HONEYBEE COLLECTED POLLEN AS ANTIBIOTICS TO CONTROL AMERICAN FOUL BROOD DISEASE, AT GIZA GOVERNORATE

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Abstract

The aim of this study was to determine the phenolic compounds and antibacterial activity components of pollen grains, obtained from different concentrations extracts of ethanol and water for controlling American foulbrood (AFB) disease. In this study four Egyptian bee pollen grains types were collected from pollen basket, namely Broad bean (*Vicia faba*), date palm (*Phoenix dactylifera*), clover (*Trifolium alexandrinum*) and maize (*Zea mays*), during spring. Identification of Phenolic composition in pollen grains samples were investigated by high performance liquid chromatography (HPLC) instrument. Two pollen extracts, using ethanol and water by three concentrations 5, 10 and 20% agent's *Paenibacillus larvae* subspecies *larvae*, formerly *Bacillus larvae* and it's potential for the control of American foulbrood disease (AFB) in vitro. Generally the concentration of 5% gave no effect neither pollen ethanol or aqueous extracts. But pollen grain water extracts gave inhibition zone diameter with different pollen grain of types (10%) and (20%) concentrations. The water extract of date palm pollen grains recorded a highly effect of inhibition zone which were 21.30& 28.65mm with 10&20% concentrations respectively. This study considers a primary step for the pollen grains on *P. larvae* spores in vitro. These products showed efforts no toxicity effects on different stage of honeybee. Suggesting them as perspective, low cost and consumer-acceptable agent for control of AFB disease in honeybee colonies.

Keywords: Honey bees, pollen grains, antioxidant, antimicrobial, American foulbrood.

INTRODUCTION

Paenibacillus larvae subspecies *larvae*, formerly *Bacillus larvae* (Heyndrickx *et al.*, 1996) a Gram-positive, spore-forming bacterium, is the causative agent of the American foulbrood (AFB), one of the most serious and destructive brood diseases to honeybees colonies. (Grimm and Mossbeckhofer, 1993) demonstrate that AFB is a problem for beekeepers world-wide. One hypothesized mechanism of resistance is built on genetically-based adult behavior. The resistant colony removed the dead brood completely, while the susceptible colony allowed some damaged brood to remain in the cells. Rinderer and Rothenbuhler (1974) demonstrated that pollen grains have a effect on the AFB mortality. Pollen contains microorganisms which act as antagonists of *P. larvae* (Brodsgaard *et al.*, 1998). The presence of pollen in the

intestine of the larvae may act to inhibit *P. l. larvae* growth (Reiche *et al.,* 1996). Other investigated substances include various saturated and unsaturated free fatty acids (Feldlaufer *et al.,* 1993) showed antibiotic activity.

Consequently, various strains of *P. larvae* showed resistance to antibiotics, such as oxytetracycline- HCl (OTC), have been discovered in Argentina as well as in many United States areas (Miyagi *et al.*, 2000).

Pollen, as well as other bee products, has gained increased attention for its therapeutic properties, as antibacterial effects (Proestos, *et al.*, 2005). The therapeutic action has been attributed to several phenolic compounds with antioxidant activity, present in these products. In addition, pollen contains significant amounts of polyphenolic substances, mainly flavonoids (Almeida-Muradian *et al.*, 2005). The polyphenols are involved in plants growth; they also supply resistance against pathogens. Some authors state the necessity of additional inquiries to evaluate the relative composition of polyphenolic substances in pollen and extracts of it, as well as the differences in their specifications as (Serra and Escola, 1997). The polyphenols constitute one of the most numerous metabolic groups of plants and are integral part of people and animals diet.

Our study leads to identification of highly potent natural products effective against AFB in vitro, no toxicity to honey bees and commercial availability suggesting them as perspective, low cost and consumer-acceptable agents for control of AFB.

MATERIALS AND METHODS

The first hybrid of Carniolan bees' race *Apis mellifera carnica* was used in this experiment.Twelve honey bee colonies were chosen in the apiary at Dokki, Giza. Broad bean (*Vicia faba*), date palm (*Phoenix dactylifera*), clover (*Trifolium alexandrinum*) and maize (*zea mays*) pollen grains were collected in 2016 as orbicular pellets removed from the pollen baskets on the hind legs of bees as they passed through pollen traps attached to honeybee hives. Broad bean, date palm, clover and maize pollen grains were readily recognized from standard reference sources. All pollen grains materials were put sealed in glass jars and stored at – 20 °C shortly collection until use.

Preparation of ethanolic extracts (PEE) from pollen grains

The pollen grains samples 20g were milled homogenized and extracted individually using 100 mL of ethanol solution 70% as extraction solvent in different concentrations 5, 10 and 20 % PEE at temperature of 70ºC for 30 min.

The supernatant was separated and the solid residue was re-extracted. Then, the ethanol extracts of pollen grains were combined and stored at 5ºC for the analysis. All samples were extracted in twice.

Preparation of aqueous extracts (PAE) from pollen grains

The pollen grains samples 20g were milled, homogenized extracted individually using 100 ml of distilled water extraction solvent in different concentrations 5, 10 and 20% PAE at temperature of 70ºC for 30 min with constant agitation. The supernatant was separated and the solid residue was re-extracted. Then, the water extracts of pollen were combined and stored at 5ºC for the used. All samples were extracted in duplicate.

Estimation and Identification of phenolic compounds:

Preparing of 10 % pollen solution, one g of pollen grains was dissolved in10ml ethyl alcohol 70%, and then kept in closed glass tubes for analysis by HPLC instrument. Identification of phenolic compounds of the pollen samples was performed by a JASCO, using a hypersil C_{18} reversed- phase column (250 X 4.66 mm) with 5 µm particle size. All chemicals and solvents used were in HPLC spectral grade. Twenty three standard phenolic compounds were obtained from Sigma.

Preparation and isolation of bacteria

Paenibacillus larvae subspecies *larvae*, formerly *Bacillus larvae* bacteria were isolated from an infected brood comb according to diagnosis reported by (Shimanuki and Knox, 2000). The bacterial spores collected from the dried remains of infected bee larvae. were taken to prepare fresh inoculums. Streak was taken using sterile cotton swap and was suspended in 9 ml of sterile dist. water in a screw-capped. Then the suspension was heat and shocked at -80°C for 10 min. to kill any non-spore-forming bacteria. 0.2 ml of the stock suspension was used for bioassay (Mahesh and Satish, 2008).

Preparation of the inoculums after isolation of *Paenibacillus larvae* bacteria, it was grown in Columbia sheep blood agar and incubated for 3days at 37 °C. The bacterial culture was transferred to a liquid medium (brain-heart-infusion (Oxoid®) and incubated for 48 hours at 37 °C. Then 1 ml aqueous of suspension was frozen at -70°C until required. The verification of *Paenibacillus larvae* was made using catalase-test, (Plagemann, 1985) "Plageman" test with Columbia sheep blood agar slants, and then inoculated with 40 ml of autoclaved brain-heart-infusion with 1ml defrosted bacteria suspension. After a heat-shock at 77 °C for 10 min, the suspension was incubated for 48 hrs. at 37°C, when the suspension reached an optical extinction of 0.22–0.23.

Measurement of Bioassay

The diameters of inhibition zones were measured after 72 hrs. We did not make any correction for disk diameter. The total diameter of the zone was recorded. Negative controls consisted of disks treated with 50% ethanol, and commercial sensitivity test disks (BBL, Becton Dickinson Microbiology).

Statistical analysis:

Data of all treatments were analyzed in a randomized complete block design (ANOVA) by MSTAT-C version 1.41 (Sendecor and Cochran, 1980). and using graph pad prisma version 3.03 for windows, software. All means were compared by Duncan's multiple range test at level (0.05).

RESULTS

The obtained data in Table (1) and Figs. (1, 2, 3 & 4) showed that the Phenolic compounds present in pollen grains which subjected of HPLC separation; composition. The four types of pollen grains Broad bean (*Vicia faba*), Date palm (*Phoenix dactylifera*), clover (*Trifolium alexandrinum*) and maize (Zea *mays*) were dependent on available source in the collecting area. Twenty three compounds were determined in the tested samples.

In the broad bean pollen grains the phenolic compounds were thirteen both, salicylic acid, *p*-Coumaric acid, daidzin and phenol were 45.940, 41.808, 38.697&13.447 mg/100g respectively, while genstin and daidzein were 4.282 &1.041 mg/100g pollen grains respectively, and found fewer than one mg/100g both of pyrogallic acid, *P*-OH benzoic acid, quercetin, caffeic acid, 3, 5 di-methoxy benzyl, eugenol and galangin.

In the date palm pollen grains the phenolic compounds were ten both of twenty three compounds were determined in the tested samples, phenol and daidzin were 1772.703 & 123.250 mg/100g respectively, while pyrogallic acid, vanillin, salicylic acid and gallic acid were 27.186, 11.152, 8.913 & 1.791 mg/100g pollen grains respectively. caffeic acid, kaempferol, *P*-OH benzoic and 3,5 Dimethoxy benzyl alcohol recorded 0.887, 0.695, 0.076 & 0.003 mg/100g pollen grains respectively.

In the clover pollen grains the phenolic compounds were eleven both of twenty three compounds in the tested samples, catechin, *p*-Coumaric acid, daidzin, pyrogallic acid and cinnamic acid phenolic compounds were 106.234, 34.844, 15.442, 12.246 & 10.529 mg/100g respectively, while *P*-OH benzoic, caffeic acid, gestein and ferulic acid recorded 6.516, 2.818, 1.902 & 1.081 mg/100g respectively and content traces of kaempferol and eugenol recorded 0.063& 0.004 mg/100gm pollen grains respectively.

Maize pollen grains consists of phenolic compound nine both of twenty three compounds in the tested samples, and were consisted of less than mg/100g phenolic compound than others tested type pollen grains. Phenolic compound
p-coumaric acid, daidzin, cinnamic acid and *P*-OH benzoic were 34.844, 15.442, 10.529 & 6.516 mg/100g respectively. Caffeic acid, genistein and ferulic acid were 2.818, 1.902 & 1.081 mg/100g respectively, while kaempferol and eugenol were 0.063 & 0.004 mg/100g respectively.

Table 1. The phenolic compounds in the main pollen grains (broad bean, date palm, clover and maize) (mg/100g)

Phenolic compound \ Pollen grains	Broad bean	Date palm	Clover	maize
Gallic acid: 3,4,5-Trihydroxybenzoic acid $C_7H_6O_5$	0.000	1.791	0.000	0.000
p-OH benzoic acid *4-Hydroxybenzoic acid $C_7H_6O_3$	0.681	0.076	6.516	6.516
Caffeic acid: *3-(3,4-dihydroxyphenyl)prop-2-enoic acid $C_9H_8O_4$	0.143	0.887	2.818	2.818
*phenol: C_6H_6O	13.447	1772.703	0.000	0.000
p-Coumaric acid: *3-(4-hydroxyphenyl)-2-proponic acid $C_9H_8O_3$	41.808	0.000	34.844	34.844
Salicylic acid : *2-hydroxybenzoic acid $C_7H_6O_3$	45.940	8.913	0.000	0.000
Ferulic acid: *3-(4-hydroxy-3-methoxy-phenyl)prop-2- enoic acid $C_{10}H_{10}O_4$	0.000	0.000	1.081	1.081
Cinnamic acid: * (E)-3-phenylprop-2-enoic acid $C_9H_8O_2$	0.000	0.000	10.529	10.529
Quercetin: *2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy- chromen-4-one $C_{15}H_{10}O_7$	0.322	0.000	0.000	0.000
Eugenol $C_{10}H_{12}O_2$	0.002	0.000	0.004	0.004
Chrysin: *5,7-dihydroxy-2-phenyl-chromen-4-one $C_{15}H_{10}O_4$	0.000	0.000	0.000	0.000
Galangin: *3,5,7-trihydroxy-2-phenyl-chromen-4-one $C_{15}H_{10}O_5$	0.0004	0.000	0.000	0.000
Pinostrobin: *5,7-dihydroxy-2-phenyl-chroman-4-one $C_{15}H_{12}O_4$	0.000	0.000	0.000	0.000
Vanillin: *4-hydroxy-3-methoxy-benzaldehyde $C_8H_8O_3$	0.000	11.152	0.000	0.000
3,5-Dimethoxybenzyl Alcohol $C_9H_{12}O_3$	0.035	0.003	0.000	0.000
Catechin : *(2s,3r)-2-(3,4-dihydroxyphenyl)chromane-3,5,7-triol $C_{15}H_{14}O_6$	0.000	0.000	106.234	0.000
Daidzin: *7-hydroxy-3-(4-hydroxyphenyl)chromen-4-one $C_{15}H_{10}O_4$	38.697	123.250	15.442	15.442
Genstin: *4',5,7-Trihydroxyisoflavone; Sophoricol $C_{15}H_{10}O_5$	4.282	0.000	0.000	0.000
Daidzein: *7-(-D-Glucopyranosyloxy)-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one $C_{21}H_{20}O_9$	1.041	0.000	0.000	0.000
Genistein :, 7-Dihydroxy-3-(4-hydroxyphenyl) chromen-4-one $C_{15}H_{10}O_5$	0.000	0.000	1.902	1.902
Pyrogallic acid: *benzene-1,2,3-triol $C_6H_6O_3$	0.988	27.186	12.246	0.000
Rutin *Rutoside $C_{27}H_{30}O_{16}$	0.000	0.000	0.000	0.000
Kaempferol; *3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one $C_{15}H_{10}O_6$	0.000	0.695	0.063	0.063

*IUPAC name

Table 2. Antibacterial activity of pollen types (5, 10 and 20% conc.) on *Paenibacillus larvae,* meandiameter of inhibition zone (mm) of 3 samples .

Pollen types	Pollen ethanol extract70%			Mean / pollen ethanol extract	Pollen aqueous extract			Mean / pollen aqueous extract	General Mean	$LSD_{(0.05)}$
	5%	10%	20%		5%	10%	20%			
Broad bean	0.00	21.00	25.80	15.60 b	0.00	17.00	23.80	13.60 f	14.60 B	0.101
Date palm	0.00	15.75	25.50	13.75 e	0.00	21.30	28.65	16.65 a	15.20 A	
Clover	0.00	17.71	26.70	14.80 c	0.00	17.10	25.40	14.17 d	14.49 C	
Maize	0.00	15.75	20.75	12.17 i	0.00	16.40	22.65	13.02 h	12.59 D	
pollen basket	0.00	0.00	0.00	0.00 k	16.40	22.50	0.00	12.97 h	6.48 F	
bee bread	0.00	13.00	17.00	10.00 j	17.50	22.20	0.00	13.23 g	11.62 E	
Mean	0.00	13.87	19.29	11.05 B*	5.65	19.42	16.75	13.94 A*	12.50	

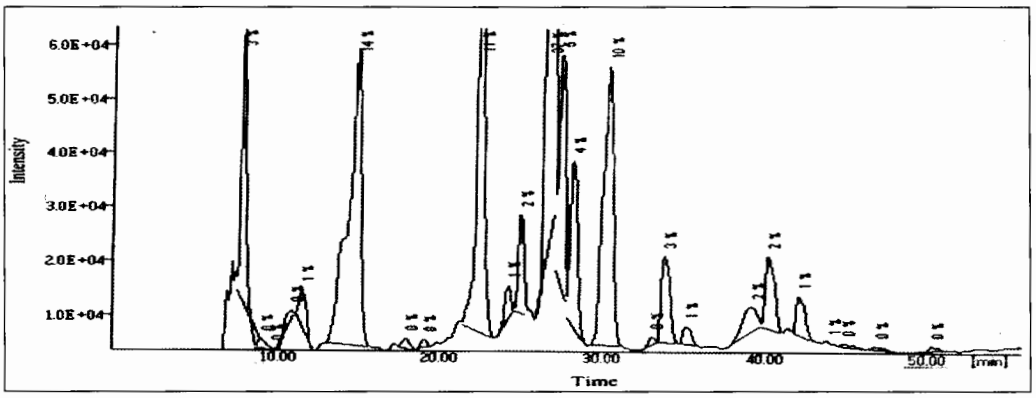

Fig. 1. HPLC chromatograme of Phenolic compounds separation in ethanolic extract of the date palm pollen grains

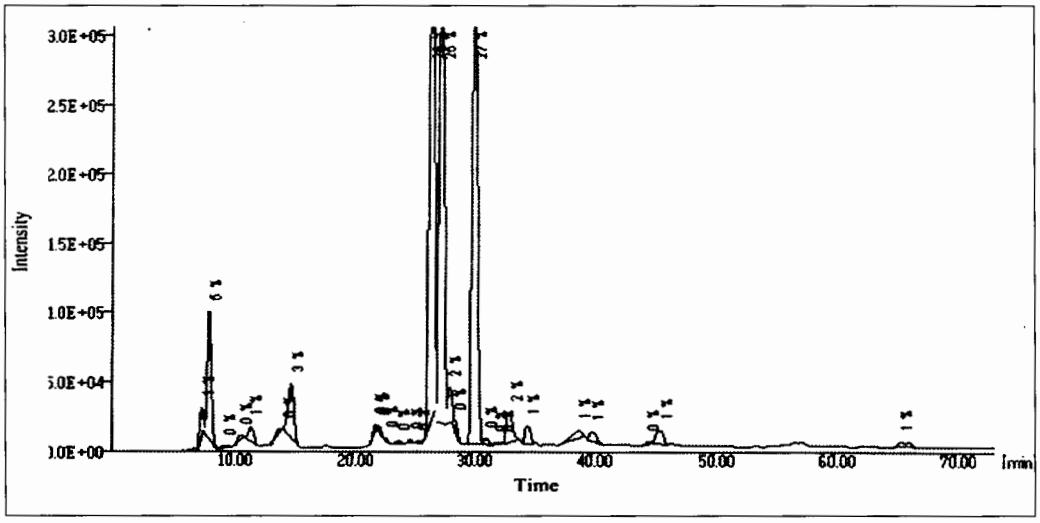

Fig. 2. HPLC chromatograme of Phenolic compounds separation in ethanolic extract of the broad bean pollen grains

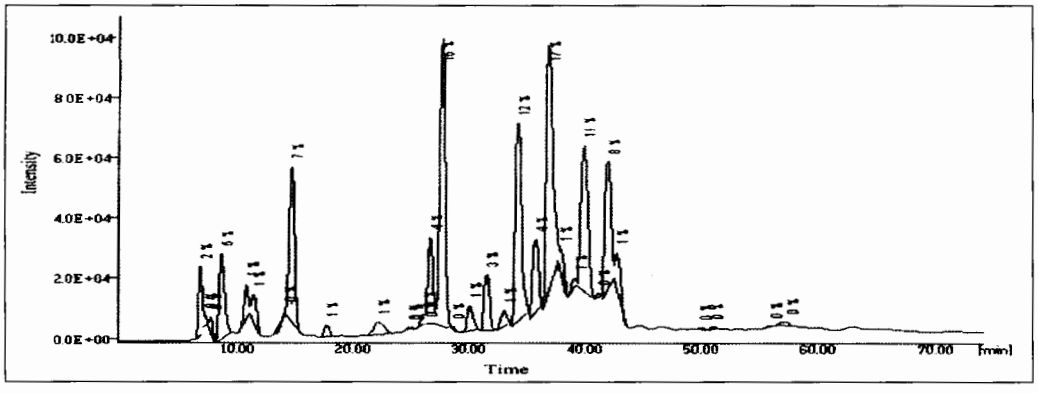

Fig. 3. HPLC chromatograme of Phenolic compounds separation in ethanolic extract of the clover pollen grains

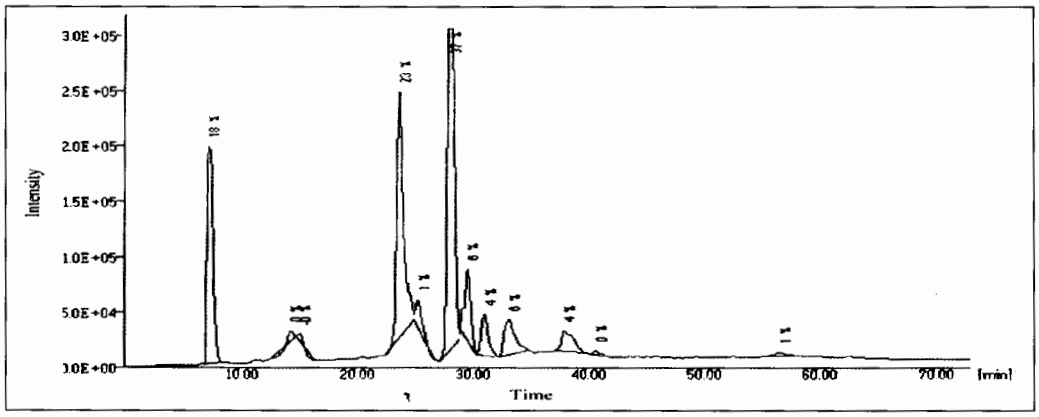

Fig. 4. HPLC chromatograme of Phenolic compounds separation in ethanolic extract of the maize pollen grains

The obtained results in (Table 2) showed that the total phenolic compounds content of pollen extracts were dependent on the solvent type aqueous or ethanolic with different concentrations (5, 10 & 20%), as antibacterial activity against P. *larvae* bacteria. The concentration 5% showed not effect neither with pollen ethanol or aqueous extracts. But the diameter of inhibition zone of pollen types at 20% concentration showed effect against on *P. larvae* growth, while alcohol pollen basket extract showed not effect.

Generally the aqueous pollen extract giving higher inhibition zone diameter with different pollen types at 10% and 20% concentrations. Generally the extract of date palm induced 15.20 mm and induced 21.30, 28.65 mm with 10 and 20% concentrations of ethanolic and aqueous extracts respectively. On the other hand, the ethanolic extract of clover pollen grains gave an inhibition zone which was 17.71 & 26.7 mm with 10 & 20 % concentrations respectively, and broad bean were 21 & 25.8 mm with 10 & 20 % concentrations, whereas the aqueous extract of clover pollen grains recorded 17.1, 25.4 mm with 10 & 20 % concentrations respectively. The aqueous extract of broad bean pollen grains gave an inhibition zone which was 17.0 & 23.8 mm with 10 & 20 % concentrations respectively. In the end we can say found high significant among pollen alcohol extract differences types 10 % & 20 % concentrations in inhibition zone diameter respectively. Also significant among Pollen aqueous extract different types 10 & 20% concentrations) respectively.

The chemical profile obtained through the HPLC technique revealed compounds with high polarity, probably phenolic acids, and a large variety of more polar compounds, probably flavonoids. The chromatographic profile of bee pollen extracts indicated the presence, on average, of approximately 23 different compounds. The bee pollen samples showed a high variability of qualitative and

quantitative composition for phenolic compounds (Table 1 & Fig 1, 2, 3 and 4). As different extracts exhibited different antibacterial activities, there may be different kinds of phenolic content in different pollen extract when comparing the chromatograms. Clover phenolic compounds chromatogram showed the highest number of peaks (phenolic compounds) which reflect to make the ethanolic extract of clover pollen was the more effective on the paenibacillus larvae bactria than the other ethanolic pollen extracts, it might be due to the high content of catechin (106) in this extract which absent in the other pollen extract, on the other hand the chromatogram of maize pollen ethanolic extract showęd low number of peaks (phenolic compounds) it may be the reason of the low antimicrobial activity against paenibacillus larvae, in addition the different patterns of sensitivity are due to different phenolic compounds in pollen. According to chemical evidences outlined in the chromatograms obtained the bee pollen has a different chemical composition. Several peaks at different retention times showed a complex composition. The composition of phenolic compounds in bee pollen loads, similarly as in the case of other chemical compounds of bee pollen strictly depends on botanical origin and according to the results obtained; pollen seems to have interesting biological properties.

DISCUSSION

The Phenolic compounds present in pollen grains which subjected of HPLC separation; composition. The four types of pollen grains Broad bean (*Vicia faba*), Date palm (*Phoenix dactylifera*), clover (*Trifolium alexandrinum*) and maize (Zea *mays*) were dependent on available source in the collecting area. Several authors have found many kinds of phenolic and flavonoid compounds in pollen (Proestos, *et al.,* 2005 and Mao *et.al.*, 2013) our analysis of pollen extracts revealed that p-coumaric acid can induce detoxification genes; moreover, RNA-seq analysis demonstrates that it up-regulates a select suite of genes required for defense against pesticides and pathogens.

Salicylic acid (SA, 2-hydroxybenzoic acid) is a key signaling molecule that mediates plant defense against a variety of pathogens in a number of species. Its accumulation is required for the establishment of local and systemic required resistance (SAR) responses (Dempsey *et al.*, 1999). Also their antimicrobial action corresponds directly to chain length of the alcohol component, i. e. it increase with the chain length. Their action is fungi static or similar to benzoic and ascorbic acid. The phenolic group makes them more effective then benzoates against bacteria in general, gram- positive stains in particular.

Flesar *et al.,* (2010) studied in total, 26 natural compounds of various chemical classes (flavonoids, alkaloids, terpenoids) and 19 crude extracts from selected plants were tested in vitro for antibacterial activity against three strains of *P. larvae*, by the broth micro dilution method. (Molan *et al.,* 1988) reported that, honey from different floral sources varies greatly in their antibacterial activity. Reported significant differences between different kinds of floral honey in their activities on *S. aureus* at dilutions of 1/4, 1/8 and 1/16 original strength.

Among the individual substances, sanguinarine (MIC 4 μg/ml), followed by thymoquinone, capsaicin, trans-2-hexenal and nor dihydroguaiaretic acid (MIC 4–32 μg/ml) possessed the strongest antibacterial effect (Kroyer and Hegedus, 2001) achieved 8.2 mg/g of polyphenolics substances in natural bee-collected flower pollen, which could be significantly increased in the extracts (21.4-24.6 mg/g) with the highest content of total polyphenols in the ethanol extract. Phenolic constituents (total phenols, phenylpropanoids, flavonols and anthocyanins) and antioxidant ability were determined in bee pollen of 12 plant species. Great variability of phenolic contents was observingly in the pollen of investigated species. Total antioxidant activity differed to a large extent (0.8–86.4% inhibition of lipid peroxidation) however, in most of the examined pollens; it was high and corresponded with the phenylpropanoid level. Molan and Russell (1988) found that pollen present in honey could be the source of the antibacterial aromatic acids. The antimicrobial effects of various extracts from different parts of *Phoenix daylifera* from Iran have been studied, and the results showed that this plant, particularly the pollen part, may be used in the treatment of infections, including gram positive bacteria (Khider *et al.*, 2013) according to date Palm Pollen from various extracts exhibited antimicrobial activity against several strains, with *S. aureus* and *E. coli* being the most sensitive strains, followed by *L. monocytogenes*, *S. enteritidis* and *P. aeruginosa*.

CONCLUSION

The present study indicate that the antibacterial activity against *P. larvae*, perhaps back other biological properties of pollen grains types, correlated with their pollen ethanol and aqueous concentrations but mostly to their chemical composition which can be variable vegetal source.

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حبوب اللقاح المجموعة بواسطة نحل العسل كمضادات حيوية لمكافحة مرض تعفن الحضنة الأمريكى بمحافظة الجيزة

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تهدف هذه الدراسة الى تقدير الفينولات ومضادات البكتريا الموجودة فى بعض حبوب اللقاح المختبرة وتأثيرها المثبط على البكتيريا المسببة لمرض تعفن الحضنة الأمريكي *Paenibacillus larvae* في طوائف نحل العسل بمحافظة الجيزة. تم اختبار ٤ أنواع من حبوب اللقاح المجموعة بواسطة نحل العسل من المصادر النباتية المختلفة، وهي حبوب لقاح الفول (*Vicia faba*) وطلع النخل (*Phoenix dactylifera*) والبرسيم (*Trifolium alexandrinum*) والذرة (*zea mays*) خلال فصل الربيع ٢٠١٧ وتم تحديد المركبات الفينولية فيها من خلال جهاز الـــ HPLC بعد عمل مستخلص كحولي (الإيثانول) ومستخلص مائي تحت ثلاث تركيزات مختلفة ٥ % و ١٠ % و٢٠ % .

أظهرت النتائج عدم وجود فروق معنوية بين كل من المستخلصين الكحولي والمائي لحبوب اللقاح المختبرة في تأثيرها على البكتيريا (AFB) مع تركيزي ١٠ % و٢٠ % و لم يعطي تركيز ٥ % من كلا من مستخلصى حبوب اللقاح الايثانولى والمائى أى تأثير تثبيطى

أعطى المستخلص المائى لجميع أنواع حبوب اللقاح المستخدمة فى التجربة بتركيز ١٠% و٢٠% مساحات تثبيطية وكان أعلاها المستخلص المائى لطلع النخل بتركيز ١٠% و٢٠% حيث كان قطر التثبيط ٢١,٣٠ مم و٢٨,٦٥ مم على التوالي. وبالتالي يفضل استخدام المستخلص المائي عن المستخلص الكحولي وذلك لتقارب التأثير على بكتيريا تعفن الحضنة الامريكي في المعمل. ويمكن إعزاء الفعل التأثيري للمستخلصات المختبرة لمجموعة الفينولات الموجودة في كل حبوب اللقاح المختبرة كل على حدة. وتعتبرهذه الدراسة خطوة أولى لمعرفة تأثير حبوب اللقاح على جراثيم بكتيريا تعفن الحضنة الأمريكى معمليا حيث أن هذه المنتجات غير سامة لأطوار النحل المختلفة ومتاحة تجاريا ومنخفضة التكاليف.