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In vitro, evaluation effect of *Punica granatum, Artemisia herba-alba,* and *Callistemon viminalis* extracts against chalkbrood disease

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Abstract

Chalkbrood is one of the most dangerous diseases of the honeybees, *Apis mellifera*. It causes a significant reduction in brood and honey production thus, leading to an economic loss in apiculture. In present study, the pathogenic fungus was isolated from infected larvae in Northern Governorate-Palestine and identified based on morphological and cultural characters as *Ascosphaera apis*.

Crude aqueous extracts of three different plant species including *Punica granatum, Artemisia herba-alba,* and *Callistemon viminalis,* were evaluated *in vitro* for their antifungal activities against *A. apis*. Results showed that extract of *P. granatum* was the most active one among all. The *C. viminalis* demonstrated a moderate activity with MICs of 5%. The remaining extract of, *A. herba-alba,* was totally inactive against *A. apis*. For further evaluation of the antifungal properties of the different plant extracts, the radial mycelial growth inhibition test was also performed on agar media supplemented by plant extracts to achieve different concentrations of 5%, 10% and 20%. The results showed that the average radial mycelia growth of *A. apis* was significantly reduced by *P. granatum* and *C. Viminalis* extracts ($P < 0.05$). Contrary to this, the remaining extract of *A. herba-alba,* was found to have weak antifungal activities at all concentrations tested. In order to assess the antifungal effect of the *P. granatum* extract on *A. apis,* the growth of profile for the fungus was followed at extract free agar media after incubation at different concentrations of *P. granatum* extract.

The results found that the extract of *P. granatum* flower exhibits a fungistatic effect because it causes changes on the normal growth profile of *A. apis* at the different concentrations tested (5%, 10% and 20%).

Since the extract of *P. granatum* flower was proved to be the most effective *in vitro* against *A. apis*, its toxicity to worker bees was evaluated. The results of the toxicity test showed that, the flower extract of *P. granatum* was not lethal to adult workers at low concentrations tested since the a cumulative mortality percentages were 4.4%, 1.1 % and 3.6% at 0% 12.5% and 25% concentrations respectively.

In conclusion, results from these findings suggest that the aqueous extract of *P. granatum* flower may be used as natural antifungal agents to inhibit growth of *A. apis*. These findings however need to be progressed to field applications to evaluate the efficacy of the most active antifungal plant extract identified in this study against the causative agent of chalkbrood disease in an apiary system.

Keywords: MIC, plant extracts, fungistatic, *ascosphaera apis*, *apis mellifera*, *punica granatum, artemisia herba-alba, callistemon viminalis,* chalkbrood

Introduction

Honeybees (*Apis mellifera* L.) are one of the most well-known, popular and economically beneficial insect on earth (Delaplane, 2010) [7].

Palestine had approximately 66,733 honeybee colonies in 2008 (PCBS, 2008) [19]. Through the years 2007-2008, Gaza Strip has produced 46 tons of honey.

Uncontrolled and heavy use of pesticides in Gaza Strip (Safi *et al.*, 2000) [20] may contribute to obstruction in the apiculture.

This beneficial insect however, may attacked during the different stages of its life cycle by a number of pathogens like bacterial, fungal, viral, and parasites (Delaplane, 2010) [7]. Some of these pathogens may affect adult bees, while others, which are called as brood diseases, affect the immature stages of the bees such as larva and pupae stages (Palmer-Jones, 1967) [18].

Beekeepers in Gaza strip declared, that many of these diseases were reported in Gaza strip since 1980s (Lord, 1994) [14].

Chalkbrood is a common fungal disease of the honeybee (*A. mellifera*) brood. It is caused by the spore forming fungi *Ascosphaera apis* (Maassen ex Claussen) Spiltoir and Olive.

Infection of the larvae mainly occur when spores within the larval food, provided by adult worker bees, are ingested, but can be also occurred as a result of growth of the fungus through the cuticle. Since larvae are the most susceptible stage throughout bees. Life cycle, infected colonies can be easily recognized by the presence of white and/or black mummified larvae. As *A. apis* is heterothallic fungi, diseased larvae are white if a single strain is successful in colonizing a larva (Christensen and Gilliam, 1983) [5]. On the other hand, diseased larvae are black due to the presence of spore producing fruiting bodies that are brown to black when mature (Anderson *et al.*, 1998) [1] and occurs when a diseased larva is simultaneously infected with both + and -strains of *A. apis* that have mated (Gilliam *et al.*, 1978; Flores *et al.*, 1996; Moeller and Williams, 2006) [11, 10, 16]. Although chalkbrood is not usually fatal to honeybee colonies it can cause considerable losses in both bee numbers and colony productivity (Bailey,

1963) [2], thus causing high economic losses through reduced honey production.

The development of chalkbrood disease is usually connected with the prevalence of the infectious materials (fungal spores) in honeybee colonies and in the hive environment (Flores and Gutierrez, 1997) [9].

Extracts isolated from several plants have been proved to have antifungal (Satish *et al.,* 2007) [21], antibacterial (Saxena and Gomber, 2006) [22], anti-inflammatory (Schinella *et al.,* 2002) [23], and antioxidant activities (El-Massry *et al.,* 2002; schinella *et al.,* 2002) [23].

(Chantawannakul *et. al.* 2005) [3], has demonstrated an antifungal action of the aqueous extracts of some medicinal plants such as cinnamon and betel piper against *A. apis*.

Taking into consideration the importance and the safe of naturally occurring compounds found in most plants, the use of their extracts is still of great interest as possible antifungal agent fundamentally active against the chalkbrood disease.

Therefore, the purpose of the present study was to evaluate *in vitro* the antifungal activities of the crude extracts of three plant species including *Punica granatum, Artemisia herba-alba, and Callistemon viminalis,* against *A. apis*, the causative agent of chalkbrood disease in honeybee larva. The plants were mainly selected on base of their ethno-medicinal properties, where they showed antimicrobial activity when tested against bacteria, fungi and yeasts. Furthermore, these plant species could be easily found in the studied area.

Materials and Methods

Fungus isolation and identification

Larvae showing characteristic symptoms of chalkbrood disease (white mummified larvae) were collected from naturally infected hives from a local apiary at Beit Lahia city, Northern Governorate, Palestine. This was done for isolation and identification of *A. apis* to be used throughout the study.

The isolation and cultivation technique followed here was previously described by (Davis *et al. 2003*) [6]. Mummified larvae were dabbed onto the agar surface at various points around the PDA-0.4 YE plate. The plates were incubated at 30ºC in a biological incubator.

For identification of fungus, purified cultures were examined macroscopically and microscopically depending on their morphological traits, mycelium growth, colony texture and ascospore production.

Preparation of Plant material and plant extracts

The selected parts of different plants: *Artemisia herba-alba* whole plant, *Callistemon viminalis* Leaves, *Punica granatum* flowers. Plant parts were shade dried at room temperature (20-25ºC) for 7 days. Plant powders (20 g) were extracted by soaking overnight in 80 ml distilled water i.e. 20% (W/V). The mixture was boiled and filtered through a piece of cheesecloth gauze. The filtrates were centrifuged at 3000 rpm for 5 min to remove plant debris. The supernatants were sterilized by filtration through sterilized, disposable filter unit with a pore size of 0.45µm into sterile cups. All extracts were preserved at 4ºC for further investigation. This preparation is designated as 100% crude plant extract and used to examine their effects on A. apis.

Determination of the Minimum Inhibitory Concentration (MIC)

Crude extracts of the different plants were screened in order to test their antifungal activity, using the Minimum Inhibitory Concentration (MIC) method on PDA+0.4 y media as described by (Liu *et al.* 1991) [13].

The lowest concentration of plant extract that inhibit visible growth of fungi on the agar plate was considered as the minimal inhibitory concentration (Hornitzky, 2001; Davis and Ward, 2003) [12, 6].

Preparation of spore suspension

Spore suspension was obtained by washing the ascospores that formed on the surface of plates with 5-10ml of 0.01% sterile Tween 80. The suspension was collected in a sterile 100ml Erlenmeyer flask. The density of spore suspension was adjusted a final concentration of approximately 50×10^6 spore/mL.

Preparation of plant extracts media

Plant extracts were separately incorporated into molten PDA+0.4 Y media to obtain final concentrations of 0.625, 1.25, 2.5, 5, 10 and 20%.

Inoculation and incubation of plates

After solidification, equal volumes (100 μL) of spore suspension containing approximately 5×10^5 spores of *A. apis* was inoculated at the surface of agar plates.

All plates were incubated at 30ºC for 8 days. Plates were visually inspected for the presence of growth of *A. apis* colonies. The lowest concentration at which no visible growth of *A. apis* was observed is considered as the MIC of that extract. Control plates free of any extract were also prepared.

Evaluation of antifungal activity of plant extracts by radial growth

After solidification, wells of 5 mm diameter were made in the center of agar medium with a sterile glass pipette. The control samples were PDA+0.4 Y medium without plant extract. Two perpendicular lines passing by the center of the dish and intersecting directly above the center of the hollow were drawn on the cover of the Petri dish.

Preparation of the inoculums

Five mm diameter agar discs covered with the fungus mycelium were cut out from the stock plates and used as inocula for the bioassay.

The discs were aseptically transferred and placed in the central well of the dish. Plates incubated in a dark at 30°C.

The radial mycelial growth for all treatments and control were measured by averaging the length of the four radii (mm) of the mycelium ring from the center of the inoculum disc to the outer growing edge of the mycelia along the two perpendicular lines that had been previously drawn on the cover of each plate. Measurements were determined every 24 hr for 4 days.

The percentage of inhibition of fungi growth was also calculated with respect to the control using the following formula (Nwachukwu and Umechuruba 2001).

Inhibition Percentage (%) $=\frac{rc-rt}{rc} \times 100$

Where;
rc = Average radius of the fungi grown in the control (mm).
rt = Average radius of the fungi grown in the extract (mm)
There were four replicates for each concentration and seven replicates for the control.
Plants showing best antifungal activity against *A. apis* were chosen for further analysis.

Statistical Analysis and calculations
Antifungal activities of plant extracts, expressed in terms of radial growth using descriptive statistics, the mean and standard deviation of radial growth of the fungus.
The effect of the different concentrations of plant extracts on fungal growth was analyzed by one-way analysis of variance (one-way ANOVA) and the significance of the differences between means was determined by using the Tukey honest significant difference (HSD) test at 5% level.

Evaluation of the toxicity of *P. granatum* extract on *A. mellifera*
The objective of this experiment was to assess the oral toxicity of *P. granatum* extract on adult honeybee workers to evaluate the potential of this extract as safe effective treatment for chalkbrood disease in the colony.

Procedure
i) Collection and housing of bees
An average of 350±20 adult worker bees, were brushed into each cage (140 × 140 × 70 mm) with a nylon mesh on the walls for air circulation (Figure 1). Every cage was equipped with an artificial bee feeder made of 9 × 3 cm plastic cylinder fixed on the floor at the corner of the cage.

Fig 1: Cages used in evaluation the effect of *P. granatum* extract on honeybees (140 × 140 × 70 mm dimension)

ii) Feeding conditions
Four different concentrations of pomegranate extract 12.5, 25, 50 and 100% were
prepared in distilled water. Sugar (sucrose) was dissolved in each concentration to make 50% solution (50% w/v sucrose in pomegranate extract solution). The control was composed from 50% sucrose solution only.
Before the assays, the bees were starved for 2 hr. Feeders were then filled with 10 ml of the target concentrations and bees were allowed to feed. After 24 hr of starting, feeders were removed and the amount of the syrup consumed by bees in each cage was determined. Bees were then fed on 50% (w/v), freshly prepared sugar syrup *ad libitum* only.

iii) Bee's mortality
Immobile bees or those that fallen down at the floor of the cage and showed no response to mechanical stimuli were scored as dead. Mortality was checked daily and dead bees were counted.

Results
Morphological characteristics
The isolated fungus was identified on basis of cultural and morphological characteristics as *A. apis* as illustrated in figure 2

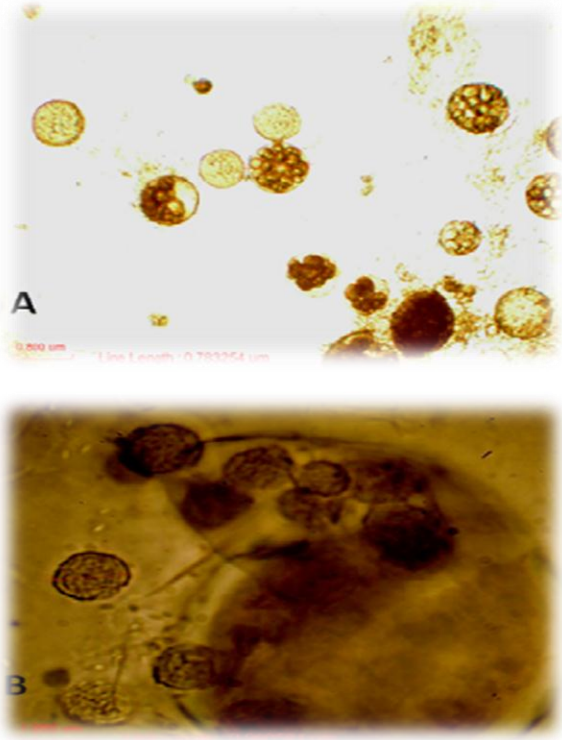

Fig 2: (A) Images of mature ascoma have a dark-brown appearance. The transparency of the ascoma walls allows observation of a number of small spherical shaped asci at 10×. (B) Ascospores out of ruptured ascoma at 40×.

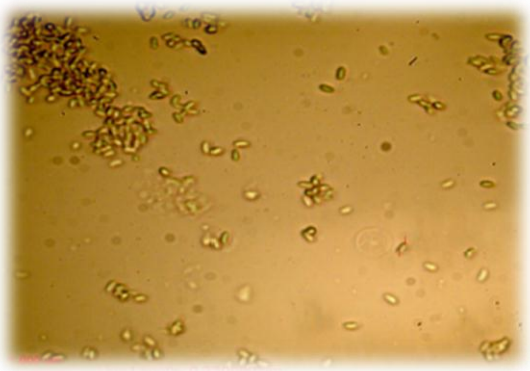

Fig 3: Isolated spores of *A. Apis* at 40 ×.

The fungal mating test was performed and the formation of ascospore were clear in the conjugation line in the middle of the plate (Figure 4.).

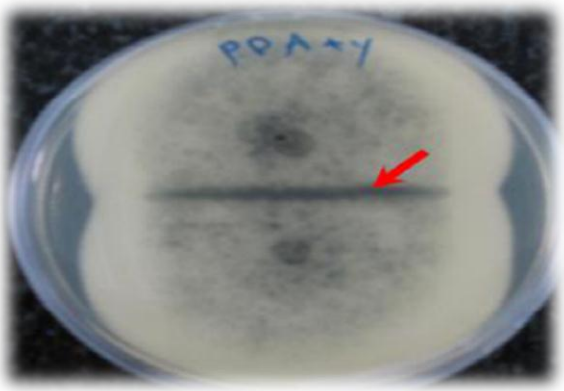

Fig 4: Cultural colony of *A. apis*, the red arrow pointed to the ascospores formed due to congregation of (+) and (-) types of the two separated fungal colony.

Minimum Inhibitory Concentrations (MICs) of the various plant Extract

The Minimal Inhibition Concentrations of the different extracts after 8 days against *A. apis* are outlined in Table 1.

Table1: Minimal Inhibition Concentrations of the different extracts after 8 days against *A. apis*

Used plant	Minimal Inhibition Concentration MIC %
Artemisia alba	> 20
Punica granatum	0.625
Callistemon viminalis	5

Among the tested plant extracts, *P. granatum* was the most active extract against *A apis* with MIC of 0.625%. Other *C. viminalis* exhibited moderate MIC against *A. apis* with MICs of 5%. The remaining extract *A. herba- alba*, was ineffectual against *A. apis* in this *in vitro* test system The photograph below (figure 5) are typical growth presentations of *A. apis* in the *in vitro* system employed in this investigation.

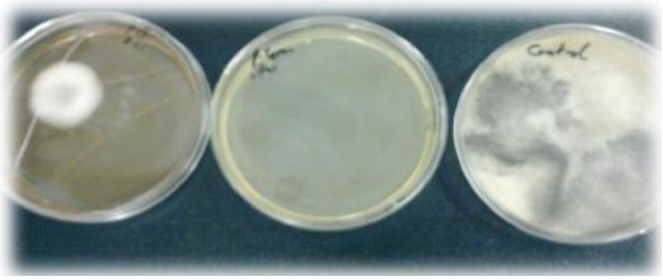

Fig 5: A pictorial presentation of the inhibitory effect of *P. granatum* MIC compared to the control after 8 days of incubation

Antifungal activity of plant extracts

Similar to the results of MIC test, the most active antifungal extract in this experiment was that of *P. granatum*, where the average radial mycelia growth of *A. apis* was significantly reduced ($P < 0.05$) by this extract at 2nd, 3rd, and 4th days of incubation at the different concentrations tested when compared with cultures grown on untreated media. (Table 2.).

Table 2: The effect of *P. granatum* extract over time on the radial growth of *A. apis* where 20, 10 and 5 represent extract that was diluted; 0=control

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2^a	5.0±0.9^a	4.6±1.0^a	4.3±0.5^a
2nd	14.9±1.6^a	7.3±0.6^b	6.8±1.8^b	5.4±0.4^b
3rd	24.8±1.9^a	7.6±0.9^b	9.0±1.1^b	6.0±0.9^b
4th	32.6±1.5^a	8.4±0.5^b	9.5±1.3^b	6.2±1.1^c

The value with a different letter in the same row is significantly different ($P<0.05$)

Table 3: The effects of *C. viminalis* plant extracts over time on the radial growth of *A. apis* where 20, 10 and 5 represent extract that was diluted; 0=control

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2^a	7.6±0.7^b	8.2±0.4^b	7.6±0.3^b
2nd	14.9±1.6^a	15.8±1.7^a	13.8±0.4^a	13.6±0.3^a
3rd	24.8±1.9^a	26.7±1.7^a	24.4±1.0^a	20.9±0.6^b
4th	32.6±1.5^a	34.7±0.8^a	30.3±0.3^b	25.3±1.4^c

The value with a different letter in the same row is significantly different ($P<0.05$)

The effect of the different concentrations 5, 10, 20% of *C. viminalis* extract are presented in Table 4.5. The radial mycelia growth of *A. apis* was significantly reduced at the 3rd day at 20% and at 4th day at 10% and 20% concentrations ($P < 0.05$).

Table 4: The effects of *A. herba-alba* plant extracts over time on the radial growth of *A. apes*. Where, 20, 10 and 5 represent extract that was diluted; 0 = control.

Time in days	Concentration %			
	0	5	10	20
1st	5.0±1.2^a	7.6±1.2^b	7.8±0.7^b	8.0±0.9^b
2nd	14.9±1.6^a	17.3±1.1^b	17.3±0.7^b	17.8±0.8^b
3rd	24.8±1.9^a	28.1±1.2^b	28.2±0.5^b	28.9±0.9^b
4th	32.6±1.5^a	36.1±0.9^b	36.9±1.1^b	38.6±0.8^c

The value with a different letter in the same row is significantly different ($P<0.05$)

The stimulatory effect of *A. herba-alba* on the mycelium growth of the *A. apis* at all concentrations tested across the four incubation days is clear. The stimulation was found to be associated with the increase of the concentration of the extract (Table 4).

Results indicated that the maximum inhibition percentages observed after 4 days of incubation were at 20% for *P. granatum*, Mycelium growth was inhibited by *P. granatum* extracts at all concentrations.

Table 5: Percentage of radial growth inhibition of *A. apis* during four days of incubation on agar media treated with plant extracts.

Percentage of inhibition (%)												
	First day			Second day			Third day			**Fourth day**		
Plant name	5%	10%	20%	5%	10%	20%	5	10	20	5	10	20
Punica	-0.4	+8.4	+14.7	+50.8	+54.6	+63.6	+69.2	+63.7	+75.8	+74.3	+70.9	+81.1
Arte alba	-51.8	-56.8	-60.6	-16.5	-16.0	-19.8	-13.2	-13.7	-16.5	-10.8	-13.3	-18.5
Callistemon	-53.0	-64.3	-53.0	-6.4	+7.1	+8.3	-7.6	+1.4	+15.8	-6.4	+7.2	+22.4

+: growth inhibition: no growth inhibition.

The nature of antifungal effect of *P. granatum* extracts

The average radial mycelial growth of *A. apis* when subcultured from treated plates into extract free media with respect to time of incubation are presented in (Figure 6.)

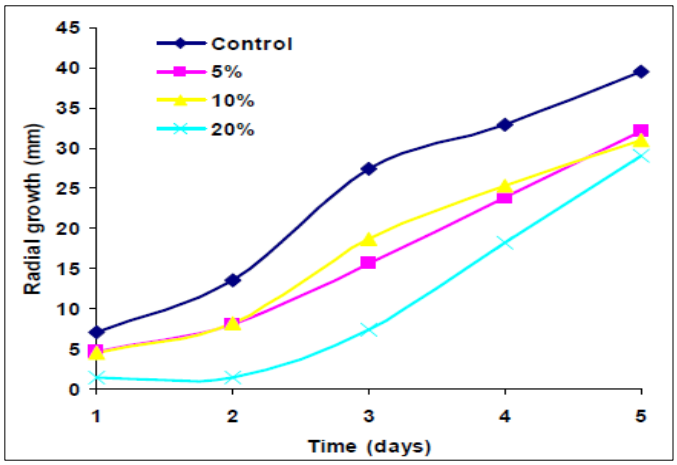

Fig 6: Growth profile for *A. apis* on PDA with 0%, 5%, 10% and 20% concentrations of *P. granatum* flower extract.

Such difference between the three tested concentrations was gradually decreased over the time. After five days of incubation no significant difference was detected between plates subcultured with mycelium discs from 5% and 10% ($P = 0.527$) and between 10% and 20% ($P = 0.096$).

Toxicity of *P. granatum* extract on *A. mellifera*

The results of the effect of different concentrations of *P. granatum* extract on worker bees though 96 ^{hr}. period are presented in (Figure 7).

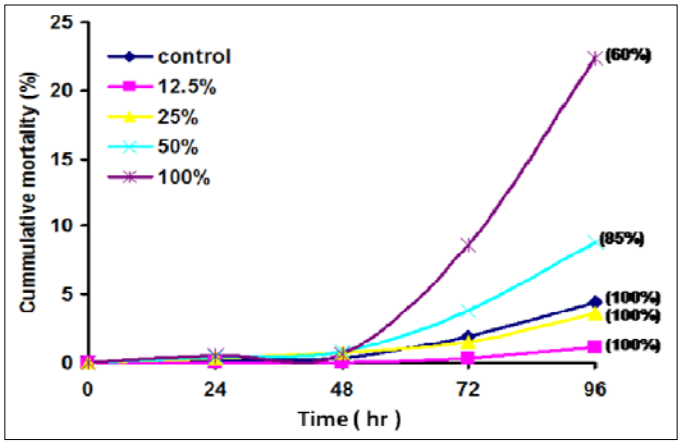

Fig 7: Cumulative mortality curves (%) of bees during 4 days period after feeding on four different concentrations of *P. granatum* extract as compared to control. Values between brackets are the percentages of the amounts of *P. granatum* extract-sucrose mixture consumed by bees in each treatment.

The highest percentage of cumulative mortality was recorded after 96 ^{hr}. and ranged from
1.1% to 22.4% at the different treatments, while the highest percentage of mortality (22.4%) was recorded at the highest concentrations tested (100%) after 96 ^{hr}. of the test.

Discussion

Identification of *Ascosphaera apis*

The morphological properties of the isolated colonies are in general agreement with previous reports (Chorbiñski and Rypuùa, 2003) [4]. Specifically, the ascospores growth was visible on 6-8 days after inoculation. Sizes of spores, ascospore and ascocyst diameters of the *A apis* agreed well with previously reported measurements (Gilliam *et al.*, 1978; Chorbiñski and Rypula, 2003) [11]. Moreover, the mycelium was white, compact and aerial with septated haypha (Chorbiñski and Rypuùa, 2003) [4]. Since it is a heterothallic fungi, mating tests have been used to diagnose species as A. apis (Christensen and Gilliam, 1983) [5]. When cultures were paired with two types, a black line of ascoma was observed due to congregation between (+) and (-) types of the two separated fungal colony. This behavior of the fungus was previously confirmed by (Spiltoir and Olive 1955) [25].

Plant extract administrated antifungal activity

Punica granatum

The aqueous crude extract of *P. granatum* flower had the highest inhibition on fungal growth with a mean diameter of 6.2 mm at the concentration of 20% at the 4th day compared to 32.6 mm of the control (Table 2).

Callistemon viminalis

C. viminalis exhibited low activity against *A. apis* where the MIC was 5% (Table 1) and the radial mycelia growth were significantly reduced ($P < 0.05$) at the 3rd day at 20% and at 4th day at 10% and 20% only (Table 3).

Plant extract of *Artemisia herba-alba* did not demonstrate antifungal activity

The weakness of antimicrobial activity of *A. herba-alba* extracts has been demonstrated by **(Seddik *et al.* 2010)** who found that the antibacterial activity of *A. herba-alba* aqueous and ethyl acetate extracts was ranged from weak to no effect against selected bacterial strains.

The nature of antifungal effect of *P. granatum* extract

An alternations in the normal growth profile of *A. Apis* was determined after exposure to different concentrations of *P. granatum* flower extract (5%, 10% and 20%) compared to the control (0 concentrations), and this alternation still significant, even after 5 days of incubation on extract free media.

The toxicity of *P. granatum* flower extract on *Apis mellifera*

The level of ingestion of syrups containing low concentrations of *P. granatum* flower extract was similar to that of pure sugar syrup (control) which may indicate that such addition of the extract did not affect the attraction of bees to this newly formulated product. When the *P. granatum* flower extract was administered in sugar syrup by systemic way, the result showed minimal mortality of *A. mellifera* when fed on low concentrations of *P. granatum* extract. At higher concentrations however, the mortality may reach to high levels.

In the groups which received high concentrations of *P. granatum* flower extract (8.8% and 22.4%), low consumption was accompanied by the highest bee mortality rate. The fact may have been due directly to starvation, which may be interpreted by the high viscosity of these high concentrations of the extract.

Based on the results, it is worth to mention that, these results are most relevant to adult worker. It is possible that the adult workers could feed the extract to larvae, and that the larvae may be more sensitive.

Even in the presence of such dilution effect the highest safe concentration, 25%, is still 40 times higher than the recorded MIC (0.625) of *P. granatum* extract for *A. apis* spores.

Conclusion

- The study confirm without any doubt, the presence of chalkbrood disease in honeybee colonies in Gaza strip.
- The extract of *P.granatum* (pomegranate) flowers show the best effect against *A.apis* followed by bottlebruch plant extract, therefore *P.granatum* has a fungistatic effect on the fungus growth.
- The pomegranate extract was not found to be lethal at low concentrations to adult workers responsible upon feeding the colony members.

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