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Propolis as a natural antibiotic to control American foulbrood disease in honey bee colonies

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American FoulBrood (AFB) is one of the most severe bacterial diseases that affect larvae of honey bee *Apis mellifera*, causing a decrease of bee population and colony production and due to the serious effects associated with AFB disease and the problems related to the use of antibiotics, it is necessary to develop alternative strategies for the control of the disease. The aim of this study was to determine, under field conditions, the effectiveness of tylosin and three kind of ethanolic extract propolis (Chinese, Egyptian and old wax comb extract propolis) for controlling AFB disease in honey bee colonies. Identification of Phenolic composition of the ethanolic extract samples were investigated by high performance liquid chromatography (HPLC) instrument. Laboratory studies were conducted to determine the LC_{50} (half lethal concentration) and LT_{50} (half lethal time) values were determine. In field trials the colonies were inoculated with AFB disease for three weeks before initiation of trial or treated with tylosin, 0.1, 0.05 and 0.025% ethanolic extract propolis (EEP) which extracted from Chinese propolis, Egyptian propolis, old wax comb extract and fed with sugar syrup only for three weeks at one week intervals. Field assays showed that the treatment of beehives affected with AFB disease by tylosin 1% and Egyptian EEP in concentration of 0.1 and 0.05% had elimination of clinical symptoms at 100% of reduction rate.

Key words: American foulbrood, ethanolic extract propolis (EEP), high performance liquid chromatography (HPLC), honey bee disease, natural treatments.

INTRODUCTION

The causative agent of American Foulbrood (AFB) disease is *Paenibacillus larvae*, a Gram positive and spore-forming bacterium that is distributed worldwide (Genersch et al., 2006). Lodesani et al. (2005) reported that AFB is a virulent brood disease and is caused by *P. larvae*, which has a long-lived, resistant spore that can remain dormant for many years in combs and honey. AFB is spread by the exchange of infected honey and combs among colonies, either by the beekeeper tools or by robber bees. If no measures are taken by the beekeeper the colony is very likely to be destroyed by the infection, thus becoming a source of contagion for the whole apiary.

Macrocyclic lactone tylosin, (which has current US Food and Drug Administration approvals for agricultural uses) may soon gain approval for the control of AFB. This antibiotic inhibits ribosomal protein synthesis and has recently shown a good efficacy, while other substances

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failed to cure AFB in colonies with a high level of spores. The β- lactams (penicillins and cephalosporins), while active *in vitro*, are apparently not effective in the field. No antibiotic is capable of acting through the thickened wall of the bacillus spore and for this reason antibiotics are said to 'mask' the infection for the whole duration of their use; usually the disease reappears when the treatment is interrupted because the spores remain viable for several decades or longer (Marco et al., 2006).

A common strategy for the prevention and treatment of affected colonies is the use of antibiotics, particularly oxytetracycline hydrochloride (Hansen and Brodsgaard, 1999). However, several problems may be associated with its extended use. Chemical residues can persist in honey affecting its quality for human consumption while application of antibiotics may reduce the lifetime of bees and raise the risk of resistant strains emergency (Shuel and Dixon, 1960; Martel et al., 2006). Al Zen et al. (2002) reported that tylosin applied in a confectioner's sugar dust was effective in reducing and eliminating symptoms of OTC-resistant AFB disease in the apiary of the study and treated hives with tylosin was significantly reduced to no diseased hives. Resistance to this and other macrolides together with lincosamides and streptogramin B occurs in Gram-positive bacteria and was first shown, in *Staphylococcus aureus* (Lai et al., 1973). The presence of *P. larvae* OTC- resistant strains has been reported so far in Argentina, the United States, Italy, New Zealand and United Kingdom (Alippi, 1996; Miyagi et al., 2000; Evans, 2003).

Propolis is a natural product derived from plant resins and collected by honey bees to seal the walls and entrance of the hive and contributes to protect the colony against different pathogens (Ghisalberti, 1979), propolis has several biological properties such as antibiotic, antifungal, antiviral, anti-inflammatory activities (Manolova et al., 1985; Marquee, 1995; Drago et al., 2000; Tichy and Novak, 2000; Santos et al., 2003).

Furthermore, different compositions and amounts of the active substances are detected in separate samples of propolis (Bankova et al., 2002). The findings of various studies confirm that chemical composition of propolis depends on trees and plants available to the bees, on the season in which it is collected, on the geographical area, and other factors (Kartal et al., 2002; Abd El Hady and Hegazi, 2002). The volatile substances (aromatic oils) determine the flavor of propolis, and the variety of flavor depends on the geographical area and assortment of plants (Bankova, 1994). Flavonoids comprise the major part of biologically active substances in propolis (Havsteen, 2002). Furthermore, propolis has been found to contain phenolic acids (for example, prenilic derivates of cinnamic and coumaric acids), characterized by very potent antimicrobial activity (Hegazi et al., 2000). The antimicrobial and anti-inflammatory activity of European propolis is associated with the presence of flavonoids, flavones, and phenolic acids and their derivates (Bankova, 2005). Due to the serious effects associated with AFB and the problems related to the use of antibiotics, the aim of the present work was to evaluates the biological activities of the EEP as a natural antibiotic, as well as its chemical composition for develop a new control strategy of this disease.

MATERIALS AND METHODS

Propolis samples

Three propolis samples were used, the first sample was Egyptian propolis (E.) which collected by glass slides (plaques of transparent glass close to the internal and lateral walls, near the little boxes 1 and 10 (Breyer, 1995) from honey bee colonies located in the apiary of Beekeeping Research Department, Plant Protection Research Institute, Agriculture Research Center at Dokki, Giza governorates, Egypt, through two years (2006-2007) and the second sample was Chinese propolis (C.) which imported from China and purchased commercially in Egyptian market and the third sample was old wax combs (W.) which collected from experimental apiary.

Preparation of EEP solution

Extraction procedures

Finely ground propolis was extracted by maceration at room temperature, with occasional shaking, in the proportion of 10 g of (C, E and W) propolis to 100 ml of solvent (ethanol 80%v/v), extracts were obtained after 7 days of maceration and the ethanolic extracts were then filtered in Whatmann N° 1 filter paper and incubated at room temperature until ethanol evaporated and the product obtained a honey-like consistence are referred to as EEP according to the method reported by Ildenize et al. (2004).This extract was diluted in sugar syrup 1:1 (1 kg of sugar in 1 L of water) at a final concentration of 2, 1, 0.5, 0.1, 0.05 and 0.025% EEP (w/v).

Identification of phenolic compounds in EEP by HPLC instrument

Identification of individual phenolic compounds of the three kind of EEP was performed on a HPLC instrument, 1 g sample was soaked in 20 ml of ethanol (80%v/v) and filtered through 0.45 μm filter membrane prior to HPLC analysis. High performance liquid chromatography, analytical HPLC was run on HPLC (JASCO, Japan), equipped with a pump (model PU-980) and a UV detector (UV-970). Separation was achieved on a hypersil BDS C18 (Thermo Hypersil-keystone, Germany) reversed-phase column (RP-18, 250 × 4.6 mm) with 5 μm particle size, a constant flow rate of 0.7 ml min^{-1} was used with two mobile phases: (A) 0.5% acetic acid in distilled water at pH 2.65 and solvent (B) 0.5% acetic acid in 99.5% acetonitrile, the system was run with a gradient program: 100% A (0 min); 0% B (0 min);100 to 50% A (50 min); 0 to 50% B (50 min), using an UV detector set at wavelength 254 nm. Phenolic compounds of each sample were identified by comparing their retention times with those of the standards mixture chromatogram. The concentration of an individual compound was calibrated and calculated on the basis of peak area measurements, and then converted to g phenolic /100 g fresh weight. All chemicals and solvents used were HPLC spectral grade and obtained from

sigma (st. Louis, USA) and Merck - (Munich, Germany chemical companies), 24 components which presented the identical UV spectrum as standards compounds.

Detection of the half lethal concentration and half lethal time, (LC_{50} and LT_{50}) of EEP on worker honey bees

Susceptibility of worker honey bees to EEP was detected using a technique developed by (Maggi et al., 2010). Hybrid carniolan race (F1) bees were collected from frames in healthy colonies from the experimental apiary through July and August, 2007. Tests were conducted using 100 workers of honey bee 1 day old removed from the emergence boxes and placed in special wooden cages (16 × 12 × 6 cm) with wire screened side and glass fronts. The workers fed with 10 ml of different (C, E and W) EEP concentrations 2, 1, 0.5, 0.1, 0.05 and 0.025% in sugar syrup (1:1) were placed into each box and a negative control was performed using sugar syrup without EEP and the assay was carried out by 4 replicates then boxes were incubated at 32°C and 65% RH. Along the experiment period, the feeding solution had been changed daily and dead bees were counted and discarded. At the end of the experiment, bees were sacrificed and mortality percentages were corrected according to natural mortality (Abbott, 1925), and subjected to probit analysis according to the method of Finney (1952).

Determination of diagnosis of American foulbrood disease in honey bee colonies

The AFB infection was determined by number of infected larvae per colony according to diagnosis reported by Shimanuki and Knox (2000). Infected colonies spotty brood have been found, capping tend to be darker, concave larvae colored and extended length wise in the cell and contents of the cell rope out forming fine elastic thread up to 30 mm (Nikola, 2001). Larvae that have died of American foul brood disease exhibit a "ropy" condition that can be demonstrated by inserting a matchstick or similar implement into the dead and mass and drawing out the material into a threadlike projection longer than 2.5 cm (Morse and Nowogrodzki, 1990).

Field experiment

The efficiency of EEP to control the AFB on *P. larvae* artificially infected colonies was evaluated on hybrid carniolan race (F1) colonies which located in the experimental apiary through year 2008, Forty-four apparently healthy colonies (without clinical symptoms of AFB) and untreated with any antibiotics before were used. Colonies consisted of three brood, two honey and pollen combs were present in each hive and all hives were inoculated two weeks before initiation of trial. The inoculation process consisted of removing 100 cells of actively diseased brood from a local commercial apiary and agitating them in sucrose solution 50%. All hives were then fed, with 500 ml of this syrup/slurry mixture until all was consumed. At initiation, AFB disease evaluation was determined by removing brood frames from each individual hive and categorizing (Hitchcock et al., 1970) infected larvae (diseased cells) per hive were count every week. After 3 weeks we have thirty-three colonies had approximately 100 diseased cells/colony (sever degree). The thirty-three Colonies were divided into five groups in a randomized design, group one, Tylosin (T.) as positive control consisted of a confectioner's sugar dust, which made by combining 200 mg of tylosin tartrate with 20 g confectioner's sugar (a dose found efficacious in a previous study). The full 20 g of this dust were applied on 3 colonies by sprinkling over end of top bars for once a week for three weeks, for a total dose of 600 mg tylosin tartrate over 3 week. Group two, Chinese propolis (C.) feeding with 500 ml of 0.1, 0.05 and 0.025% C. EEP solution, 3 colonies for each concentration for three weeks at one week intervals. Group three, Egyptian propolis (E.) and Group four, old wax comb extract propolis (W.) were used the same methodology and doses of group two. Group five, (Con.) as a control, 500 ml of sugar syrup 1:1 were performed once a week, during 3 consecutive weeks. The all treatment groups were reassessed from June to August, 2008. All these colonies were recorded with regard to their disease rating prior to the all treatments and subsequently evaluated 30 days after the third treatment, AFB disease re-examined by removing brood frames from each individual hive and the infected larvae (diseased cells) per hive were count and colonies with no visible signs of AFB disease at this time were considered recovered. The reduction percentage (rate) of infection was calculated according to the equation given by Henderson and Tilton (1955).

Reduction percentage of infection

$$= 1 - \frac{\text{n in Control before treatment } \mathbf{x} \text{ n in treatment after treatment}}{\text{n in Control after treatment } \mathbf{x} \text{ n in treatment before treatment}} \times 100$$

Where: n, number of diseased cells/colony.

Statistical analysis

For each evaluation data were compared by analysis of variance (ANOVA) and means were separated by least significance test at L.S.D0.05 on the other hand the data in Table 4 were transformed by Arcosin (angular transformed) according to Sokal and Rohef (1995).

RESULTS

Separation of phenolic compounds in three kind of EEP by HPLC

Phenolic compounds might be responsible for the biological activity in the three kind of EEP (Table 1). HPLC analysis was used to give information about the chemical composition of E.EEP, C.EEP and W.EEP. The phenolic compounds content found in E.EEP were salicylic acid, caffeic acid, ferulic acid, quercetin ,pinocembrin, pinostrobin, genistein and daiazein higher than that in C.EEP and W.EEP, in addition the phenolic compounds found in C.EEP were phenol, para hydroxy benzoic acid, p. coumaric acid, 3,5 dimethoxy benzyl alcohol, trans – cinnamic acid, chrysin, galangin, daidzin, acacetin higher than that in E.EEP and W.EEP, on the other hand in W.EEP were pyrogallic acid, protocatechuic acid, catechines, higher than that in E.EEP and C.EEP. It is evident from (Table 1) that composition of phenolic constituents were different in the three kinds of EEP and E.PEE were contained more phenolic compounds than in the C.PEE and W.PEE.

Table 1. Phenolic compounds concentration of three kinds of ethanolic extract propolis (E.EEP, C.EEP and W.EEP) determinate by HPLC.

Phenolic compound.		mg/100 g		
		C.EEP	E.EEP	W.EEP
Phenol: *phenol	C_6H_6O	3.757	15.968	0.000
Pyrogallic acid: *benzene-1,2,3-triol	$C_6H_6O_3$	0.00	0.00	31.710
Resorcinol: *benzene-1,3-diol	$C_6H_6O_2$	1.11	0.00	0.00
Salicylic acid: *2-hydroxybenzoic acid	$C_7H_6O_3$	15.72	716.80	15.13
para hydroxy benzoic: *4-hydroxybenzoic acid	$C_6H_6O_3$	9.18	11.60	0.000
Protocatechuic acid: *3,4-dihydroxybenzoic acid	$C_7H_6O_4$	29.66	54.60	25.45
Vanillin: *4-hydroxy-3-methoxy-benzaldehyde	$C_8H_8O_3$	0.00	0.00	13.60
p-Coumaric acid: * 3-(4-hydroxyphenyl)-2-proponic acid	$C_9H_8O_3$	1.25	0.00	0.00
Coumarine: * chromen-2-one	$C_9H_6O_2$	5.88	38.64	0.000
Caffeic acid: *3-(3,4-dihydroxyphenyl)prop-2-enoic acid	$C_9H_8O_4$	7.119	10.77	7.97
Trans-Cinnamic acid: * (E)-3-phenylprop-2-enoic acid	$C_9H_8O_2$	32.58	38.64	2.04
Ferulic acid: *3-(4-hydroxy-3-methoxy-phenyl)prop-2- enoic acid	$C_{10}H_{10}O_4$	1.56	193.55	0.00
Quercetin: *2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy- chromen-4-one	$C_{15}H_{10}O_7$	0.00	98.11	0.00
Pinocembrin: *2S)-5,7-dihydroxy-2-phenyl-chroman-4- one	$C_{15}H_{12}O_4$	0.00	73.70	0.00
Chrysin: *5,7-dihydroxy-2-phenyl-chromen-4-one	$C_{15}H_{10}O_4$	67.03	53.29	1.73
Galangin: *3,5,7-trihydroxy-2-phenyl-chromen-4-one	$C_{15}H_{10}O_5$	70.13	63.51	1.95
3.5 dihydroxy isoflavone: *3.5-Dihydroxy-3-(4- hydroxyphenyl)chromen-4-one	$C_{15}H_{10}O_5$	0.546	16.79	0.0393
Pinostrobin: *5,7-dihydroxy-2-phenyl-chroman-4-one	$C_{15}H_{12}O_4$	0.00	76.79	0.00
Daidzin: *7-hydroxy-3-(4-hydroxyphenyl)chromen-4-one	$C_{15}H_{10}O_4$	0.199	25.09	0.269
Genistein: *5,7-dihydroxy-3-(4- hydroxyphenyl)chromen-4-one	$C_{15}H_{10}O_5$	9.90	87.40	0.00
Catechines: *(2R,3S)-2-(3,4-dihydroxyphenyl)chroman-3,5,7-triol	$C_{15}H_{14}O_6$	9.10	12.13	29.60
Acacetin: *5,7-dihydroxy-2-(4- methoxyphenyl)chromen-4-one	$C_{16}H_{12}O_5$	48.32	11.60	1.93
Phenolphthalein: *2-[(4-hydroxyphenyl)-(4-oxo-1-cyclohexa-2,5-dienylidene)methyl]benzoic acid	$C_{20}H_{14}O_4$	10.44	14.85	10.44
Daidzein: *7-(-D-Glucopyranosyloxy)-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one	$C_{21}H_{20}O_9$	42.97	50.97	0.00
Total Peak Area		42533437	112470140	11014825

*IUPAC name: E.EEP: Egyptian ethanolic extract propolis, C.EEP: Chinese ethanolic extract propolis, W.EEP: Wax comb extract ethanolic extract propolis.

Median lethal concentration and time of using EEP on honeybee workers

The objectives of the present study are to determine the acute oral toxicity, expressed as half lethal time and concentration (LT_{50} and LC_{50}) of three kind of EEP (C., E. and W.) on honeybee workers and to evaluate the safe concentration of them to be applied on colonies infected with AFB, about the LT_{50} data. Table 2 demonstrated that there were significant differences among the concentration 2, 1 and 0.5% of three tested kind of EEP and there was no significant difference in 0.1, 0.05 and 0.025% of three kind of EEP in comparison to control (0.00% of EEP), so the high concentration of C., E. and W.EEP (2, 1 and 0.5%) had effected toxically on honeybee worker (oral administration), on the other hand the low concentration of C., E. and W.EEP (0.1, 0.05 and 0.025%) had a safely effect on honeybee workers. Data in Table 3 demonstrated that the W.EEP was more toxic (LC_{50} = 1.404) than C.EEP (LC_{50} = 15.047) and E.EEP (LC_{50} = 8.223), in addition there are a significant deference among the three kind of EEP in LC_{50}, lower and upper limit of LC_{50} were reported in the table.

The reduction percentage (rate) of infection

The effect of EEP on the counts of infected larvae per hive was assessed by feeding, result obtained are summarized in Table 4, it is clear that tylosin and 0.1 and 0.05% E.EEP had a high significant positive influence on controlling the growth of *Paenibacillus larvae* with 100% reduction rate. The C.EEP and W.EEP group had a significant deference when compared with untreated (Con.) in three concentrations 0.1, 0.05 and 0.025%, with the mean rate of reduction 69.13, 64.98 and 40.66, for C.EEP group, respectively. In addition the reduction rates

Table 2. Median lethal time at least at 50% (LT_{50}) of C., E. and W.EEP on honey bee workers.

Concentration of EEP. Soluble in sugar solution 50% (% (w/v))	C.EEP	E.EEP	W.EEP
	LT_{50} (day)		
2.000	$12.40^{b} \pm 0.11$	$10.60^{b} \pm 0.49$	$9.10^{d} \pm 0.72$
1.000	$12.10^{b} \pm 0.23$	$11.20^{b} \pm 0.26$	$8.70^{d} \pm 0.46$
0.500	$13.80^{b} \pm 0.46$	$11.10^{b} \pm 0.89$	$10.80^{c} \pm 0.14$
0.100	$19.90^{a} \pm 0.86$	$18.50^{a} \pm 0.40$	$16.00^{b} \pm 0.26$
0.050	$19.70^{a} \pm 0.69$	$18.70^{a} \pm 0.75$	$21.06^{a} \pm 0.80$
0.025	$20.30^{a} \pm 0.40$	$19.30^{a} \pm 0.66$	$20.60^{a} \pm 0.40$
0.000	$19.80^{a} \pm 0.63$	$19.80^{a} \pm 0.17$	$19.80^{a} \pm 0.63$

E.EEP: Egyptian ethanolic extract propolis, C.EEP: Chinese ethanolic extract propolis, W.EEP: Wax comb extract ethanolic extract propolis.

Table 3. Median lethal concentration (LC_{50}) of C., E. and W.EEP on honey bee workers.

	C.EEP	E.EEP	W.EEP	F	P	$L.S.D_{0.05}$
LC_{50} (%)*	$15.04^{a} \pm 0.16$	$8.223^{b} \pm 0.20$	$1.404^{c} \pm 0.19$	139.598	0.000	1.998
Upper limit %	131.07 ± 0.08	31.63 ± 0.16	4.41 ± 0.15			
Lower limit %	5.04 ± 0.06	3.73 ± 0.18	0.72 ± 0.10			

*percentage of ethanolic extract propolis in sugar syrup (50% w/v), E.EEP: Egyptian ethanolic extract propolis, C.EEP: Chinese ethanolic extract propolis, W.EEP: old wax comb extract ethanolic extract propolis.

in W.EEP group were 87.95, 57.29 and 60.67%, respectively. Therefore, from mentioned results it could be concluded that the two investigated concentration (0.1 and 0.05% E.EEP) had inhibitory effect on viability and growth of *Paenibacillus larvae* under filed conditions.

DISCUSSION

The present investigation is a systematic study to evaluated using the ethanolic extract propolis, EEP for controlling AFB disease infected colonies. The differences observed in the propolis composition in the three kinds of EEP, it may be due to the deferent in vegetal source available in the collecting area (Egypt and China) (Table 1), the chemical composition of propolis is dependent on its geographical location; as a result, its biological activity is closely related to the vegetation native to the site of collection (Park et al., 2002; Christov et al., 2005). The antibacterial activity of EEP could be related to the chemical composition of propolis, which includes phenolic compounds (flavonoids and aromatic acids), terpenes and essential oils among others (Forcing, 2007). The antibacterial and antifungal activities of European and Uruguayan propolis are mainly due to flavonones, flavones, phenolic acids and their esters while in the case of Brazilian propolis such activities are due to prenylated o-coumaric acids and diterpenes (Ghisalberti, 1979; Kujmgiev et al., 1993; Marquee, 1995; Kanazawa et al., 2002; Bankova, 2005).

Results in Tables 2 and 3 indicated that the high concentration of EEP (2, 1 and 0.5%) affected toxically on honeybees that may be due to the anti nutritive compound like phenolic compound which occurs in propolis in high concentration so we cannot use it in field experiment. The ANFs (Anti-nutritive factors) which have been implicated in limiting the utilization of shrub and tree forages include non-protein amino acids, glycosides, phytohemagglutinins, polyphenolics, alkaloids, triterpenes and oxalic acid, ANFs may be regarded as a class of these compounds which are generally not lethal and they diminish animal productivity but may also cause toxicity during periods of scarcity or confinement when the feed rich in these substances is consumed by animals in large quantities (Agenda and Tshwenyane, 2003).

The present work reports the systematic study about the use of the ethanolic extract of propolis for the treatment of *P. larvae*-affected bee colonies. These results (Table 4) indicate that EEP has a direct *in vivo* antibacterial activity against *P. larvae* vegetative cells and that very low concentrations of propolis were required to inhibit its growth and these results are based on the compounds soluble in organic solvents (phenolic compounds) these compounds are responsible for the main part of the biological activity of propolis, on other hand it is important to note that the concentration of ethanolic extract propolis were significantly different, especially in regards to the active components. These results are in accordance with previous works that reported the antibacterial activity of EEP against

Table 4. Evaluation of the effect of EEP administered by feeding on the mean number of infected larvae per hive and reduction rate.

EEP.	Concentration of EEP. soluble in sugar solution 50% (w/v)	Number of Infected larvae (diseased cells) per hive		Reduction rate (%)
		Before	After	
	0.100	117	171	$69.13^{c} \pm 4.32$
C. EEP	0.050	134	205	$64.98^{cd} \pm 4.22$
	0.025	87	245	$40.66^{e} \pm 2.43$
	0.100	115	0	$100.0^{a} \pm 0.00$
E. EEP	0.050	111	0	$100.0^{a} \pm 0.00$
	0.025	111	41	$91.47^{b} \pm 0.56$
	0.100	113	65	$87.95^{b} \pm 0.86$
W. EEP	0.050	110	222	$57.29^{d} \pm 0.63$
	0.025	106	200	$60.67^{d} \pm 5.083$
T.	1.000*	105	0	$100.00^{a} \pm 0.00$
Con.	0.000**	92	444	$0.00^{f} \pm 0.0000$
F				113.544
P				0.000
$L.S.D_{0.05}$				7.388

* 200 mg of tylosin tartrate with 20 g confectioner's sugar, ** sugar syrup 1:1 without EEP, T.: Tylosin tartrate, E.EEP: Egyptian ethanolic extract propolis, C.EEP: Chinese ethanolic extract propolis, W.EEP: old wax comb extract ethanolic extract propolis, T: Tylosin. Con: Control.

diverse pathogens (Drago et al., 2000; Garedcw et al., 2004). Antibacterial effect of propolis was also demonstrated, since a significant decrease in the number of *P. larvae* spores/g of honey was found in naturally infected beehives treated with EEP. The proposed mechanism of action, includes the oral ingestion of EEP by adult honeybees and its delivery to larvae with feeding, facilitating the interaction and direct antibacterial effect on *P. larvae* vegetative cells, the addition of honey to the larval diet is around the third day of the larval stadium, coinciding with germination and multiplication of vegetative cells of *P. larvae* (Shuel and Dixon, 1960; Hansen and Brodsgaard, 1999).

The site(s) and number of hydroxyl groups on the phenol ring are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Weissman, 1963). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman, 1987). Flavones are phenolic structures containing one carbonyl group their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Tsuchiya et al., 1996). Simuth et al. (1986) reported that the mechanism of propolis action on microorganisms seems to be complex with respect to those components which are presently known. The inhibition of cell division and of cross wall separation of daughter cells by EEP led to the formation of pseudo-multicultural streptococci. This effect could be due to the blockage of the so-called splitting system of the cross wall as was demonstrated by *S. aureus* during treatment with trimethoprim (Nishino et al., 1987). The inhibition of cell division observed in the presence of EEP suggested that this natural drug would act like nalidixic acid which is known to inhibit DNA replication and, indirectly, cell division and propolis inhibited the synthesis and secretion of proteins from the bacterial cells (Nintendo et al., 1994).

Karina et al. (2008) propose that this mechanism cannot prevent the infection of new larvae with *P. larvae* spores, but can inhibit the replication of vegetative cells in the larval gut. Moreover, we cannot rule out a possible indirect effect of the propolis due to the stimulation of the bee immune system. Several authors have reported the stimulating effect of propolis in the innate and adaptive immune response of mouse, bovines and humans. *In vitro* and *in vivo* assays demonstrated that propolis activates macrophages, increasing their microbecide activity, enhances the lytic activity of natural killer cells and stimulates antibody production (Forcing, 2007). Enhancement of the defense response of honeybees by propolis could also be important for the control of other honeybee diseases (Evans et al., 2006). The mixture and

combined effects of its different components decrease the chance of propolis-resistant bacterial strains emergency, due to the several target sites probably present in a bacterial cell (Rios et al., 1988; Denyer and Stewart, 1998). The present findings indicate that the antibacterial activity, and perhaps other biological properties of propolis, could not be correlated with their propolis concentration but mostly to their chemical composition which can be variable according to the collection site and vegetal source.

Conclusion

The aim of this study develop a new strategy for controlling American FoulBrood (AFB) disease by using a natural antibiotic collected by honeybees from plant resins which is called propolis, to avoid using a common antibiotic (tylosin and oxytetrecycline) for its several problems, chemical residues, reduce the life time of bees and the risk of resistant strains emergency. Thirty-three colonies had a sever degree of AFB disease which is located in experimental apiary of beekeeping research department, plant production research institute, Egypt were treated by several concentration of three kind of propolis ethanolic extract (Chinese, Egyptian and old wax comb propolis) soluble in sugar solution 50%. Result indicated that tylosin, 0.1 and 0.05% of Egyptian propolis ethanolic extract eliminating of AFB clinical symptoms at 100% of reduction rate. This result could be related to the chemical composition of propolis which includes a high active phenolic compounds.

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