Physiochemical and antimicrobial properties of four Egyptian honeys with reference to American foul Brood disease

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Abstract: American Foul Brood disease (AFB) is one of the most severe bacterial diseases that affect honey bee larvae *Apis mellifera*, therefore, causing bee colony decrease. The aim of the present study was to evaluate the effectiveness of selective honey types against *Paenibacillus larvae larvae*. .Results showed that different honey types at different concentrations (5,10 and 20%) showed antibacterial activity against *P .larvae larvae* bacteria with variable degrees reached to 27.75mm .In spite of the antibacterial activity of camphor honey, which has high level of Hydroxymethyl furfural(HMF) and adulterated honey with different level of sucrose did not show any inhibitory effect on *P. larvae larvae* . Results of physical analysis showed that the specific gravity, viscosity and color in all honey types were ranged between (1.3-1.44), the viscosity ranged from 13.6 to 87.8. the Electrical conductivity (EC) values of samples were < 0.012 mS/cm), the total soluble solids (T.S.S) percentage ranged between 77.0 to 83.2% and moisture contents ranged from (17.0 to 23.0%). The chemical characteristic of honey samples compared with (HMF) and honey adulterated with sucrose indicated that all samples were acidic. Free acidity, lactones and total acidity values were also within the limit (11 mS/cm to 78 mq/kg), (1 mq/kg to 34 mq/kg) and (12 mq/kg to 93.5 mq/kg) respectively . HMF is only present in trace amounts in fresh honey.
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1.INTRODUCTION

American foulbrood (AFB) is a vicious infectious disease affecting honey bee larvae, Apis mellifera and other Apis spp. Worldwide, De Graaf et al.,(2006) caused by *Paenibacillus larvae* larvae, a Gram positive bacterium which can produce over one billion spores in each infected larvae. The infection transmitted to larvae by nurse bees or by spores remaining at the base of a brood cell, the susceptibility of larvae to AFB disease decreases with age increase, Von Der Ohe and Dustmann (1997). Exchanging combs containing the remains of diseased brood is the most common way of spreading the disease from colony to colony. The use of oxytetracyline to control AFB in north America is now in jeopardy with the advent of oxytetracycline-resistant strains throughout the new world, Alippi 1996; Miyagi et al., 2000) resistant strains were first discovered in Canada in the late 1990's, Van-Westendorp 2001; Tuckey 2002.

The control should ensure that the pathogens are reduced to a level at which they do not provoke further clinical symptoms of the disease. The control can be carried out using antibiotics or management techniques, Hansen and Brodsgaard(1997). However, residues of oxytetracycline have been found in honey from the brood nest of colonies fed antibiotic extender patties, furthermore, strains of *Paenibacillus larvae larvae* may develop resistance to sulfathiazole and oxytetracycline after continuous usage, Morse and Shimanuki(1990). Mirosamycin, another macrolide antibiotic, has been studied as a result of its high activity in this initial screen, Nakajima et al.,(1998). Ampicillin as an another antibiotic with high activity in vitro; when it was tested in beehives gave high residues in honey but very low levels in larvae, casting doubt on its utility in disease control, Nakajima et al.,(1998).Oxytetracycline hydrochloride (OTC= Terramycin®) remains the only approved drug treatment available in the United States for the prevention and control of AFB, Shimanuki(1997) . As late as 1993, no difference in sensitivity to OTC could be detected in laboratory disk diffusion assays using *P. larvae* spores collected prior to and after the introduction of OTC, Shimanuki and Knox(1994). However, in 1996 reports from Argentina indicated a possible drug resistance, Alippi (1996). Honey is well known for its antimicrobial properties Microbes that may be found in honey are primarily yeasts and spore-forming bacteria. The medicinal properties of honey has been reported and documented by bee keepers and medical practitioners alike,

Molan(1992); Bankova et al.,(2000).The healing property of honey is due to the fact that it offers antibacterial activity and its high viscosity helps to provide a protective barrier to prevent infection. Manisha and Shyamapada (2011) documented that the antimicrobial activity in most honeys is due to the enzymatic production of hydrogen peroxide .

The aim of the present work was to evaluate the antibacterial effect of four Egyptian honeys (Citrus; Clover; Cotton & Camphor) on *P. larvae larvae*. A Physicochemical properties of those four honeys were also tested against honey adulterated with HMF or sucrose.

2.Materials and Methods:

2.1.Materials:

2.1.1. Bacterial strains:

P.larvae larvae larvae bacteria was isolated from an infected brood comb.

The bacterial spores collected from the dried remains of infected bee larvae. They were taken to prepare fresh inoculums. Streak was taken using sterile cotton swap and was suspended in 9 ml sterile dist. water in a screw-capped. Then the suspension was heat shocked at -80°C for 10 minutes to kill any non-spore-forming bacteria. 0.2mL of the stock suspension was used for bioassay, Adjguzel et al., 2005; Mahesh and Satish,2008.

2.1.2. Honey samples:

-Honey samples used were:

Citrus (*Citrus spp*), Clover (*Trifolium alexandrium*), Cotton (*Gossypin barbadens*) & Camphor (*Trifolium alexandrium*).

-They were collected from various apiaries.

-Adulterated honey was used as a comparative control

2.2.Methods:

2.2.1. Preparation of the inoculums

After isolation of *P.larvae larvae 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 aliquots of suspension were frozen at – 70 °C until required. The verification of *P. larvae larvae* was made using catalase-test, Haynes(1972) with Columbia agar and the "Plageman" test with Columbia sheep blood agar slants, Plagemann (1985) and then inoculated 40 ml 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 hours at 37 °C, when the suspension reached an optical extinction of 0.22–0.23. The bacterial growth was determined after measuring the absorbance at 546 nm,Barry **(**1976).

2.2.2. Treatments preparation

After collecting of honey samples, they were stored in dark cabinet at room temperature. Then, three honey dilutions (5%, 10% & 20%) were prepared. For comparison, Five levels of Hydroxymethyl forfural (HMF) (288, 203, 138, 76.8 and 90.0 mg/kg) and three different concentrations (13, 14 & 15%) of sucrose solution were also prepared.

Samples from all treatments and dilutions were analyzed for the followings: Physical properties which includes; specific gravity, viscosity, color, electrical conductivity (%) and total soluble solids (%). Chemical properties which includes; moisture (%), pH, free acidity (mq/kg) , lactone (mq/kg), total acidity (mq/kg) , carbohydrate (%) , fructose (%), glucose (%), sucrose (%), maltose (%) and Hydroxymethyl forfural (HMF).

2.2.3. Physical properties:

a- ***The specific gravity, viscosity and the color*** were measured according to(Munro, 1943; Wedmore, 1955; White, 1978). Electrical conductivity was determined by conducterimetric assay (WTW Inolab conducterimeter), from a solution containing 10 g of honey in 75 mL of distilled water, Sancho et al., (1992) the total soluble solids (TSS%) was measured according to A.O.A.C. (1980).

b-Moisture determination: A.O.A.C. (1990a) Official Method 969.38) was ascertained by refractometry, using an Abbe refractometer (Digital refractometer Atago, Germany). All measurements were performed at 20°C, after waiting for 6 min for equilibrium, and obtaining the corresponding % moisture (g/100 g honey) from the refractive index of the honey sample by consulting a standard table for the purpose.

2.2.4. The chemical parameters:

a- ***Honey pH*** was measured, with a combined pH glass electrode connected to pH meter Basic 20, in a solution prepared with 10 g of honey in 75 mL of distilled water (NP 1309/1976). Free acidity was determined by potentiometric titration A.O.A.C. (1990a) Official Method 962.19. Honey samples were homogenized in a water bath and filtered through gauze, prior to analysis. Ten grams of honey were then dissolved in 75 mL of distilled water, and alcoholic solution of phenolphthalein added. The solution was titrated with 0.1 N NaOH. Acidity (milliequivalent of acid per kg of honey) was determined as 10 times the volume of NaOH used in titration.

b-***The total sugar content*** of the honey tested was similar with the findings of other previously studied by High Performance Liquid Chromatography (HPLC) measured the concentration of fructose, glucose, sucrose and maltose (%) according to Bogdanov and Bauman (1988)Hydroxy methyl

furfural (HMF) was determined by using the standard method A.O.A.C. (1990a) Official Method 980.23. Five grams of honey were dissolved in 25 mL of distilled water, treated with a clarifying agent (0.5 mL of Carrez I and 0.5 mL of Carrez II solutions) and volume made up to 50 mL. The solution was filtered, and the first 10 mL discarded. The absorbance of the filtered solution was measured at 284 and 336 nm against an aliquot of the filtered solution treated with NaHSO3. HMF was determined as: HMF/100 g of honey = (Abs_{284} _ Abs_{336}) x 14.97 x (5/g of sample).

3.RESULTS AND DISCUSSION

3.1.Physical analyses

Table (1) shows the results obtained for the physical parameters analyzed in the four honey samples. The specific gravity, viscosity and color in all honey types were ranged between (1.3-1.44), and the viscosity ranged from 13.6 to 87.8, all samples were found to meet honeys quality European Legislation, EU(2001).

Electrical conductivity (EC) is one of the most important factors for determining the physical characteristics of honey, Serrano et al.,(2004). It is also an important physical measurement for the authentication of uni-floral honeys, Mateo and Bosch-Reig (1998). With the exception of a single sample (0.044 mS/cm), the EC values of samples were within the allowed parameters (lower than 0.012 mS/cm) (table 1). According to Persano et al.,(1995) the nectars from some plants are stronger than others, and even low contamination of honey with ―stronger nectar can modify its sensory and physical properties. The EC values of four honey were reported to be 0.21–1.61 mS/cm in a previous study by Ouchemoukh et al.,(2007). However, our results were similar to the findings previously reported by Saxena et al.,2010; Alvarez-Suarez et al.,2010.

TSS is a measure of the combined content of all inorganic and organic substances in honey in the molecular, ionized or micro-granular (colloidal solution) suspended forms. Our data represented in table (1) reveled that, the total soluble solids (T.S.S) percentage ranged between 77.0-83.2%. These results demonstrated that there is a good correlation between EC and TSS, indicating that both parameters can be used to determine honey purity EU(2001).

Honey moisture content depends on the environmental conditions and the manipulation from beekeepers at the harvest period, and it can vary from year to year, Acquarone et al.,(2007). High moisture content could accelerate crystallization in certain types of honey and increase its water activity to values where certain yeasts could grow. Moisture contents of honey samples ranged from (17.0 to 23.0%) which are well below to the imposed limit of 620% EU(2001). There were no significant differences, between humidity values obtained for the four honey samples. These results are indicative of good storage ability of these honeys, since high moisture content could lead to fermentation during storage as shown in table (1).

Table (1) Physical Properties of Egyptian Citrus; Clover; Cotton & Camphor honeys.

Parameters	Honey samples					
	Citrus	Clover	Cotton	Camphor	high level of HMF value	high level of Sucrose value
Specific gravity	1.35±0.03	1.41±0.006	1.40±0.09	1.42±0.01	1.31±0.06	1.40±0.003
Viscosity(Poise)	31.66±9.99	66.93±11.40	46.10±28.92	46.10±16.70	46.10±16.78	53.10±21.55
EC (%)	0.01±0.003	0.02±0.009	0.013±0.006	0.03±0.007	0.003±0.003	0.01±0.00
T SS (%)	78.73±1.20	81.33±1.52	80.00±2.00	80.47±1.51	81.33±0.73	88.83±0.44
Moisture (%)	21.00±1.32	18.10±0.74	19.63±1.21	18.77±0.88	19.00±1.32	19.00±0.58
Total	26.53±7.86	33.56±9.30	29.43±8.58	29.36±8.63	33.69±8.70	30.87±9.17

3.2.Chemical analysis

The data represented in table (2) refers the chemical characteristic of honey samples compared with honey adulterated with (HMF) or sucrose. All of the tested honey samples were acidic in nature, with pH values that varied between 3.5 and 4.4 (table 2). These values were similar to those previously reported for other honey samples from India, Brazil, Spain and Turkey, which were reported to have pHs between (3.49 to 4.70), Azeredo et al.,(2003). The low pH of honey inhibits the presence and growth of microorganisms. These parameters have great importance during the extraction and storage of honey, as they influence the texture, stability and shelf life of honey, Terrab et al.,(2002) . A highly acidic honey sample indicates the possible fermentation of sugars into organic acids, Saxena et al.,(2010).

Free acidity, lactones and total acidity values are also within the limit (11 mS/cm to 78 mq/kg), (1 mq/kg to 34 mq/kg) and (12 mq/kg to 93.5 mq/kg) respectively, data shown in table (2) . The free acidity

of honey may be explained by taking into account the presence of organic acids in equilibrium with their corresponding lactones, or internal esters, and some inorganic ions, such as phosphate. High acidity can be indicative of fermentation of sugars into organic acids, Kayacier and Karaman(2008) . None of the samples exceeded the limit allowed, which may be taken as indicative of freshness of all honey samples.

The total sugar content of the honey tested was similar with the findings of other previous studies, Ouchemoukh et al.,(2007).None of the samples exceeded the highest limit set for total sugar content by the European community directive as shown in (table 2). These samples do not only meet the standards but also correspond to the levels observed in other studies,(Andrade et al.,1999; Rodriguez et al., 2004; Kucuk et al., 2007). No significant differences were observed between reducing sugars (glucose and fructose) values obtained for the four analyzed honey samples. Higher sucrose contents in clover honey could be the result of an early harvest of honeys, i.e., the sucrose has not been converted to fructose and glucose, Azeredo et al.,(2003). The values obtained for honey samples are among the limits given by the European directive for this parameter.

HMF formation results from the acid-catalyzed dehydration of hexose sugars with fructose being particularly susceptible. In addition, HMF is only present in trace amounts in fresh honey, and its concentration has been reported to increase with storage and the prolonged honey heating. HMF is thus an essential parameter used to indicate honey purity. The HMF concentrations of the remaining honey samples were similar, ranging from 12.0 to 32.6 mg/kg (table 2). Notably, all HMF concentrations were within the recommended range set by Alimentarus Codex and Alinorm(2000)at 80 mg/kg. The values are also within the allowed maximum limit of 40 mg/kg, as recommended by the Turkish Alimentarus codex (2003) for honey samples from tropical countries.

Our results were agreement with HMF content of honey samples from different countries. Ajlouni and Sujirapinyokul (2010)reported low HMF concentrations of two unprocessed Australian honey samples, Grey box and Banksia (1.35 and 1.12 mg/kg, respectively). The HMF concentrations of some Australian honeys, such as rainforest, Homebrand and Mallee honey, were reported to be (2.2, 17.7 and 34.0 mg/kg) respectively, Fallico et al.,(2004). High HMF formation may occur due to overheating, exposure to high temperatures, Ajlouni and Sujirapinyokul (2010) or the type of sugar present in the honey, as well as the fructose/glucose ratio, Fallico et al.,(2006).Overall, the low HMF concentrations of the tested honey confirm that these samples are of good quality.

Table (2) Chemical Properties of Egyptian Citrus; Clover; Cotton & Camphor honeys.

Parameters	Honey samples					
	Citrus	Clover	Cotton	Camphor	high level of HMF value	high level of Sucrose value
pH	3.6-3.8	3.3-4.1	3.6-4.3	3.9-4.23	3.5-4.4	3.4-3.9
Free acidity (mq/ kg)	26.0-35.0	11.0-78.5	16.5-55.0	38.6-49.1	0.19-82.5	18.5-26.5
Lactone (mq/kg)	3.3 -34.0	1.0-3.5	1.0-22.5	10.5-13.4	4.5-7.5	1.0-7.5
Total acidity (mq/ kg)	38.3-60.0	12.5-79.5	17.5-77.5	52.0-60.4	30.5-93.5	19.5-34.0
Fructose (%)	35.1-41.0	36.8-40.0	35.7-37.0	38.9-40.37	37.6-40.0	30.0-36.6
Glucose (%)	27.7-30.0	29.0-31.0	29.1-31.0	32.0-33.4	21.0-34.2	23.5-29.3
Sucrose (%)	0.75-3.9	2.5-4.2	1.1-2.6	1.6-3.97	0.6-4.5	13.0-17.7
Maltose (%)	5.0-9.0	3.2-7.4	2.0-2.6	2.6-4.97	0.0-5.5	1.0-6.0
HMF(mg/kg)	3.0-23	7.7-32.6	5.0-15.0	3.98-12.0	76.0-288.0	3.84-18.5

3.3. Antibacterial activity

The different honey types at different concentration (5, 10 and 20%) showed an antibacterial activity against a *P. larvae larvae* bacteria with variable degrees reached to 27.75 (table 2). Data shows that the antibacterial activity of camphor honey, which has high level of HMF and adulterated honey with different level of sucrose did not show any inhibitory effect on bacterial growth of *P. larvae larvae* . By using of the least significant differences (LSD) test for multiple comparisons, it is apparent that the clover honey inhibition zone (12.5mm and 17.75 mm) was the highest significant effect on *P. larvae larvae* than other treatment at 5 and 10% honey, respectively (P=0.004,LSD=6.64) and (P=0.000,F=50.95,LSD=2.73). At 20% honey,

cotton honey the inhibition zone, was 27.75mm showed the highest significant inhibitory effect on *P.larvae larvae* growth compared to other honey treatment (P=0.018, F=4.28, LSD=12.75). The inhibition zone diameter of different honey types at 20% concentration significantly showed the strongest effect on *P. larvae larvae* compared to other concentrations (5 and 10%).

Overall the differences seen in the antimicrobial activity of the different honey types could be due to the variations in the level of hydrogen peroxide that arises in honey and in some cases to the level of non-peroxide factors. Hydrogen peroxide can be destroyed by components of honey; it can be degraded by reaction with ascorbic acid and metal ions, and by the action of the enzyme catalase which comes from the pollen and nectar of certain plants Almahdi Melad et al.,(2003). Also, very large differences have been found between honeys from different floral sources, e.g. in the thermal stability of their glucose oxidase content and in the sensitivity of the hydrogen peroxide-producing enzyme to denaturation by light because of a photosensitizing component that comes from some floral sources, (Allen et al.,1991;Bogdanov, 1997 ; Taormina et al.,2001) reported that darker colored honeys are generally more inhibitory than light colored honeys, due to their higher content of minerals and antioxidants. Gheldof et al., (2002) reported that the antioxidant capacity of honey appeared to be a result of combined activity of a wide range of compounds including phenolics, peptides, organic acids, enzymes, Maillard reaction products, and possibly other minor components.

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 microdilution method. 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. These data were conserved with the data obtained by McCarthy(1995) who reported that, honey from different floral sources varies greatly in their antibacterial activity. Rybak and Szczęsna (1996) found that the minimum concentrations of honey which inhibit the growth of *B.subtilis* were 5-10%. Molan et al.,(1988) 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. Radwan et al.,(1984) reported that honey from *Acacia mellifera* inhibits the growth of *E.coli* ,Molan and Russell (1988)found that pollen present in honey could be the source of the antibacterial aromatic acids.

Table (3) Antibacterial activity of honey types (5, 10 and 20% honey) on *Paenibacillus larvae larvae*, the inhibition zone diameter (in mm).

Honey types	5%	10%	20%	P	F	LSD
Camphor	0.0	12.5	17.75	0.002	28.15	2.421
Citrus	5.5	14.75	18.75	0.0001	20.123	4.88
Clover	12.5	17.75	21.5	0.002	10.304	5.241
Cotton	5.5	14.0	27.75	0.008	7.489	11.63
HMF(288mg/kg)	0.0	14.0	18.0			
HMF 203	0.0	11.0	13.0			
HMF 138	0.0	0.0	0.0			
HMF 76.8	0.0	11.0	14.0			
HMF 90.0	0.0	0.0	0.0			
Sucrose 13 %	0.0	0.0	0.0			
Sucrose 14 %	0.0	0.0	0.0			
Sucrose15 %	0.0	0.0	0.0			
P	0.004	0.000	0.018			
F	6.410	50.95	4.28			
LSD	6.64	2.78	12.75			

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