Vol 1 No 4 December 2020 Pages 13-18 e-ISSN 2708-5694

Journal of Agriculture, Food and Environment (JAFE)

Journal Homepage: http://journal.safebd.org/index.php/jafe

http://doi.org/10.47440/JAFE.2020.1403

Original Article

Phenolic contents and antimicrobial activity of some kinds of Libyan honey

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Article History

Received: 25 September 2020

Revised: 25 November 2020

Accepted: 09 December 2020

Published online: 31 December 2020

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Keywords

Phenolic compounds, antimicrobial activity, Floral origin, Libyan honeys, Authenticity.

A B S T R A C T

The phenolic contents of 6 Libyan honey varieties of different floral sources were determined. Honey samples included the 5 mono-floral kinds of honey, *Ziziphus louts*, *Citrus medica*, *Thymus capitatus*,*Amygdaluscommunis* and *Commiphormyrrha*, while the multi-floral honey was Rabia (spring) honey. The analysis of phenolic compounds was performed using High Pressure Liquid Chromatography (HPLC). Twenty three phenolic components in the different kinds of honey were determined. The highest number of phenolic components were found in the darker honey, *Thymus* and *Commiphor* followed by *Citrus*, Rabia and *Ziziphus*, respectively. The least number of phenolic components were detected in *Amygdalus* (only 4). *p*-Hydroxybenzoic acid was found in all studied honey varieties, while rutin was not detected in any of the honey samples analyzed. Gallic acid and chrysin were found only in *Thymus* honey, Caffeic acid, salicylic acid and pinostrobin were only in *Commiphor* honey, while catechin, daidzein and pyrogallic were detected only in *Citrus* honey. The antimicrobial effect on *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Enterococcus faecalis, Pseudomonas aeuroginosa, Escherichia coli, Bacteriods spp., Sarcina spp.* and *Candida albicans*was studied. All honey samples inhibited the growth of *Escherichia coli* with different degrees, where P<0.001. Among all bacteria, *Bacteroids* spp. and *Klebsiella pneumoniae* were the most resistant against most honey samples.

Introduction

Honey is a complex natural food produced from the honey bee *Apismellifera* feeding on plant nectar of blossoms, exudates of trees and plants, or from honey bees feeding on honeydew produced by hymenoptraninsects. Honey is a saturated solution of sugar of 31% glucose and 38% fructose, and its colour and flavor vary considerably depending on its botanical and geographical origin (Gheldof *et al.*, 2002) and of moisture content of about 17.7% (Nagai *et al.*, 2006). In addition to the minor component of phenolic acids, flavonoids, glucose oxidase, catalase, ascorbic acid, carotenoids, organic acids, and α-tocopherol (Ferreres *et al.*, 1993). Honey contains at least 181 components (White 1975).Phenolic compounds are common in plants and collected by honey bees with nectar (Scalbert *et al.*, 2005; Fiorani *et al.*, 2006; Pyrzynska and Biesaga 2009). Some phenolic compounds have been shown to exhibit antibacterial, antiviral, anti-inflammatory, anticarcinogenic, antiatherogenic, antithrombotic, Immune-modulating and analgesic activity (Evers *et al.*, 2005; Harris *et al.*, 2006; Nasuti *et al.*, 2006 and Viuda-Martos *et al.*, 2008). Phenolic contents, free amino acids, volatile compounds, trace elements as well as physiological and chemical characters have been used to determine the botanical and geographical origin of honey (Senyuva *et al.*, 2009; Ioannis *et al.*, 2014 and Youngsu *et al.*, 2015). Mohamed *et al.*, (2017) studied the physiological characteristics and total phenolic compounds contents of some Libyan honey collected from the local markets of Banghazi city in east Libya. The samples included the four mono-floral honey, *Ziziphus louts, Thymus capitatus, Eucalyptus sp.* and *Arbutuspavari*, and the multi-floral honey Al-Rabia. They found that the total phenolic compound content of the samples ranged from 97.67-123.50 mg gallic acid / 100g of honey, with a mean value 100.64 ± 11.93 mg gallic acid / 100 g.

The use of honey for the treatment of diseases and wounds has been mentioned since ancient times (2100-2000 BC), where Aristotle (384-322 BC) described pale honey for sore eyes and wounds (Mandal and Manda 2011 and Vallianou *et al.*, 2014). The healing effect of honey could be due to its

physical and chemical properties (Rusell *et al.,* 1999 and Snow and Manley-Harris 2004) and to its antioxidant and antimicrobial activity (Escuredo *et al.,* 2012; Isidorov *et al.,* 2015; Almasaudi *et al.,* 2017 and Leyva-Jimenez *et al.,* 2019). A possible reason for its activity depends on its ability to generate hydrogen peroxide by the bee derived enzyme glucose dehydrogenase (Saleh *et al.,* 2011). Microorganisms such as *Staphylococcus aureus, Staphylococcus epidermis, Micrococcus luteus, Streptococcus uberis, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae* are frequently isolated from human and animal skin wounds (Naser *et al.,* 2003; and Altoparlak *et al.,* 2005). Abd-El Aal *et al.,* (2007) found that honey has stronger inhibitory effect (85.7%) than the commonly used antimicrobial agents on gram-negative bacteria *Pseudomonas aeruginosa, Enterobacter sp.* and *Klebsiella.* A 100% inhibition was recorded for the methicillin-resistant gram positive bacteria *Staphylococcus aureus.* The antimicrobial activity of honey against *Bacillius cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Morganiellamorganii, Micrococcus luteus, Escherichia coli and Candida albicans; Enterococcus faecalis* and the pathogenic fungi *Candidiaalbicans* has been studied by many authors (Mercan *et al.,* 2007).

The present work was aimed at to quantify the total phenolic contents of 6 Libyan kinds of honey of different floral sources and to evaluate their antimicrobial effects on *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Enterococcus faecalis, Pseudomonas aeuroginosa, Escherichia coli, Bacteriods spp., Sarcina spp.* and *Candida albicans.*

Materials and Methods

The present investigation was carried out at the Beekeeping Research Section, Plant Protection Research Institute, Giza, Egypt.

Honey samples

Six types of Libyan honey of mono and multi-floral sources were collected from selected beekeepers during the harvesting periods and from local markets in western Libya. The honey of mono-floral sources were *Ziziphus louts*, *Citrus medica*, *Thymus capitatus, Amygdaluscommunis Commiphormyrrha*, while the honey of multi-floral source was Rabia (Spring) honey. Honey samples were kept in dark at room temperature prior to analysis. The samples were investigated microscopically to determine their containing of pollen grain types.

Determination of phenolic compounds contents

The analyses of phenolic components in six types of Libyan honey and their potential for floral authentication were evaluated. The analyses included 23 standard flavones (Gallic acid, *p*-Hydroxybenzoic acid, Caffeic acid, Phenol, *p*-coumaric acid, Salicylic acid, Ferulic acid, Cinnamic acid, Quercetin, Chrysin, Galangin, Pinostrobin, Vanillin, 3,5 dimethoxy benzyl alcohol, Catechin, Daidzin, Genstin, Daidazein Gestein, Pyrogallic, and kaempherol). Extraction of phenolic compounds from honey samples was carried out using ethyl alcohol, where one g of honey was dissolved in 10ml ethyl-alcohol 70% to prepare a final concentration of 10 % honey solution, and then kept in closed glass tubes for analysis.

HPLC Identification

The identification of phenolic compounds of the honey samples was performed by a JASCO, using a hypersil C_{18} reversed-phase column (250 X 4.66 mm) with 5 µm particle size.

Injection using a Rheodyne injection valve with a 50 µl fixed loop was used. A constant flow rate of 1 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 elution gradient was linear starting with (A) and ending with (B) over 35 min, using a µv detector set at wavelength 254 nm. Phenolic compounds of each sample were identified by comparing their relative retention times with those of the standards mixture chromatogram. The concentration of individual compounds was calculated based on the peak area measurements and then converted to µg phenolic g^{-1} dry weight. All chemicals and solvents used were in HPLC spectral grade. 23 standard phenolic compounds were obtained from Sigma (St, Louis, USA) and Merck-Schuchard + (Munich, Germany) chemical companies.

Estimation weight % of phenolic compounds

The scanning of identified phenolic compounds extracted in honey samples by (HPLC) analysis is the estimation of weight % for these compound was calculated as follows:

Weight % phenolic = 100 X (PH/PH^*) X (v/v^*) X (w^* x w)

Where: PH: area for sample

PH^*: area of standard

V: volume of sample

V^*: volume of standard

W^*: weight of standard

W: Weight of sample**.**

Bacterial strains

Bacterial strains and *Candida albicans* were kindly donatedby the Microbial Genetic Department, Genetic Engineering and Biotechnology Division, National Research Center, Giza, Egypt.

Assay of antimicrobial activity

The antimicrobial activity of honey samples was determined by the disc diffusion method (Collins *et al.,* 1995). A concentration of 20% of each kind of honey in distilled water was prepared in a clean sterile test tube and kept in a refrigerator at 4^oC to be used for the microbiological test.

Preparation of the microbial culture

The tested organisms were inoculated in the appropriate liquid media and incubated at 37^oC for 24 hours. The microbial culture was used for the preparation of seed layer by inoculating the agar medium with 2% (v/v) of the microbial culture, thoroughly mixed, and immediately used as the seed layer of plates.

Preparation of plates

The appropriate agar medium was distributed at the rate of 7 ml portion in Petri dishes. After solidification 5 ml of the seeded agar was distributed over the surface of the base layer and left for 15 min to solidify. The previously prepared filter paper discs (each disc was moistened with exactly 0.05 ml of the diluted honey) placed side down on the seeded agar and gently pressed with a tip of sterile forceps. Discs were placed symmetrically around the center of the dish. Plates were incubated at 37^o C for 24 hours. For *P. aeruginosa* and for

M. leutus, plates were incubated at 30 °C. Antimicrobial activity was determined by measuring the diameter of inhibition zones around the discs to the nearest mm.

Three replicates were prepared for each honey sample. As a positive control method, the antibiotic tetracycline (30 µg) was used, while sucrose sugar solution (20%) was used as a negative control method.

Statistical analysis

Results are expressed as mean ± standard deviation. ANOVA was applied at a confidence level of 95%.

Results

The samples of analyzed honey, their local names and their floral sources are listed in the table (1). In our study of 23 phenolic components were found in the different honey samples as shown in table (2) and graph (1). Gallic acid and traces of chrysin were found to be characteristic for *Thymus*. Caffeic acid, salicylic acid and pinostrobin for *Commiphor*. Catechin, daidzein and pyrogallic for *Citrus*, while *p*-Hydroxybenzoic was detected in all honey samples. The highest number of phenolic components were found in the darker honey *Thymus* and *Commiphor* followed by *Citrus*, Rabia and *Ziziphus*, respectively. Only 4 phenolic components were detected in *Amygdalus*.

Table 1. Types and floral sources of Libyan honeys

Nr. of samples	Local name of honey	Floral source
Sample 1	Sidr	*Ziziyphus louts*
Sample 2	Limon	*Citrus medica*
Sample 3	Zater	*Thymus capitatus*
Sample 4	Lose	*Amygdaluscommunis*
Sample 5	Morr	*Commiphormyrrah*
Sample 6	Al Rabia	*Multiflora*

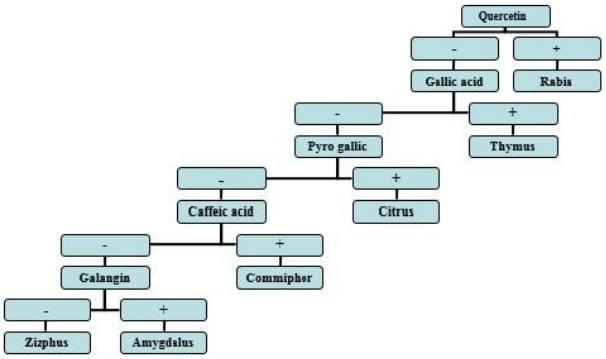
Table 2. The phenolic contents detected in Libyan honey (µg/100g)

Chemical Name	Chemical formula	Sidr µg/100g	Citrus µg/100g	Zater µg/100g	Lose µg/100g	Morr µg/100g	Al rabia µg/100g
Gallic acid	$C_7H_6O_5$	0.00	0.00	18.34	0.00	0.00	0.00
p-Hydroxybenzoic acid	$C_7H_6O_3$	251.30	83.85	154.44	69.07	1248.17	251.70
Caffeic acid	$C_9H_8O_4$	0.00	0.00	0.00	0.00	143.64	0.00
Phenol	C_6H_6O	0.00	3416.60	14737.98	0.00	9037.58	6173.74
p-Coumaric acid	$C_9H_8O_3$	2387.71	1055.94	513.37	0.00	0.00	2068.42
Salicylic acid	$C_7H_6O_3$	0.00	0.00	0.00	0.00	1524.34	0.00
Ferulic acid	$C_{10}H_{10}O_4$	0.00	269.13	2520.43	0.00	0.00	0.00
Cinnamic acid	$C_9H_8O_2$	342.41	0.00	0.00	0.00	350.26	0.00
Quercetin	$C_{15}H_{10}O_7$	0.00	0.00	0.00	0.00	0.00	45.05
Euganol	$C_{10}H_{12}O_2$	0.00	0.00	82.41	0.81	0.00	0.00
Chrysin	$C_{15}H_{10}O_4$	0.00	0.00	0.55	0.00	0.00	0.00
Galangin	$C_{15}H_{10}O_5$	0.00	0.00	0.00	1.99	0.00	0.28
Pinostrobin	$C_{16}H_{14}O_4$	0.00	0.00	0.00	0.00	40.13	0.00
Vanillin	$C_8H_8O_3$	522.23	8.44	0.00	0.00	290.20	0.00
3,5-Dimethoxybenzyl alcohol	$C_9H_{12}O_3$	0.00	0.47	0.00	0.00	0.00	10.53
Catechin	$C_{15}H_{14}O_6$	0.00	428.44	0.00	0.00	0.00	0.00
Daidzin	$C_{21}H_{20}O_9$	2746.43	0.00	0.00	11943.00	2626.99	0.00
Gestin	$C_{15}H_{10}O_5$	205.80	0.00	245.65	0.00	0.00	1293.85
Daidazein	$C_{15}H_{10}O_4$	0.00	1647.53	0.00	0.00	0.00	0.00
Genistein	$C_{15}H_{10}O_5$	0.00	0.00	75.02	0.00	295.61	0.00
Pyro gallic acid	$C_6H_6O_3$	0.00	46.22	0.00	0.00	0.00	0.00
Rutin	$C_{27}H_{30}O_{16}$	0.00	0.00	0.00	0.00	0.00	0.00
Kaempferol	$C_{15}H_{10}O_6$	0.00	0.00	27.50	0.00	17.44	0.00

Table 3. The diameter (in mm) of inhibition zones and standard deviation of different bacterial strains by honey samples compared to control

Honey samples / Bactria strains	*Zizyphus*	*Citrus*	*Thymus*	*Amygdalus*	*Commiphor*	*Rabia*	*Tetracycline*	*Sucrose*
Escherichia coli	21.0 ± 1.17^{c}	11.31±1.15^{b}	10.66±0.57^{b}	5.33±0.57^{a}	11.33±1.15^{b}	22.33±0.57^{c}	0.00	0.00
Enterococcus faecalis	0.00	0.00	0.00	22.66±0.57^{c}	12.00±1.00^{b}	12.0±0.00^{b}	20.66±1.15^{c}	0.00
Staphylococcus aureus	12.0 ± 0.0^{b}	0.00	5.33±0.57^{a}	0.00	0.00	21.33±1.15^{c}	21.0±1.17^{c}	0.00
Pseudomonas aeruginosa	11.33±1.15^{b}	11.31±1.15^{b}	0.00	11.0±0.00^{b}	0.00	0.00	0.00	0.00
Bacillus subtilis	0.00	0.00	6.33±1.15^{a}	11.33±0.57^{b}	0.00	5.00±0.00^{a}	20.0±0.55^{c}	0.00
Bacteroids spp.	6.00±0.00^{a}	11.55±1.12^{b}	0.00	0.00	0.00	0.00	0.00	0.00
Sarcina spp.	5.82±0.43^{a}	0.00	19.8±1.15^{c}	20.0±0.55^{c}	21.33±1.15^{c}	11.5±0.50^{b}	22.0±0.00^{c}	0.00
Klebsiella pneumoniae	19.8±1.32^{c}	0.00	0.00	0.00	0.00	21.0±0.00^{c}	5.66±0.57^{a}	0.00
Candida albicans	5.66±0.57^{a}	10.14±1.55^{b}	20.66±1.15^{c}	21.33±1.15^{c}	0.00	5.66±1.15^{a}	21.33±1.15^{c}	0.00

Different letters indicate significant difference ($P < 0.01$).



Graph 1. Phenolic contents as a marker and discriminant Libyan honey

In the present study *p*-Hydrobenzoic ranged from 83.85 µg/100 g in *Citrus*, 1248.17 µg/100 g in *Commiphor*. phenol from 3416.59 µg/100 g *in Citrus* to 14737.98 µg/100g in *Thymus*, *p*-Coumaric acid from 513.37 µg/ 100g in *Thymus* to 2387.71 µg/ 100g in *Ziziphus*. Ferulic acid was found only in *Citrus* (269.13 µg/ 100g) and in *Thymus* (2520.43 µg/ 100g), while cinnamic acid was detected in both *Ziziphus* and *Commiphor* (4324.11 µg/100g and 3502.63 µg/100g, respectively). Traces of euganol were found in *Amygdalus* (0.81 µg/100g), while its amount in *Thymus* measured 82.41 µg/100g. Traces of galangin were found in both Rabia and *Amygdalus* (0.28 µg/100g and 1.99 µg/100g, respectively). The amount of detected vanillin ranged from 8.44 µg/100g in *Citrus* to 290.20 µg/100g in *Commiphor*, 3,5 dimethoxybenzyl ranged from 0.47 µg/100g in *Citrus* to 10.53 µg/100g in Rabia, daidazin ranged from 2626.99 µg/100g in *Commiphor* to 11943.0 µg/100g in *Amygdalus*, genstin ranged from 2456.45 µg/100g in *Ziziphus* to 1293.85 µg/100g in Rabia, gestein ranged from 75.02 µg/100g in *Thymus* to 295.61 µg/100g in *Commiphor* and kaempherol ranged from 17.44 µg/100g in *Commiphor* to 275.04 µg/ 100g in *Thymus*.

The results of the inhibition effects of different honey samples in comparison to control are shown in table (3).It was observed that all honey samples inhibited the growth of *Escherichia coli* with different degrees, where P<0.001. The lowest effect was recorded for the *Amygdalus* honey with an inhibition zone of 5.33±1.15 mm, while the greatest effects were shown by Rabiaand*citrus* honey with inhibition zones of 22.33± 0.57 mm and 21.0±1.17 mm, respectively. Among all bacteria, *Bacteroids* spp. and *Klebsiella pneumoniae* were the most resistant against most honey samples, while five out of the six honey samples inhibited the growth of *Sarcina spp.* Except *Commiphor*, all honey samples inhibited the growth of the fungus *Candida albicans. Commiphor* honey inhibited only 3 out of the nine tested microorganisms, while *Zizyphus* and Rabia honey inhibited seven of them.

Escherichia coli, Pseudomonas aeruginosa and *Bacteriods spp.* were found to be resistant to the antibiotic tetramycine (+ve control), while 20% sucrose sugar solution (-ve control) had no inhibitory effect on all bacterial strains.

Discussion

Floral source, geographical origin, seasonal and environmental factors and processing affect the honey phenolic composition and antioxidant activities (Al-Mamary *et al.*, 2002; Gheldof *et al.*, 2002; Yao *et al.*, 2003; 2005; Ioannis *et al.*, 2014 and Youngsu *et al.*, 2015).

In the present study both benzoic was found in all studied honey varieties, while rutin was not detected in any of the honey samples analyzed. Gallic and chrysin were found only in *Thymus* honey; caffeic acid, salicylic acid and pinostrobin only in *Commiphor* honey; while catechin, daidzein and pyrogallic acid were found only in *Citrus* honey. Quercetin was detected only in multi-floral honey. Our results showed that phenolic contents can be used as a marker for the studied honey varieties. Studying the phenolic contents of *Robinia* honey samples in Croatia, Kenjerić *et al.*, (2007) reported that quercetin, kaemperol and chrysin raged from 2.9 to 29.9, 5.7 to 23.8, and 21.1 to 231.1 µg/100g, respectively. Myricetin was not detected in any of the analyzed honey samples. Martos *et al.*, (1997) studied the flavonoids composition of 13 Tunisian honeys (eucalyptus, thyme, rosemary, orange, grape, sunflower and multifloral honey) and propolis. They reported that flavonoid contents varied significantly between 20 and 2,400 µg/g. Quercetin and kaempferol were detected in linden and heather honey studied by Mechalkiewicz *et al.*, (2008). Quercetin ranged from 2.0 to 2.6 mg/kg in linden honey and 0.39 to 0.41 mg/kg in heather honey. Respective values for kaempferol were 1.5 to 1.9 mg/kg in linden honey and from 0.28 to 0.32 mg/kg in heather honey. Ioannis *et al.*, (2014) studied phenolic compounds of Greek thyme honey from the different geographical origin and found that quercetin ranged from 0.58 mg/kg (in a honey sample from Irakleio) to 69.00 mg/kg (from Hania), kaempferol ranged from 50.01 mg/kg (from Lakonia) to 61.38 mg/kg (from Hania), chrysin ranged from 0.01 mg/kg (from Hania) to 5.60 mg/kg (from Kefalonia), myricetin ranged from 0.74 mg/kg (from Hania) to 244.67 mg/kg (from Kefalonia) and syringic acid from 1.56 mg/kg (from Irakeio) to 195.4 mg/kg (from Hania).

Dark coloured *Commiphor* and *Thymus* honey were found to have the highest number of phenolic compounds among the studies of honey varieties (10 phenolic compounds). This result agrees well with the findings of Bertoncelj *et al.*, (2007), who stated that dark coloured varieties of honey have higher levels of phenolic compounds and antioxidant activities, and with the results of Youngsu *et al.*, (2015), who found that the dark colour of chestnut honey showed the higher levels of total phenolics than light coloured acacia honey. Ferreira *et al.*, (2009) studied the total phenolic contents of Portugues honey and reported 132.17 mg/kg for light coloured honey, 168.44 mg/kg for amber honey and 204.24 mg/kg for dark honey. According to the study of Mohamed *et al.*, (2017) on the total phenolic compounds contents of some Libyan honey from Banghazi city (Eastern Libya), Arbutus honey (*Arbutus pavari*) which have the highest optical density value, exhibited the highest phenolic compounds content. Further research studied on physical and chemical characteristics, organic acids, proteins, enzymes and antimicrobial effects of Libyan honey are recommended.

The antimicrobial activity of honey is mainly contributed to the high osmolarity and acidity. Besides, hydrogen peroxide, volatiles, organic acids, flavonoids, phenolic compounds, wax, pollen, propolis are important factors that provide antimicrobial properties to honey. Shin and Ustunol (2005) stated that the sugar composition of honey from the different floral source is responsible for the inhibition of various intestinal bacteria. According to Moumbe *et al.*, (2013) the minor components of honey including proteins, minerals, phytochemicals and antioxidants are responsible for the

antimicrobial activity of honey in the treatment of infections, burns, wounds and ulcers.

Our results are in agreement with other published studies, showing that some kinds of honey have an inhibitory effect against the fungus *Candida albicans* and the bacteria *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Enterococcus faecalis, Pseudomonas aeuroginosa, Scherichia coli, Bacteriods spp., Sarcina spp.* (Mercan *et al.,* 2007, Almasaudi *et al.,* 2017and Leyva-Jimenez *et al.,* 2019). Who reported that honey was effective against gram-positive bacteria *Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis* and gram-negative bacteria *Escherichia coli* and *Pseudomonasaeruginosa.* The inhibitory effect of honey against *S. aureus*, *E. coli* and *K. pneumonia* is of great importance due to the fact that Streptococcus species and coliforms are recognized pathogens. In this work, the growth of *Pseudomonas aeruginosa* was inhibited by 3 honey samples. This type of bacteria is always found in wounds, especially those related to burns causing a variety of systemic infections, particularly in victims with severe burns (Yau *et al.,* 2001). Irish *et al.,* (2011) noted that temperature, the time of storage, and the nature of the flower's nectar may explain the different antimicrobial activities of different kinds of honey.

Our data are in agreement with the findings obtained by McCary (1995), who reported that honey from different floralsources 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, which causes the component to act individually or synergically to prevent bacterial resistance (Cooper *et al.,* 2010). In addition to pollen, propolis is also found in honey. 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).

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