19.7 ± 0.5	116.0 a
	50	14.5 ± 1.4	91.3 b
	25	9.3 ± 0.8	58.3 c
	12.5	8.7 ± 0.5	53.3 c
	6.25	8.2 ± 0.2	49.0 c
Tylosin	0.2 g $	16.4 ± 0.9	100.0 b
Doxycycline	0.2 g	16.0 ± 0.8	97.6 b
Tylo-Doxcine	0.2 g	16.9 ± 0.9	103.0 b

*: I. Z (mm) ±S.D: Average of inhibition zone ± Standard deviation.
#: R. E (%): Average of relative efficiency, significance letters apply within column only.
$: Recommended field application rate of Tylosin.

Regarding the two plants belonging to Family Myrtaceae, data in Table (5) illustrate that the essential oil of *Suzgium aromaticum* petals (at concentration of 100 %) had antibacterial activity (with R.E. of 109.1 %) which did not significantly differ from that of Tylosin. Meanwhile, the diluted essential oil of petals (at concentration of 50 %) had R.E. of 95.5 % with no significant difference, compared with that of Tylosin, and when it diluted to be at concentrations of 25, 12.5 and 6.25%, the antibacterial activities of them decreased significantly to be with R.E. of 79.3, 65.2 and 55.5 %, respectively. The obtained data in **Table (6)** show that the essential oils extracted from the leaves, seeds of *Eucalyptus camaldulensis* and the mixture of them (at concentration of 100 %) recorded 69.0, 72.3 and 62.2 % R.E., respectively. Meanwhile such essential oils (at all lower concentrations) exhibited low levels of antibacterial activity. In regard to the chosen plant of Family Anacardiaceae, data in **Table (7)**

Table (5): Antibacterial activity (inhibition zone, mm) of five concentrations of *Suzgium aromaticum* **essential oil against** *P. l. larvae* **by the disc diffusion method and their relative efficiency (R.E.) percentages.**

Parts / Antibiotics	Concentrations %	I. Z (mm) ± S. D.*	R. E (%). #
Flower petals	100	21.2 ± 0.8	109.1 a
	50	17.5 ± 0.4	95.5 b
	25	15.0 ± 0.0	79.3 c
	12.5	12.2 ± 0.2	65.2 d
	6.25	10.2 ±0.2	55.5 d
Tylosin	0.2 g $	18.7 ± 3.9	100.0 ab
Doxycycline	0.2 g	18.6 ± 1.0	99.5 b
Tylo-Doxcine	0.2 g	19.3 ± 0.8	103.3 ab

*: I. Z (mm) ±S.D: Average of inhibition zone ± Standard deviation.
#: R. E (%): Average of relative efficiency, significance letters apply within column only.
$: Recommended field application rate of Tylosin.

Table (6): Antibacterial activity (inhibition zone, mm) of five concentrations of *Eucalyptus camaldulensis* **essential oil against** *P. l. larvae* **by the disc diffusion method and their relative efficiency (R.E.) percentages.**

Parts / Antibiotics	Concentrations %	I. Z (mm) ± S. D.*	R. E (%). #
Leaves	100	13.7 ± 0.2	69.0 bc
	50	11.7 ± 0.2	58.0 b-e
	25	8.5 ± 0.0	42.9 ef
	12.5	7.3 ± 0.2	38.6 f-h
	6.25	4.7 ± 3.3	24.9 gh
Seeds	100	14.3 ± 0.2	72.3 b
	50	12.2 ± 0.5	60.0 b-d
	25	9.0 ± 0.0	45.9 d-f
	12.5	7.7 ± 0.2	40.4 gf
	6.25	7.0 ± 0.0	36 f-h
Leaves and seeds	100	12.3 ± 0.2	62.2 b-d
	50	10.5 ± 0.4	52.2 c-f
	25	8.5 ± 0.4	42.9 ef
	12.5	7.3 ± 0.2	38.6 f-h
	6.25	4.7 ± 3.3	23.4 h
Tylosin	0.2 g $	19.7 ± 0.8	100.0 a
Doxycycline	0.2 g	19.0 ± 0.7	96.5 a
Tylo-Doxcine	0.2 g	19.8 ± 0.6	101.0 a

* : I. Z (mm) ±S.D: Average of inhibition zone ± Standard deviation.
#: R. E (%): Average of relative efficiency, significance letters apply within column only.
$: Recommended field application rate of Tylosin.

Table (7): Antibacterial activity (inhibition zone, mm) of five concentrations of *Schinus terebinthifolius* **essential oil against** *P. l. larvae* **by the disc diffusion method and their relative efficiency (R.E.) percentages.**

Parts / Antibiotics	Concentrations %	I. Z (mm) ± S. D.*	#R. E (%).
Leaves	100	12.7 ± 0.2	70.4 c
	50	11.0 ± 0.0	64.1 cd
	25	9.0 ± 0.0	54.3 d-f
	12.5	8.0 ± 0.0	49.6 ef
	6.25	0.0 ± 0.0	0.0 g
Seeds	100	11.2 ± 0.2	62.0 c-e
	50	10.2 ± 0.2	59.2 c-f
	25	8.0 ± 0.0	48.3 f
	12.5	7.3 ± 0.5	45.6 f
	6.25	0.0 ± 0.0	0.0 g
Leaves and seeds	100	11.7 ± 0.2	64.0 cd
	50	10.0 ± 0.0	58.2 c-f
	25	8.0 ± 0.0	48.3 f
	12.5	7.7 ± 0.5	47.7 f
	6.25	0.0 ± 0.0	0.0 g
Tylosin	0.2 g $	17.2 ± 1.1	100.0 b
Doxycycline	0.2 g	18.2 ± 0.7	106.6 a
Tylo-Doxcine	0.2 g	18.0 ± 1.2	105.3 ab

* : I. Z (mm) ±S.D: Average of inhibition zone ± Standard deviation.
#: R. E (%): Average of relative efficiency, significance letters apply within column only.
$: Recommended field application rate of Tylosin.

recorded 70.4, 62.0 and 64.0 % R.E., show that the essential oils extracted from the leaves, seeds of *Schinus terebinthifolius* and the mixture of them (at concentration of 100 %), respectively. Meanwhile such essential oils (at all lower concentrations) exhibited very low levels of antibacterial activity against the pathogen. Concerning the three plants belonging to Family Lamiaceae, Fig. (1) illustrate that the essential oil of *Ocimum bacilicum* leaves (at concentrations of 100 %) had antibacterial activity (with R.E. of 111.9 %) which did not differ significantly from that of Tylosin, and when it diluted to be at concentrations of 50, 25, 12.5 and 6.25%, the antibacterial activities decreased significantly to be with R.E. of 71.7, 53.0, 50.2 and 27.9 %, respectively.

In addition, the essential oil of seeds was effective only when it was crude with 104.9 % R.E., but at lower concentrations exhibited low levels of efficiency. Data of Table (8) reveals that the essential oil of *Mentha piperita* leaves and stem (at concentrations of 100 %) had antibacterial activity (with R.E. of 122.8 %) significantly higher than that of Tylosin, while lower concentrations exhibited significantly lower antibacterial activities. In addition, the essential oil of *Mentha pulegium* leaves plus stem (at concentrations of 100 %) had antibacterial activity (with R.E. of 97.6 %) with no significant difference from that of Tylosin, while lower concentrations exhibited significantly lower antibacterial activities. On the other hand, as illustrated in Fig. (2), the essential oil of *Thymus vulgaris* showed the highest antibacterial activity compared with all tested essential oils tested in present work. Such essential oil at concentration 100 % resulted in inhibition zone area of 47.5 mm, which recorded 245.4 % R.E. Also, when tested at concentrations of 50, 25 and 12.5 % it showed 198.4, 178.3 and 163.4% R.E., in respect, which were significantly higher, in inhibiting the growth of *P. l. larvae* bacteria, than that exhibited by any tested antibiotic. However, at concentration of 6.25 %, the antibacterial activity was decreased, and did not differ significantly from that of control.

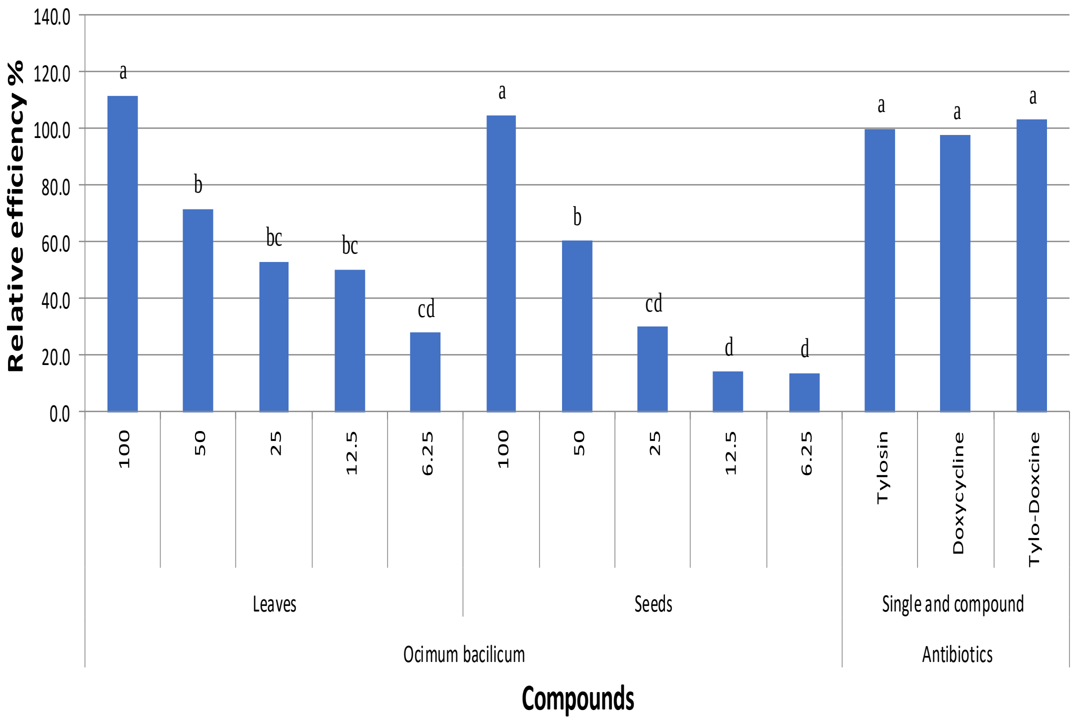

Fig. (1): Antibacterial activity (relative efficiency of Tylosin, %) of *Ocimum bacilicum* essential oil (at five concentrations) against *P. l. larvae* based on inhibition zones estimated by the disc diffusion method.

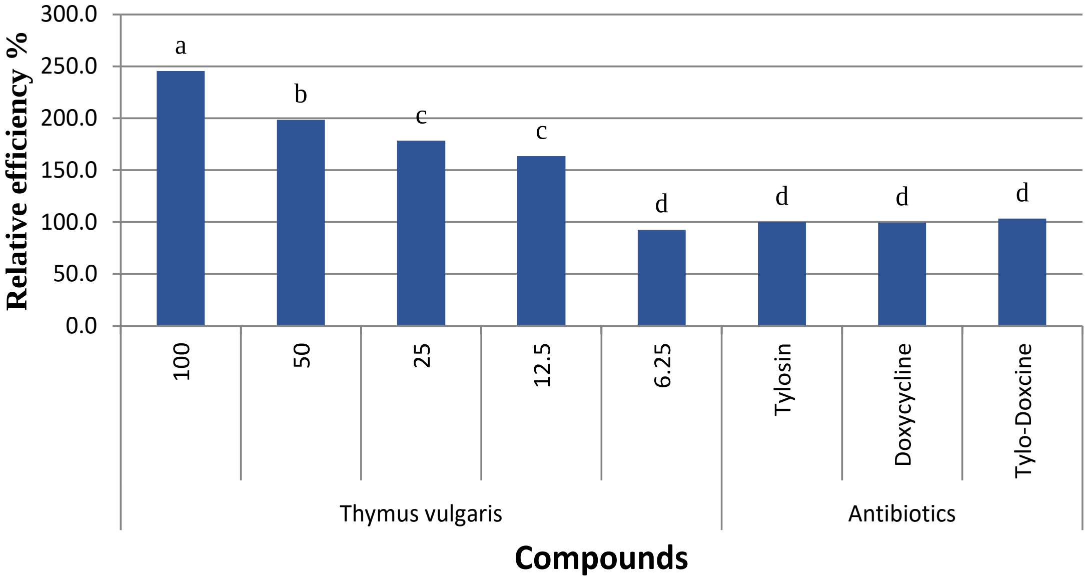

Fig. (2): Antibacterial activity (relative efficiency of Tylosin (%) of *Thymus vulgaris* essential oil against *P. l. larvae* based on inhibition zones estimated by the disc diffusion method.

Table (8): Antibacterial activity (inhibition zone, mm) of five concentrations of *Mentha piperita* and *M. pulegium* essential oils against *P. l. larvae* by the disc diffusion method and their relative efficiency (R.E.) percentages.

Plant essential oil		*Mentha piperita*		*Mentha pulegium*	
Parts / Antibiotics	**Concentrations**	**I. Z (mm) ± S. D.***	**R. E. (%) #**	**I. Z (mm) ± S. D.***	**R. E. (%) #**
Leaves + stem	100%	23.0 ± 0.5	122.8 a	18.2 ± 0.6	97.6 b
	50%	15.0 ± 0.0	83.4 c	12.2 ± 0.2	67.6 c
	25%	12.3 ± 0.2	69.3 cd	10.5 ± 0.0	59.0 c
	12.5%	9.7 ± 0.2	54.9 de	8.7 ± 0.2	49.1 d
	6.25%	8.5 ± 0.4	50.0 e	7.2 ± 0.2	28.4 d
Tylosin	0.2 g $	17.8 ± 1.0	100.0 b	17.8 ± 1.0	100.0 b
Doxycycline	0.2 g	18.4 ± 1.0	103.1 b	18.4 ± 1.0	103.1 ab
Tylo-Doxcine	0.2 g	18.8 ± 0.9	105.6 b	18.8 ± 0.9	105.6 a

*: I. Z (mm) ± S.D: Average of inhibition zone ± Standard deviation.

#: R. E (%): Average of relative efficiency, significance letters apply within column only

4. DISCUSSION

American foulbrood (AFB) is considered a serious bacterial disease to honey bee colonies. It is caused by *Paenibacillus larvae larvae* bacteria. For many years, certain antibiotics were used to control this disease.

Increasing reports of antibiotics resistance in the pathogen (Miyagi *et al*., 2000; Evans, 2003; Lodesani and Costa, 2005), contamination of bee products related to antibiotics use (Bogdanov, 2006; Martel *et al*., 2006) and negative impacts on honey bees (Peng *et al*., 1992) induced searching for safe alternative strategies for disease control (Alippi *et al*., 2005). Also, antibiotics are legally banned for use in beekeeping in several countries due to its potential contribution in the spread of CCD. Recently, considerable attention has been paid to develop safe alternative strategies for the control of AFB. Due to its contents of phenolic and terpenoid compounds having antimicrobial activities (Conner, 1993), essential oils (EOs) are considered the most important alternative to antibiotics. Several attempts have been made to investigate the *in vitro* antibacterial activity of plant EOs against *P. l. larvae* (Fuselli *et al*., 2008; Awad, 2011 and Santos *et al*., 2012). To investigate the antibacterial activity of potential essential oils, according to previous experiments, against the pathogen (Eid *et al*., 2019) when diluted, we designed and conducted different assays. Local isolate of *P. l. larvae* was tested for their sensitivity to five concentrations (100, 50, 25, 12.5 and 6.25 %) of potential essential oils in present experiments. Different parts of ten plants belonging to four families: Rutaceae, Myrtaceae, Anacardiaceae and Lamiaceae, were used to extract fifteen essential oils. The efficiencies, in inhibition the growth of *P. l. larvae* bacteria, of these crude oils and four mixtures of some of them beside their diluted concentrations were evaluated using disk diffusion assay.

Concerning the three plants belonging to Family Rutaceae, obtained results indicated that the efficiencies, in inhibiting *P. l. larvae* growth, of *C. limon* essential oil from leaves, peels and mixture of them (at tested concentrations) varied from weak to very high. The most effective one was the crude oil of leaves (inhibition zone of 34.2 mm with 213.5 % R.E). The mixture of crude oils occupied the 2nd place in inhibiting the bacteria (inhibition zone of 26.7 mm with 166.7 % R.E). The 3rd rank was occupied by leaves essential oil (at 50 % concentration) which exhibited antibacterial activity higher than that of Tylosin. Furthermore, the antibacterial activities against *P. l. larvae* of *C. reticulate* essential oil from leaves, peels and mixture of them (at tested concentrations) varied from weak to high, while those of *C. sinensis* essential oil from leaves varied from moderate to high. Regarding the two plants belonging to Family Myrtaceae, the efficiencies, in inhibiting the growth of pathogen, of *S. aromaticum* essential oil from petals (at tested concentrations) varied from moderate to high, while those of *E. camaldulensis* essential oil from leaves, seeds and mixture of them varied from weak to moderate. Although the effects at concentration of 100 % were moderate, dilutions were performed (50, 25, 12.5 and 6.25 %) because of the potentiality of *Eucalyptus camaldulensis* essential oils in the controlling many honey bee diseases. In addition, considering the four plants belonging to Family Lamiaceae, the antibacterial activities of *O. bacilicum* essential oil, regardless plant tissue or concentration, varied from weak to high. Moreover, *M. piperita* and *M. pulegium* essential oils from leaves plus stem varied from moderate to high, and from weak to moderate, in respect.

On the other hand, results of these experiments emphasized the high efficiency of the crude essential oil from *T. vulgaris* whole plant, beside its tested diluents, in inhibiting the growth of *P. l. larvae*. It was most effective treatment achieving inhibitory activity (inhibition zone of 47.5 mm with 245.5 % R.E.) almost 2.5-fold more than that exhibited by any control antibiotic. In addition, diluting the oil to be the half concentration significantly decreased its activity to be (inhibition zone of 36.3 mm with 198.4 % R.E.) approximately double that of any tested antibiotic. Moreover, the same trend was observed when the concentration decreased to be 25 or 12.5 %, which still exhibiting high activities (inhibition zones of 33.5 and 30.5 mm with 178.3 and 163.4 % R.E., respectively). With respect to *S. terebinthifolius* plant belonging to Family Anacardiaceae, the efficiencies of essential oils extracted from leaves and seeds, and mixture of them (at tested concentrations) varied from weak to moderate, except it at the lowest concentration (6.25 %) which had no effect.

Our findings of *Citrus limon* oils were in accordance with that of Badr Al-Deen *et al*. (2013) and Eid *et al*. (2019) who found that peels oil showed high antimicrobial activity especially against Gram-positive bacteria. This antimicrobial activity may be attributed to the components D-limonene, terpenes, sesquiterpene, oxygenated monoterpene, linalool, acid esters, aliphatic hydrocarbons and other unidentified hydrocarbons (Hasija *et al*., 2015). In addition, fruits and leaves of Citrus species contain various biologically active compounds as flavonoids (Othman *et al*., 2016). In addition, this study confirmed the potential usefulness of essential oils extracted from *Sayzygium aromaticum* (Hateet *et al*., 2013), as microbiostatic, antiseptic or as disinfectant agent. On the other hand, strong inhibitory effects of essential oils from plants belonging to Family Lamiaceae against all tested *P. l. larvae* strains and other pathogenic bacteria (Roussenovae, 2011; Boruga *et al*., 2014; Zaidi and Dahiya, 2015; and Eid *et al*., 2019). Finally, we could include that some essential oils extracted by hydro-distillation method revealed considerable antibacterial activity against *P. l. larvae in vitro* when tested by diffusion disc method as both of *Thymus vulgaris* whole plant and *Citrus limon* leaves. In general, the antibacterial activities of evaluated essential oil were concentration-dependent.

Authors' contributions

All authors contributed in conceptualization, methodology, software, validation, formal analysis investigation, resources, data curtain, writing the original draft preparation, writing, review, editing, supervision and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Competing interests

All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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النشاط المضاد للبكتيريا لمستخلصات زيوت طيارة ضد مسبب لمرض تعفن الحضنة الأمريكي (*Paenibacillus larvae larvae*)

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ملخص

تم اجراء الدراسة الحالية لبحث النشاط المضاد البكتيري معمليا لمستخلصات زيوت طيارة محتملة بخمسة تركيزات (100 ، 50 ، 25 ، 12.5 ، 6.25 %) ضد *Paenibacillus larvae larvae* (المسبب المرضي لتعفن الحضنة الأمريكي). تم استخدام أجزاء مختلفة من عشرة نباتات تتبع أربع عائلات نباتية (السذابية والآسية والبطمية والشفوية) في هذه التجربة لاستخلاص خمسة عشر زيت طيار منها. وتم تقييم فاعلية تلك الزيوت الخام بجانب أربعة مخاليط من بعضها في تثبيط نمو بكتيريا *P. l. larvae* وذلك بطريقة نشر القرص disc diffusion. وأشارت النتائج المتحصل عليها إلي أن فاعليات الزيوت الطيارة المستخلصة من أوراق وقشور ثمار الليمون *Citrus limon* ومخلوطهما (بالتركيزات المستخدمة) في تثبيط نمو بكتيريا *P. l. larvae* تباينت من الضعيفة إلي العالية جدا. وكان الزيت الطيار المستخلص من الأوراق هو الأكثر تأثيرا حيث أدي لمنطقة تثبيط مقدارها 34.2 مم وذلك بفاعلية نسبية مقدارها 213.5 % بالنسبة للتيلوزين. وبالنظر للأربعة نباتات التابعة للعائلة الشفوية أكدت النتائج الفاعلية العالية للزيت الطيار الخام المستخلص من نبات الزعتر كاملاً وكذلك لكل مخففاته المستخدمة في تثبيط نمو بكتيريا *P. l. larva*. وقد كان هذا الزيت الخام هو المعاملة الأعلي تأثيراً حيث كان له نشاط تثبيطي (منطقة تثبيط مقدارها 47.5 مم) بفاعلية نسبية مقدارها 245.5 %. كذلك تم ملاحظة نفس الاتجاه عند خفض التركيز إلي 50 أو 25 أو 12.5 % حيث ظل الزيت الطيار في إظهار نشاطات عالية (مناطق تثبيط مقدارها 36.5 ، 33.5 ، 30.5 مم بفاعلية نسبية مقدارها 198.4 ، 178.3 ، 163.4 % علي الترتيب). وعامة كانت النشاطات المضادة للبكتيريا للزيوت الطيارة التي تم تقييمها معتمدة علي التركيز.

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