Saudi Journal of Biological Sciences 24 (2017) 1001–1006

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

In vitro evaluation of the effects of some plant essential oils on *Ascosphaera apis*, the causative agent of Chalkbrood disease

Mohammad Javed Ansari [a,*], Ahmad Al-Ghamdi [a], Salma Usmani [b], Khalid Ali Khan [a], Abdulaziz S. Alqarni [c], Manpreet Kaur [d], Noori Al-Waili [e]

[a] Bee Research Chair, Department of Plant Protection, College of Food and Agriculture Sciences, King Saud University, PO Box 2460, Riyadh 11451, Saudi Arabia
[b] Department of Biochemistry, D.K.M. College for Women, Thiruvalluvar University, Vellore, Tamilnadu 632004, India
[c] Department of Plant Protection, College of Food and Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia
[d] Forest Botany Division, Forest Research Institute, Dehradun, India
[e] Waili Foundations for Science and Trading, New York, USA

ARTICLE INFO

Article history:
Received 3 April 2016
Accepted 6 April 2016
Available online 30 May 2016

Keywords:
Essential oils
Antifungal activity
Antibiotics
Ascosphaera apis
Chalkbrood disease
Biological control

ABSTRACT

Ascosphaera apis is one of the major fungal pathogens of honey bee broods and the causative agent of Chalkbrood disease. The factors responsible for the pathogenesis of Chalkbrood disease are still not fully understood, and the increasing resistance of *A. apis* to commonly used antifungal agents necessitates a search for new agents to control this disease. The *in vitro* antifungal activities of 27 plant essential oils against two isolates of *A. apis* (Aksu-4 and Aksu-9) were evaluated. Out of the 27 plant essential oils tested, 21 were found to be effective in killing both isolates of *A. apis*. Based on their minimum fungicidal concentration (MFC) values, the effective oils were grouped into three categories: highly effective, moderately effective and minimally effective. Mountain pepper oil, Kala Bhangra oil, spearmint oil, babuna oil, betel leaf oil, carrot seed oil, cumin seed oil and clove bud oil were highly effective, with MBC values between 50.0 μg/mL and 600.0 μg/mL. Mountain pepper was the most effective essential oil, with an MBC value of 50.0 μg/mL. Citral and caryophyllene containing oils were the most effective with MIC 50 ppm. The essential oils tested exhibited significant antimicrobial activities against both strains of *A. apis*, and they may contain compounds that could play an important role in the treatment or prevention of Chalkbrood disease of honeybee.

1. Introduction

The honeybee (*Apis mellifera*), is an important pollinator of various crops and plant species worldwide. The total annual global economic worth of pollination amounts to an estimated 153 billion euro, representing 10% of the value of the global agricultural production (Gallai et al., 2009). A mysterious decline in honeybee colonies has gained attention worldwide as they are threatened by various pathogens globally (van_Engelsdorp and Meixner, 2010). The most contagious and destructive diseases that affects honeybee honey brood is Chalkbrood, which is caused by the fungus *Ascosphaera apis* (Maassen ex Claussen) (Spiltoir, 1955). *A. apis* causes significant losses in terms of both bee numbers and colony productivity (Zaghloul et al., 2005). This disease is now found throughout the world, and there are indications that Chalkbrood incidence may be on the rise (Aronstein and Murray, 2010).

A. apis only produces sexual spores and is heterothallic, thus spores are only produced when mycelia of the two opposite mating types come together and fruiting bodies are formed (Aronstein et al., 2007). Honeybee larvae primarily get infected by ingesting sexual spores of *A. apis* with their food. Spores germinate in the lumen of the gut (Bailey and Ball, 1991). Germination of spores requires very specific conditions that are found in the larval gut environment (Bignell and Heath, 1985). Infected larvae rapidly reduce food consumption, and then stop eating altogether. Theantana and Chantawannakul (2008) recently identified several enzymes produced by *A. apis*, some of them implicated in assisting the pathogen in penetration of the peritrophic membrane of the bee larval midgut. Fungal spores present on all surfaces within

* Corresponding author.
E-mail address: mjavedansari@gmail.com (M.J. Ansari).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2016.04.016
1319-562X/

the beehive (Puerta et al., 1995), and remain viable for many years, providing a continual source of infection. In addition to environmental conditions, interaction between biotic factors such as differences in fungal strains and the genetic background of the bees may affect the incidence and severity of the disease (Flores et al., 2005).

A broad range of chemotherapeutic compounds have been tested for their ability to control Chalkbrood (Heath, 1982; Liu, 1991; Glinski and Chmielewski, 1996; Davis and Ward, 2003). Hornitzky (2001) listed chemicals that seemed promising for controlling fungal growth either in culture or in bee colonies. Unfortunately none of the tested compounds achieved the level of control required to fight the disease. Common problems associated with synthetic pesticides and antimicrobials 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 (Glinski and Chmielewski, 1996), and an increased rate of resistance of various *A. apis* strains. Furthermore, pesticides and antifungal chemicals residues present in honey represent a major human health hazard (Frazier et al., 2008). The discovery of new antibiotics to control Chalkbrood could lead to the emergence of additional resistant *A. apis* strains. Therefore, there is great interest in the investigation of alternative and efficient Chalkbrood-controlling substances.

Over the years, a number of alternative strategies have been developed and implemented to control Chalkbrood disease (Heath, 1982; Hornitzky, 2001). A broad range of chemotherapeutic compounds have been tested for their ability to control Chalkbrood (Heath, 1982; Liu, 1991; Glinski and Chmielewski, 1996; Davis and Ward, 2003). Hornitzky (2001) listed chemicals that seemed promising for controlling fungal growth either in culture or in bee colonies. Unfortunately none of the tested compounds achieved the level of control required to fight the disease (Hornitzky, 2001). There is increased interest in investigations into new effective Chalkbrood control methods. Essential oils (EOs) from plants, herbs and spices exhibit antimicrobial activity against fungal pathogens (Hayouni et al., 2008). This antimicrobial activity is mainly due to the presence of phenolic and terpenoid compounds, which have well-known antimicrobial activity (Conner, 1993). EOs and aqueous herbal extracts have been used as remedies for infection since ancient times (Ríos and Recio, 2005; Kaufman et al., 2006).

The current knowledge of the antagonistic effect of EOs against *A. apis* is limited. some attempts have been made to investigate the antifungal activity of plant EOs and natural plant-derived antimicrobial products against *A. apis* (Hornitzky, 2001; Larran et al., 2001; Dellacasa et al., 2003; Davis and Ward, 2003; Aronstein and Hayes, 2004; Mourad et al., 2005; Boudegga et al., 2010; Kloucek et al., 2012). EOs containing citral, geraniol and citronellal were reported to have the best inhibiting effect on fungal growth *in vitro* (Calderone et al., 1994; Davis and Ward, 2003). According to the pioneering work by Boudegga et al. (2010) and Eguaras et al. (2005), some EOs from *Armoracia rusticana*, *Thymus vulgaris*, *Cymbopogon flexuosus* and *Tagetes minuta* exhibit considerable antifungal activities against *A. apis*. These findings provide a novel mode of controlling honeybee disease.

The aim of this study was to evaluate the *in vitro* antimicrobial activities of EOs from 27 plant species against 2 wild strains of *A. apis* isolated from infected honeybee colonies using an agar disc diffusion assay. The minimal inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) values of these EOs were to screen for highly effective, moderately effective, minimally effective and ineffective EOs. Some of the EOs tested in this study exhibit antimicrobial activity against some honeybee bacterial pathogens (Ansari et al., 2016); however, little information about their antimicrobial activity against *A. apis* has been published. Therefore, the present study sought to investigate the activity of 27 different plant EOs against *A. apis*.

2. Materials and methods

2.1. Isolation of A. apis and culture conditions

Two isolates of chalkbrood fungus, *A. apis* (Aksu-4 and Aksu-9) was used in this study. These strains of *A. apis* were isolated from hybrid Carniolan honeybee (*Apis mellifera carnica*) infested brood with Chalkbrood symptoms at apiaries located at Bee research unit, King Saud University Riyadh and Dirab agriculture farm, Riyadh respectively. Since these aperies with known histories of Chalkbrood disease and did not used any chemical treatment for overcoming Chalkbrood disease. Black and white colour mummies, depending on whether or not ascospores are present, were collected in the year 2013 and 2014 from the aforesaid localities and used for isolation of the causal pathogen. Black (sporulating) and white (mycelia only) mummies were washed with sterile water, cut into small pieces (0.25–0.5 cm) and surface sterilized by dipping into 0.5% sodium hypochlorite solution for 3 min then rinsed for several times in sterile water. We scraped the outer and inner surfaces of disinfested brood samples with a sterile applicator stick and plated on sterile Petri dishes on Yeast-Glucose-Starch agar (YGPSA) medium (1% yeast extract, 0.2% glucose, 0.1 M KH_2PO_4, 1% soluble starch, 2% agar) containing ampicillin (100 μg/mL) and streptomycin (12 μg/mL) to exclude growth of both Gram-positive and Gram-negative bacteria, and incubated at 35 °C incubated under 6% CO_2 for 4 d. The fungi were sub cultured on the same medium until pure culture was established. After establishment of pure culture, cells were collected by centrifugation (5000×*g* for 10 min at 4 °C), washed twice with PBS (phosphate-buffered saline, pH 7.2), and finally re-suspended in PBS. The inoculum was prepared and adjusted to an inoculation level of 1.5×10^8 CFU/mL.

2.2. Morphological and molecular identification of fungal isolates

Fungal strains were morphologically identified at 10× and 40× magnification (*Axio Lab A1*, Carl Zeiss, Germany) using reference images in Anderson and Gibson (1998). The fungal isolates were also examined morphologically by scanning electron microscope (*Leo 435*, Cambridge, USA) and compared with model description of *A. apis* as described by Chorbiński (2003). Molecular identification was carried out by PCR and sequencing of the ITS1-5.8S-ITS2 ribosomal DNA region. The primers used in PCR were derived from conserved regions of the DNA sequences reported by James and Skinner (2005) for the 5.8S ribosomal DNA. The polymerase chain reaction (PCR) was carried out in a thermocycler (*Applied Biosystems*, Singapore) according to methods described by James and Skinner (2005) with some modifications. The reaction was conducted in a volume of 25-μl. The reaction mixture contained 2.5 μl of 10× PCR buffer (10 mM Tris–HCl – pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.5 μl of 10 mM dNTPs mix, 0.1 μl Taq DNA polymerase (5 U/μl), 0.5 μl of forward primer (5′-TGTCTGTGCGGCTAG GTG-3′) and 0.5 μl of reverse primer (5′-CCACTAGAAGTAAAT GATGGTTAGA-3′) at 20 μM each (Promega), 1 μl DNA template and 19.9 μl sterile deionized H_2O. Amplification conditions were 94 °C for 10 min; 30 cycles of 94 °C for 45 s min, 62 °C for 45 s, 72 °C for 1 min; 72 °C for 5 min as the final extension. The PCR products were separated by electrophoresis on 1.5% agarose gels, and the band patterns were visualized using ethidium bromide staining.

2.3. Chemicals and plant essential oils

The EOs used in this study are summarized in Table 1, together with their main chemical constituents. Almond (*Prunus glandulosa* L.) and Clove bud (*Syzygium aromaticum* (L.) Merrill & Perry) oils were purchased from Himedia chemicals (Himedia, India), and the oils of cardamom (*Elettaria cardamomum* (L.) Maton), khus (*Vetiveria zizanoides* (L.) Nash) and carrot seed (*Daucus carota* L.) were purchased from Imperial Extracts (Ernakulam, India). Ajwain (*Trachyspermum ammi* Sprague), mountain pepper (*Litsea cubeba* Pers.), nutmeg (*Myristica fragrans* Gronov.), Rosewood (*Aniba rosaeodora* Duke) and spearmint (*Mentha spicata* L.) oils were purchased from Mother Herbs P (Ltd.), New Delhi, India. Linseed (*Linum usitatissimum* L.), betel leaf (*Piper betle* L.), Bottle brush (*Callistemon citrinus* (Curtis) Skeels), babuna (*Matricaria chamomilla* L.), Clove basil (*Ocimum gratissimum* L.), Cumin seed (*Cuminum cyminum* L.), dill (*Anethum graveolens* L. (dill)), Fennel seed (*Foeniculum vulgare* Mill.), Garland Daisy (*Glebionis coronaria* (L.) Cass. Ex Spach), garlic (*Allium sativum* L.), geranium rose (*Pelargonium graveolens* L.), Hempseed (*Cannabis sativa* L.), jojoba (*Simmondsia chinensis* (Link) C. K. Schneid.), Kala Bhangra (*Croton bonplandianus* Baill.), Palmrosa (*Cymbopogon martinii* (Roxb.) Wats.), Stone apple (*Aegle marmelos* (L.) Correa ex Roxb.) and wheat germ (*Triticum vulgare* L.) oils were obtained by the steam distillation method (Cassel and Vargarss, 2006). Chlorimidazole was purchased from Sigma (Sigma–Aldrich, India).

2.4. Screening of anti-fungal activity using Halo (growth inhibition zone) assay

The Halo (diameter of growth inhibition) of 27 plants EOs was tested using Well diffusion assay (Dellacasa et al., 2003). Two wild isolated fungal strains were used in this study. YGPSA medium plates were prepared and used for culture and storage of fungal strains. The inocula (mycelium) were excised from the border of fresh 7 day old growing *A. apis* culture with agar by a 6 mm diam-

Table 1
Essential oils and their main chemical constituents.

Plant essential oils	Botanical name	Family	Main constituents (over 10%)
Ajwain oil	*Trachyspermum ammi* Sprague	Apiaceae	Thymol (43.7%)
Almond oil	*Prunus glandulosa* L.	Rosaceae	α-Tocopherol (24.2%)
Babuna oil	*Matricaria chamomilla* L.	Asteraceae	α-Bisabolol (56.9%)
Betel leaf oil	*Piper betle* L.	Piperaceae	Safrole (24.75%), Eugenol (18.9%)
Bottle brush oil	*Callistemon citrinus* (Curtis) Skeels	Myrtaceae	1,8-Cineole (43.45%), α-Pinene (12.8%)
Cardamom oil	*Elettaria cardamomum* (L.) Maton	Zingiberaceae	α-Terpenyl acetate (46.0%), 1,8-Cineole (27.7%)
Carrot seed oil	*Daucus carota* L. subsp. halophilus	Apiaceae	Carotol (67.53%)
Clove basil oil	*Ocimum gratissimum* L.	Lamiaceae	Eugenol (41%), 1,8-Cineole (11%)
Clove bud oil	*Syzygium aromaticum* (L.) Merrill & Perry	Myrtaceae	Eugenol (74.3%), Eugenyl acetate (10.78%)
Cumin seed oil	*Cuminum cyminum* L.	Apiaceae	Cuminal (32.82%), Safranal (17.83%)
Dill oil	*Anethum graveolens* L. (dill)	Apiaceae	R-(-)-Carvone (38.89%), Apiol (30.81%), Limonene (15.93%)
Fennel seed oil	*Foeniculum vulgare* Mill.	Apiaceae	Trans-anethole (61.32%), Estragole (11.91%)
Garland daisy oil	*Glebionis coronaria* (L.) Cass. Ex Spach	Asteraceae	α-Humulene (26.8%), Camphor (12.28%), γ-Curcumene (11.82%)
Garlic oil	*Allium sativum* L.	Amaryllidaceae	Allyl trisulfide (31.68%) Diallyl disulfide (19.11%)
Geranium rose oil	*Pelargonium graveolens* L.	Geraniaceae	Citronellol (30.4%), Geraniol (17.8%)
Hempseed oil	*Cannabis sativa* L.	Cannabaceae	(E)-Caryophyllene (22.26%), Limonene (14.2%)
Jojoba oil	*Simmondsia chinensis* (Link) C. K. Schneid.	Simmondciaceae	9-Octadecen-1-ol (41.35%), 1,21-Docosadiene (20.65%)
Kala Bhangra oil	*Croton bonplandianus* Baill.	Euphorbiaceae	β-Caryophyllene (16.7%), Germacrene D (14.7%)
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%)
Nutmeg oil	*Myristica fragrans* Gronov.	Myristicaceae	β-Pinene (11.69%), α-pinene (10.06%), Sabinene (41.7)
Palmrosa oil	*Cymbopogon martinii* (Roxb.) Wats.	Poaceae	Geraniol (72%), Geranyl acetate (16%)
Rosewood oil	*Aniba rosaeodora* Duke	Lauraceae	Linalool (77.56%)
Spearmint oil	*Mentha spicata* L.	Lamiaceae	Carvone (65.10%), D-limonene (16.11%)
Stone apple oil	*Aegle marmelos* (L.) Correa ex Roxb.	Rutaceae	α-Phellandrene (39.85%), Limonene (27.54%)
Wheatgerm oil	*Triticum vulgare* L.	Poaceae	1-Eicosene (13.77%), Hexadecyl acetate (12.19%)

eter sterile cork borer. These excised discs were plated in the middle of each Petri dish. A well of a same dimension filled with 3.5 µl of pure EO and laid at a distance of 40 mm. Petri discs were then incubated at 30 °C, in darkness in incubator using aerobic conditions and the measurements were made after eight days. Halo around EO well was measured at after 5 days interval. EOs failed to inhibit the growth of *A. apis* (No Halo = No diameter of growth inhibition around well) after 5 days of incubation and were considered to be ineffective in preventing growth of this fungus.

2.5. Determination of the minimum inhibitory concentration (MICs) of plant essential oils

To determine the MIC of the effective plant EOs, 13 different doses of EOs (0, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 2000 µg/mL) were prepared in liquefied melted YGPSA medium (45 °C) before pouring into petri dishes. YGPSA agar medium, followed by manual rotation of the Erlenmeyer flask to disperse the oil in the medium. About 20 ml of the medium was poured into glass Petri-dishes (9 cm × 1.5 cm). 100 µl of spore suspension containing approximately 5×10^6 spores of both isolates of *A. apis* was added to the media plates containing different concentration of EOs. The spore suspension was spread evenly over the surface of the agar using sterile disposable cotton swab. All plates were incubated at 30 °C for 7 days and were examined morphologically for the presence and absence of the growth of *A. apis* colonies growth. All experiments were carried out in triplicate. The MIC was determined to be the lowest concentration of EO that resulted in the inhibition of the visible growth of *A. apis* after a seven days incubation, compared with the growth control (free of any plant EOs).

2.6. Determination of the minimum fungicidal concentration (MFC) of plant essential oils

EOs exhibiting inhibitory effect on the growth of *A. apis* were examined for minimum fungicidal concentration (MFC). To determine the MFC of the effective plant EOs, YGPSA agar medium with different concentrations of EOs (0, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 2000 µg/mL) were prepared by adding the appropriate quantity of essential oil/compound to the melted medium, followed by manual rotation of the Erlenmeyer flask to disperse the oil in the medium. About 20 ml of the medium was poured into glass Petri-dishes (9 cm × 1.5 cm). 100 µl of spore suspension containing approximately 5×10^6 spores of both isolates of *A. apis* was added to the media plates containing different concentrations of EOs. The spore suspension was spread evenly over the surface of the agar using sterile disposable cotton swab. All plates were incubated at 30 °C for 7 days and were examined morphologically for the presence and absence of the growth of *A. apis* colonies growth. All experiments were carried out in triplicate. Control plates (without EO) were inoculated following the same procedure. Minimal fungicidal concentration (MFC) was defined as the lowest concentration of EO that inhibits $\geqslant$99.9% of the final inoculum. The experiment was conducted with four repetitions for each EO, considering each petri dish as an experimental unity. Standard antibiotics Chlorimidazole (Sigma) dissolved in dimethyl sulfoxide (DMSO) was used as positive control.

2.7. Statistical analysis

All experiments were performed in triplicate, and the results are expressed as 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.

3. Results

3.1. Minimum inhibitory concentration (MICs) of plant essential oils

Out of the 27 essential oils tested, 20 exhibited antibacterial activity against two strains of *A. apis*, with halo ranging from 6.8 mm to 1.4 mm (Table 2). The halo produced by mountain pepper oil, Kala Bhangra oil, spearmint oil, babuna oil, betel leaf oil, carrot seed oil, cumin seed oil and clove bud oil for Aksu-4 were 6.8 ± 0.6, 5.6 ± 0.8, 5.2 ± 0.7, 4.8 ± 0.7, 4.8 ± 0.5, 4.6 ± 0.4, 4.4 ± 0.8 and 4.3 ± 0 mm, and for Aksu-9 were 6.6 ± 0.7, 5.4 ± 0.7, 5.3 ± 0.6, 4.7 ± 0.7, 4.6 ± 0.4, 4.4 ± 0.9, 4.6 ± 0.8 and 4.5 ± 8 respectively. The halo produced by clove basil, garlic, stone apple, geranium rose, bottle brush, nutmeg, palmrosa, cardamom and cennel seed oils ranged from 3.9 ± 0.3 mm to 1.7 ± 0.6 mm for both isolates, whereas those produced by ajwain, dill, hempseed and garland daisy oil ranged from 1.6 ± 0 mm to 1.4 ± 0 mm. The other oils tested—khus, rosewood, wheat germ, almond, jojoba and linseed oil—were found to be ineffective. The zone of inhibition produced by the Chlorimidazol was 8.0 mm, which was little more than to the halo produced by mountain pepper oil.

The MICs of the 21 effective EOs and Chlorimidazol against both isolates of *A. apis* were also determined. The plant oils showed concentration-dependent growth inhibition of the *A. apis* Chlorimidazol (25 µg/mL) completely inhibited the growth of *A. apis* isolates. Mountain pepper oil was the most effective, with complete inhibition occurring at 6.8 ± 0.6 and 6.6 ± 0.7 µg/mL for Aksu-4 and Aksu-9 respectively. Eight EOs completely inhibited the growth of *A. apis* at concentrations ranging from 50 µg/mL to 400 µg/mL. Nine EOs were inhibitory at concentrations between 500 µg/mL and 700 µg/mL, and the other four EOs were inhibitory at concentrations ranging from 900 µg/mL to 1000 µg/mL. The MIC of Chlorimidazol was 8.0 ± 1.6 and 8.0 ± 1.4 µg/mL for Aksu-4 and Aksu-9 respectively (Table 2).

3.2. Minimum fungicidal concentration (MFC) of plant essential oils

The MFC is the lowest concentration of EO that results in the death of 99.9% of the fungal inoculum. The MFC values for the EOs tested were greater than their respective MIC values. Based on the MFC values, effective EOs were placed into three categories: EOs having MFC values of 50–500 µg/mL were considered to be highly effective, EOs with MFC values ranging from 500 to 1000 µg/mL were considered to be moderately effective, and EOs with an MBC between 1000 and 2000 µg/mL were considered to be minimally effective. Chlorimidazol was used as a standard, and its MBC was 35 µg/mL. Mountain pepper oil, Kala Bhangra oil, spearmint oil, babuna oil, betel leaf oil, carrot seed oil, cumin seed oil and clove bud oil were highly effective, with fungicidal values ranging from 50 µg/mL to 600 µg/mL. Nine oils (clove basil, garlic, stone apple, geranium rose, bottle brush, nutmeg, palmrosa, cardamom and fennel seed oils) were moderately effective, with MBC values ranging from 700 to 1000 µg/mL. Four oils were less effective, with MBC values from 1000 to 2000 µg/mL. The other six plant oils were not effective, even at a concentration of $\geqslant$2000 µg/mL (Table 2).

4. Discussion

Since ancient times, EOs have been used for domestic and therapeutic purposes; these oils possess broad-spectrum antimicrobial properties. EOs have been selectively used to treat various microbial infections (Deans, 1991; Hammer et al., 1998; Hili et al., 1997). The antimicrobial properties of EOs suggest that these substances could be used to control *A. apis*. Recently, a few studies

Table 2
Classification and antifungal activity of selected plant oils against *A. apis*.

Group	Plant oils	Botanical name	Mean Halo[a] (mm)		MIC (μg/mL)		MFC (μg/mL)	
			Aksu-4	Aksu-9	Aksu-4	Aksu-9	Aksu-4	Aksu-9
Most effective	Mountain pepper oil	*Litsea cubeba* Pers.	6.8 ± 0.6	6.6 ± 0.7	50	50	50	50
	Kala Bhangra oil	*Croton bonplandianus* Baill.	5.6 ± 0.8	5.4 ± 0.7	50	50	100	100
	Spearmint oil	*Mentha spicata* L.	5.2 ± 0.7	5.3 ± 0.6	100	100	200	200
	Babuna oil	*Matricaria chamomilla* L.	4.8 ± 0.7	4.7 ± 0.7	200	200	200	200
	Betel leaf oil	*Piper betle* L.	4.8 ± 0.5	4.6 ± 0.4	300	300	400	400
	Carrot seed oil	*Daucus carota* L. subsp. halophilus	4.6 ± 0.4	4.4 ± 0.9	300	300	400	400
	Cumin seed oil	*Cuminum cyminum* L.	4.4 ± 0.8	4.6 ± 0.8	400	500	600	600
	Clove bud oil	*Syzygium aromaticum* (L.) Merrill & Perry	4.3 ± 0	4.5 ± 8	400	400	600	500
Moderately effective	Clove basil oil	*Ocimum gratissimum* L.	3.8 ± 0.4	3.9 ± 0.3	500	500	700	700
	Garlic oil	*Allium sativum* L.	3.6 ± 0.7	3.7 ± 0.8	500	500	700	700
	Stone apple oil	*Aegle marmelos* (L.) Correa ex Roxb.	3.2 ± 0	3.4 ± 2	500	500	700	800
	Geranium rose oil	*Pelargonium graveolens* L.	3.6 ± 0.2	3.3 ± 0.2	600	600	800	800
	Bottle brush oil	*Callistemon citrinus* (Curtis) Skeels	3.0 ± 0.2	3.0 ± 0.2	600	600	800	800
	Nutmeg oil	*Myristica fragrans* Gronov.	2.8 ± 0	2.8 ± 0.3	600	600	800	800
	Palmrosa oil	*Cymbopogon martini* (Roxb.) Wats.	2.2 ± 0	2.2 ± 0.4	700	700	900	900
	Cardamom oil	*Elettaria cardamomum* (L.) Maton	1.9 ± 0	1.8 ± 0	700	700	900	900
	Fennel seed oil	*Foeniculum vulgare* Mill.	1.7 ± 0.8	1.7 ± 0.6	700	700	900	1000
Minimally effective	Ajwain oil	*Trachyspermum ammi* Link	1.5 ± 0	1.6 ± 0	900	900	1000	1000
	Dill oil	*Anethum graveolens* L.	1.4 ± 0	1.4 ± 0	900	900	1500	1500
	Hempseed oil	*Cannabis sativa* L.	1.4 ± 0	1.3 ± 0	900	900	1500	1500
	Garland Daisy oil	*Glebionis coronaria* (L.) Cass. Ex Spach	1.5 ± 0	1.4 ± 0	1000	1000	2000	2000
Non-effective	Khus oil	*Vetiveria zizanoides* (L.) Nash	0 ± 0	0 ± 0	⩾2000	⩾2000	–	–
	Rosewood oil	*Aniba rosaeodora* Duke	0 ± 0	0 ± 0	⩾2000	⩾2000	–	–
	Wheatgerm oil	*Triticum vulgare* L.	0 ± 0	0 ± 0	⩾2000	⩾2000	–	–
	Almond oil	*Prunus glandulosa*	0 ± 0	0 ± 0	⩾2000	⩾2000	–	–
	Jojoba oil	*Simmondsia chinensis* (Link) C. K. Schneid.	0 ± 0	0 ± 0	⩾2000	⩾2000	–	–
	Linseed oil	*Linum usitatissimum* L.	0 ± 0	0 ± 0	⩾2000	⩾2000	–	–
	Chlorimidazol*		8.0 ± 1.6	8.0 ± 1.4	25	25	35	35
	Growth control (YGPS broth + propylene glycol)		0 ± 0	0 ± 0	–		–	
	Sterility control (YGPS broth + propylene glycol + test plant oil)		0 ± 0	0 ± 0	–		–	

[a] Halo = diameter of growth inhibition; MIC = minimum inhibitory concentration; MFC = minimum fungicidal concentration.

have demonstrated the activity of EOs against honeybee pathogens. Natural antibiotics based on 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. Thymol and some chemicals (sodium propionate and sorbic acid) continue to be the drugs of choice for the treatment of Chalkbrood infections, Thymol is very harmful if it comes into contact with the skin and is also corrosive. It should not be used during the honey flow and also slows down the Queen's laying of eggs (Chaieb et al., 2011; Taber et al., 1975).

We studied the *in vitro* efficacy of 27 EOs against two isolates of *A. apis* (Table 2). Out of the 27 selected EOs, 21 were found to be effective. Our findings demonstrate that these EOs might not only have the potential to control *A. apis* infection but may also represent possible alternatives to the use of thymol and some antibiotics to control Chalkbrood. Our data (Table 2) clearly demonstrate that most of the EOs used inhibited the growth of *A. apis*, which is in contrast to the results of earlier studies on EOs by Colin et al., 1989; Calderone et al., 1994; Larran et al., 2001. *In vitro* activity showed that citral and geraniol inhibited the vegetative growth of *A. apis* (Gochnauer and Margetts, 1979). Citral rich EOs of *L. cubeba* Pers, showed fungistatic activity as well as fungicide activity when assayed *in vitro* against bee pathogens *A. apis*, and promises new possibilities for effective treatment of apiaries. Similar results were obtained by Dellacasa et al. (2003), who studied the sensibility of this Chalkbrood causing pathogen towards *Aloysia gratissima* EO, rich in β-caryophyllene and germacrene. In our study, the second most effective EO was β-caryophyllene and germacrene rich EO of *C. bonplandianus*. The largest halo was obtained using mountain pepper oil (6.8 ± 0.6 mm against Aksu-4 isolate), and the lowest was obtained using Hempseed oil (1.3 ± 0 mm against Aksu-9 isolate). Therefore, mountain pepper oil has high antifungal activity against *A. apis*.

Citral rich EOs has medicinal value and has been used in the treatment of various fungal infections. It has been reported that citral rich EOs possess *in vitro* antifungal activity against *A. apis* (Davis and Ward, 2003). The antifungal activity of mountain pepper oil is due to the presence of citral (Ansari et al., 2016). In addition, Kala Bhangra is recognized as an aromatic and medicinal plant. Kala Bhangra oil is commonly used to control some fungal diseases. The antimicrobial activity of lemongrass oil is due to the presence of β-caryophyllene and germacrene compound (Jeeshna et al., 2011). Spearmint and babuna oil, which is rich in Carvone and α-bisabolol, also has strong effects against some pathogens (Tolouee et al., 2010; Soković et al., 2009). The antagonistic effects of EOs (lemon grass oil, eucalyptus oil and tea tree oil) containing citral against *A. apis* have been reported (Davis and Ward, 2003). Citral is present in some commercial products used to control the *A. apis* (Gochnauer and Margetts, 1979). In this study, we found that the majority of the EOs tested were effective and showed anti-*A. apis* activity at low concentrations. The *A. apis*

strain was highly susceptible to mountain pepper oil (*L. cubeba* Pers), Kala Bhangra oil (*C. bonplandianus* Baill.) and spearmint oil (*M. spicata* L.). Our findings are supported by the findings of Ansari et al. (2016), who reported the high antimicrobial activity of EOs from citral containing EOs. Gogoi et al. (1997) has also reported the use of mountain pepper oil against some fungi. clotrimazole is known to be effective against *A. apis* and is the drug of choice to treat Chalkbrood infection, despite the emerging resistance of *A. apis* to this antibiotic (Wolski et al., 1996). Little information is available about the mode of action of natural substances that inhibit *A. apis* growth.

5. Conclusion

In conclusion, the results presented in this paper demonstrate that most of the selected EOs have potential antifungal activities. Mountain pepper, Kala Bhangra and spearmint oils are fungistatic against *A. apis*. The use of non-toxic natural compounds could represent a natural alternative to the use of synthetic antibiotics in the control of Chalkbrood, which should therefore reduce antibiotic resistance and the levels of antibiotic residues. Further research must be conducted on these EOs to isolate the active ingredients that kill *A. apis.*

Acknowledgment

The project was financially supported by King Saud University, Vice Deanship of Research Chairs.

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