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Antimicrobial Activities of some Egyptian Bee Honeys against *Staphylococcus aureus* And *Pseudomonas aeruginosa*

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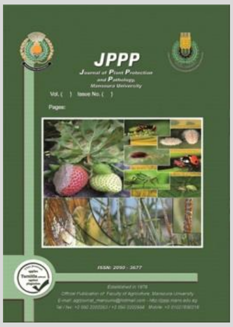

ABSTRACT

Bee honeys are natural product from the honeybee colonies which has a great nutritional and medicinal effects. In this research, different types of Egyptian bee honey were collected from 3 governorates to study their antibacterial activities related to their contents from hydroxide and non-peroxide such as phenols. The results showed that each type of bee honey had characteristics which changed by geographical collected area. All the tested bee honey samples had an inhibitory activity against of *Staphylococcus aureus* growth. The majority of tested bee honey samples had no antimicrobial effect against *Pseudomonas aeruginosa* colonies except one sample (Sesame bee honey from Asyut governorate). Hydrogen peroxide is the primarily main factor for antimicrobial activity association. Undiluted tested bee honey was the highest inhibition for growth *S. aureus* bacterial, followed by high-to-low concentrations.

Keywords: Bee honeys -antimicrobial activity - *Staphylococcus aureus* - *Pseudomonas aeruginosa*

INTRODUCTION

Nutritional and therapeutic bee honey effects have known for many thousands years. Bee honeys contained more than 200 medical compounds, besides; its inhibition properties against infected microbes growth (Allen *et al*., 1991; Greenwood, 1995; Ferreira, *et al.*, 2009 and Escuredo, *et al*., 2013).

Bee honey is a solution that is supersaturated of sugars, especially monosaccharine, and a wide range of other compounds such as minerals, proteins, amino acids, enzymes, vitamins, phenols, flavonoids, and other phytochemicals (Alqarni *et al*., 2012 and Da Silva *et al.*, 2016). The quality of bee honey is depended mainly on its chemical and biological characteristics as well as the botanical source, geographical and beekeeping practices (El-Metwally, 2015 and Solayman *et al*., 2016).

Antimicrobial activity in bee honey is the only important criterion that highlights the medical importance, especially for its local use in wound care, which has shown widespread effectiveness against a large number of microbes, including resistant species, viruses and fungi. There are multiple factors working together in the mechanism of inhibitory activity for the growth of microbes (Farkasovska *et al*., 2019 and Martinotti *et al*., 2019)

Recently, hydrogen peroxide (H_2O_2) is the main factor of the inhibitory effect against microbial growth in blossom honey and honeydew honeys. Moreover, H_2O_2 produced mainly from glucose oxidation and be accumulated when honey ripens (Bucekova *et al*., 2018 and 2019). In addition, there are other factors than peroxide that interfere with the process of inhibiting the microbes growth, such as physiochemical properties, high-acid osmosis, phenolic compounds (Kwakman *et al*., 2010 and Fea's *et al*., 2013). There are several sources of microbial contamination in bee honey such as primary sources including the digestive tracts of honeybees, pollens, air, dust, soil, and nectar, are comparatively difficult to get rid of her. In addition, secondary sources, due to honey handlers and processing, are easier to control by following the good manufacturing practices (Snowdon and Cliver, 1995).

Pseudomonas aeruginosa (Gram negative) has been a significant issue in clinic obtained diseases and causes most extreme wounds and burn infections (Roberts *et al*., 2012 and Kronda *et al*., 2013). *P. aeruginosa* has become multidrug resistant because of its capability to get new antimicrobial resistance (Camplin and Maddocks, 2014). Its action is improved by its capacity to shape biofilms and become safe and sidestep the activities of the therapeutic agents. The genus *Staphylococcus* composed of 33 species (Bergey and Holt, 1994).

Most *Staphylococci* constitute the normal flora of the skin and mucus membranes (Madigan, 2005). The most pathogenic species are *S. aureus* (Murray *et al*., 2005). Some coagulase-negative Staphylococci (CNS) strains, causative specialists of contamination in insusceptible traded off people, created protection from anti-toxins. These microscopic organisms colonize gadgets that are embedded in the human body, for example, nails, slides and mechanical joints utilized in bones, heart valves and catheters of different kinds, just as in peritoneal dialysis. It has been obtained that there was an expansion in the pervasiveness and frequency of methicillin resistant CNS and *S. aureus*, making it additionally testing to treat such diseases (Kloos and Bannerman, 1994).

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Therefore, the current research was aimed to study the relationship between some contents in honey (moisture, pH, total phenols, H_2O_2) and their inhibiting effect against growth *S. aureus* and *P. aeruginosa* bacteria in different bee honey types.

MATERIALS AND METHODS

Eight beehoney samples from different floral sources were collected directly from 3 Egyptian governorates (Asyut, El-Sharqia, and El-Faiyum) beside commercial honey bee from supermarket during 2019 (Table 1). All samples (3 replicates/sample) were stored at -20±2ºc till chemical analysis in the laboratory of apiary yard, Experimental Station, Faculty of Agriculture, Cairo University.

Table 1. Beehoney types and their sources

Source	Types of bee honey	Production date
Asyut	Clover	October, 2019
Asyut	Sesame	August, 2019
Asyut	Black cumin	April, 2019
El-Sharqia	Clover	June, 2019
El-Sharqia	Citrus	April, 2019
El-Faiyum	Clover	October, 2019
Supermarket	Clover	June,2019
Supermarket	Citrus	April,2019

In these bee honey samples the following parameter were studied

1. **Pollen analysis:** Pollen grains of all tested bee honey samples were investigated accrding to Louveaux *et al*. (1978), Ten-gram honey is dissolved in 20 ml warm water and then put in the centrifuge 2500 rolls for 10 minutes and then gets rid of the leaker and replace it with another water and then replay in the centrifuge for another 10 minutes. The entire sediment was put on a slide and spread out over an area about 20 x 20 mm, after drying by slight heating at 40 degrees add the glycerin gelatin and examination was done under the light microscope. Melissopalynology was used as a reference of pollen grain frequencies for grains constitutes >45% is very frequent, frequent from 16 to 45%, rare from 3 to15%, and sporadic <3% of the total grains (Maurizio, 1975).
2. **Physicochemical analyses:**Chemical analyses of Moisture, pH, total phenols and H_2O_2 were done in Food Safety and Quality Control laboratory, Faculty of Agriculture, Cairo University, Giza, Egypt.

a. **Moisture content (%):** was determined by digital refractometer, all measurements were performed at 20ºc (A.O.A.C., 1990).

b. **pH: Device of pH meter** (Boeco, Germany) was used for measuring pH.

c. **Total phenols:** were determined by colorimetry (UV/V) Spectrophotometer, JENWAY, England by Folin-Ciocalteu reagent. Total phenolic content was calculated from the regression equation of the standard plot ($Y=101.71x \times -0.4181$, $r^2=0.9979$) and were expressed as mg gallic acid equivalent/Kg sample (Singleton and Rossi, 1965).

d. **Hydrogen peroxidese: H_2O_2** reacts with 3.5-dichloro-2-hydroxybenzensulphonic (DHBS) acid and 4-aminophenazone (AAP) to form a choromphore (Aebi, 1984).

3. **Minimum inhibitory concentration (MIC) assay** was developed using fresh-daily serial honey dilutions (25%, 50%, 75% and 100%, v/v), aseptically prepared in nutrient broth. Samples of bee honey were maintained as stock cultures on slants of nutrient agar, with weekly transfers to new tubes. Sock cultures were used to inoculate nutrient broth cultures which served as “working cultures” in the experiments. Cultures were incubated initially for 24 hours at 37°C and then refrigerated to stall growth. The bacteria selected were chosen based on their frequent occurrence in infections. Bacterial isolates were obtained from the Department of Microbiology, the National Research Center. The bacterium to be tested was swabbed from a broth culture onto a nutrient agar plate and a well was made within the agar. For each trial 0.05 ml of honey, which had been warming in a 35-40°C water bath, was pipetted into the well. The plate was incubated for 24 hours at 37°C. After incubation the zone of inhibition surrounding each well was measured.

Statistical analysis

Data were taken in triplicates and analyzed by SAS software (SAS, 2001). One-way analysis of variance (ANOVA) was used to compare the variables in each type of honey. When significant differences were noted, Duncan's multiple range test was used to separate means and all statistical analysis was significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

1. Pollen analysis

Table (2) represents the pollen spectrum percentage of tested bee honey types. Citrus bee honey sample which was collected from Sharqia governorate include 30% of the main source of *Casuarina Sp.* as frequency pollen, following by 23% *Citrus spp.* and 20% *Phoenix dactylifera*. On contrast, most of clover bee honey samples contained *Trifolium alexandrinum* as a main source with 44.8, 80, 35, and 55% for clover Asyut, El-Sharqia, El-Faiyum, and supermarket samples, respectively. Otherwise, pollen spectrum evaluation for sesame and black cumin bee honeys showed that *Umbellifera* pollen was the main frequency source as 33 and 45% with rare of other diffrent sources. There are numerous sources for each type of honeys but with rare percentage. Maurizio (1975) reported that very frequent grains constituting is >45%, grains constituting ranged from 16-45%, rare grains constituting varied from 3-15% and sporadic is <3%. The current results confirmed the tested bee honey samples excepted market citrus samples, consider as natural honey and wide variability pollen types but in low percentage (Rateb, 2005; El Sohaimy *et al.*, 2015 and El-Metwally, 2015).

2.Physicochemical properties

a. Moisture%

There were significant diffrences (P<0.0001) in moisture percentage (Table 3) among black cumin, sesame, clover beehoney from Asyut and clover honey from El-Faiyum and El-Sharqia governorates with the other tested bee honey. The mean of moisture % ranged from 18.3±0.05 to 20.40±0.05 in clover honey (El-Faiyum governorate) and citrus honey (supermarket), respectively.

Moreover, the moisture percentage in the current research of tested honey samples appeared within the appropriate range from 18 to 20% (Codex alimentarius commission, 2001; Council Directive of European Union, 2001 and the Egyptian organization for standardization and quality control, EOSC (2005).

Moisture % is an importance factor in quality standards for honeys which is determined by the basis of quality of honey. The higher moisture content was lead to the higher probability occurrence of fermentation (Nour,1988). Moisture content is a significant measurements which impacting physicochemical properties of honey such as viscosity and crystallization, in addition to other parameters, for example, color, flavor, taste, specific gravity, solubility, and conservation. Codex alimentarius commission (2001) reported that the moisture content in honey should not exceed 20%. The moisture contentranged from 16.9 to 18.0% (average = 17.6%) in honeys harvested (n=187) in Northwest Spain (Escuredo *et al.*, 2013)). Karabagias *et al.*, 2014 stated that moisture content varied from 10.50 - 20.50% of pine honey (n=39) in Greece according to differention in reigons and seasons.

The moisture content ranged from 16 to 21% in eljabaly and citrus monofloral honeys which collected from the Apiary of the Experimental Station of Faculty of Agriculture, Cairo University (Hassanein *et al.*, 2010) and it ranged from 18.00 (marjoram honey) to 21.50% (banana honey) in Egyptian bee honeys (Farag, 2013).

Table 2. Pollen spectrum percentage of tested bee honey types

Pollen types	Asyut			El-Sharqia		El-Faiyum	Supermarket	
	Clover	Sesame	Black cumin	Clover	Citrus	Clover	Clover	Citrus
Trifolium alexandrinum	44.8	35	-	80	-	35	55	20
Phoenix dactylifera	-	10	-	5	20	25	5	5
Fam. Umbellifera	21.5	33	45	5	10	-	3	5
Citrus spp.	-	-	5	-	23	-	-	5
Eucalyptus spp.	-	-	5	-	5	22	-	5
Nigella sativa	-	-	20	-	-	-	-	-
Casuarina sp.	0.2	9	15	5	30	-	5	3
Zea mays	-	-	-	-	-	8	25	10
Fam. Chenopodoceae	-	-	5	-	6	-	-	7
Sesamum sp.	-	5	-	-	-	-	-	2
Acasia sp.	-	2	-	-	-	-	-	-
Fam. Curecubitaceae	-	-	5	-	4	13	2	5
Medicago sp.	32.7	6	-	5	-	-	2	3
Fam. Compositae	-	-	-	-	-	-	3	15

Table 3. Means of moisture, pH, total phenols(mg gallic acid equivalent / kg) and H_2O_2 (mM /100g) in tested bee honey types

Sources	Honey Type	Moisture (%)	pH	Total phenols	H_2O_2
Asyut	Clover	19.90 ±0. 05 [a]	3.80 ±0.01[f]	62.45 ±0.06 [b]	10.51 ±0.57 [e]
	Sesame	20.20 ±0. 05 [a]	3.82 ±0.00 [f]	49.23 ±0.31[c]	38.36 ±0.45 [c]
	Black cumin	19.90 ±0.05 [a]	4.51 ±0.01 [a]	137.39 ±0.08[a]	8.16 ±0.42 [f]
El-Sharqia	Clover	19.00 ±0.57 [b]	4.06 ±0.01 [c]	18.54 ±0.12 [h]	8.64 ±0.45 [f]
	Citrus	19.80 ±0.05 [a]	4.40 ±0.01 [b]	19.29 ±0.05 [g]	26.78 ±0.55 [d]
El-Faiyum	Clover	18.30 ±0. 05 [c]	3.86 ±0.01 [e]	27.89 ±0.15 [e]	48.56 ±0.60 [b]
Supermarket	Clover	19.70 ±0.05 [a]	4.04 ±0.01 [c]	26.31 ±0.30 [f]	51.71 ±0.32 [a]
	Citrus	20.40±0.05 [a]	4.02 ±0.01 [d]	37.25 ±0.33[d]	2.89 ±0.05 [h]
P-value <0.0001					

[a, b] in the same column within each factor (moisture, pH and H_2o_2) with different superscripts are significant differences (P≤0.05).

b. pH

There were significant differences (P< 0.0001) in pH (Table 3) among all tested honey samples. . Black cumin honey harvested from Asyut recorded the highest pH value (4.51±0.01), while clover honey sample from Asyut governorate give the lowest value (3.80±0.01).

High acidity of honey responsible for flavor and stability against microbial spoilage (Bogdanov *et al.*, 2008) and contained a lot of mineral (El-Metwally, 2015). Furthermore, low pH (3.2 – 4.5) of honey inhibits the presence and growth of microorganisms, wheras the optimum pH for most microorganisms is between 7.2 and 7.4 and had a great importance role during honey extraction and storage (Terrab *et al.*, 2002 and Karabagias *et al.*, 2014). The obtained results are in agreement with those of Nour (1988) who found that pH values of Egyptian honeys ranged from 3.48 to 4.95 (mean= 3.94) in citrus and sugar cane honeys, respectivetly, and varied from 3.73 to 4.60 of honey samples collected from different locations in Ekiti State, Nigeria (Kayode and Dele Oyeyemi, 2014).

As the same trend, in New Zealand, the antibacterial effect of honey was related to pH ranged from 3.0 to 4.5 (Waikato Honey Research Unit, 2012). However, bacteria have been able to resistance the effects of honey by forming biofilms (Lu *et al.*, 2014). Furthermore, honey has a mean pH of 4.4, the acidification of honey can reduce bacterial colonization (Rushton, 2007). . The other factors like, the high sugar concentration, H_2O_2 level, and the antimicrobial peptide bee defensin-1 contribute to pH as antimicrobial effect of bee honeys (Kwakman and Zaat, 2012).

c.Total phenols

There were significant differences (P<0.0001) in total phenols (Table 3) among all tested honey samples.

Black cumin honey harvested from Asyut recorded the highest total phenols value (137.39 ±0.8), followed by clover and sesame honeys (62.45±0.06 and 49.23±0.31, respectively) in the same governorate, While clover honey sample from El-Sharqia governorate give the lowest value (18.54 ±0.12).

Total phenolic content is a good criterion to determine the quality and curative properties of bee honey (Al-Mamary *et al*., 2002). These findings agreed with Aljadi and Kamaruddin, 2004 whom reported that total phenols ranged from 20 to 240 mg/100 g honeys. And also they stated that gelam and coconut honeys were 21.4 mg/g and 15.6 mg/g, respectively in Malaysian.

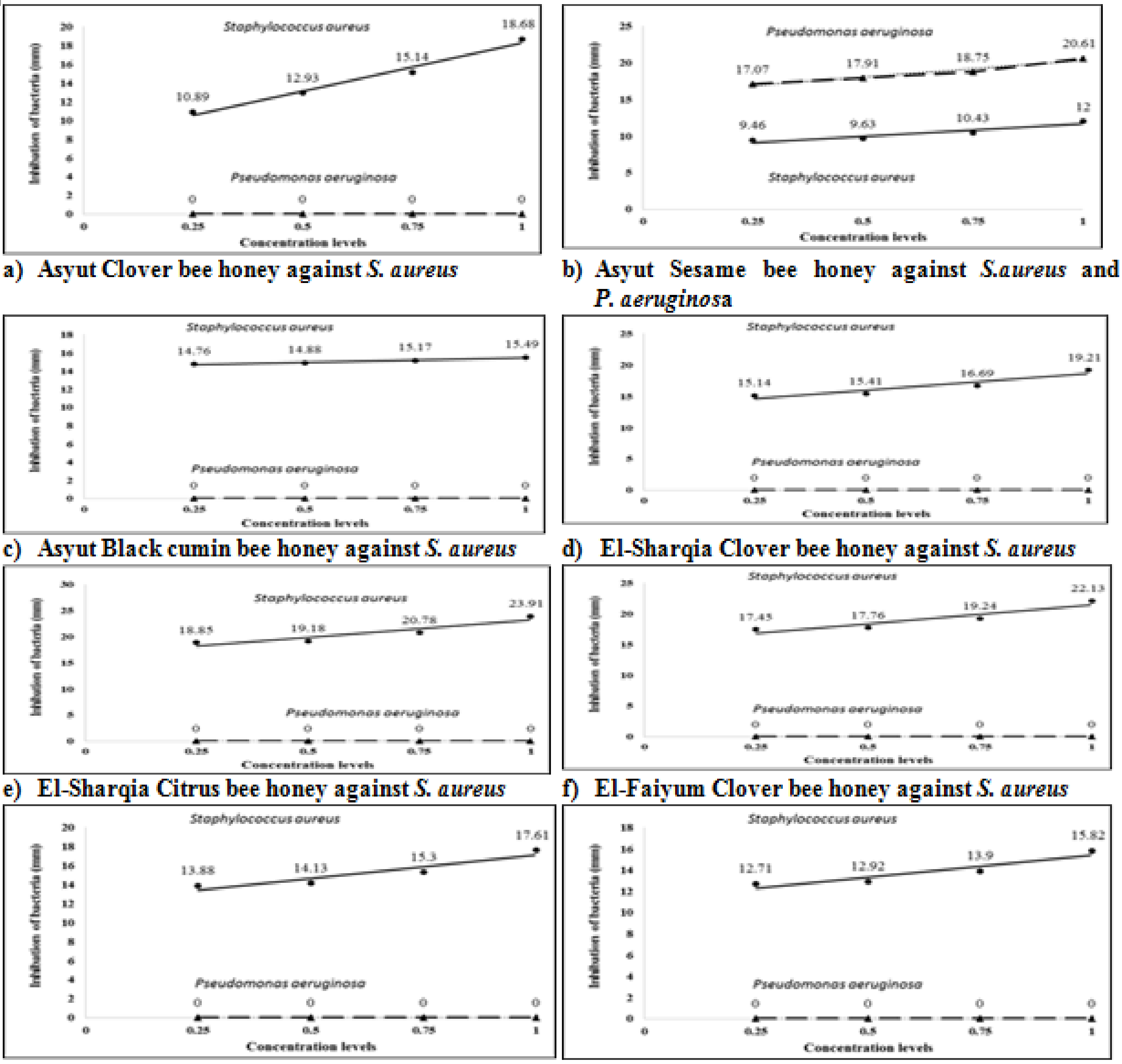
In 5 Australian honey samples, total phenolic content ranged was from 2.13 to 12.11 mg/100g (Yaoa *et al*., 2005) and ranged from 64 and 1304 mg/100g in 11 Algerian honey samples (Ouchemoukh *et al*., 2007).

d. Hydrogen peroxide (H_2O_2)

There were significant differences ($P<0.0001$) in H_2O_2 (Table 3) among all tested honey samples with wide range values. The highest value of H_2O_2 was detected in clover honey from market (51.71±0.32), then from El-Faiyum governorate (48.56±0.60) following by sesame honey from Asyut governorate (38.36± 0.45) while, the lowest value was estimated in citrus market honey (2.89 ±0.05). Both of low pH and H_2O_2 besides major antibacterial factors work together by different mechanisms to inhibit or kill bacteria in bee honeys (Bucekova *et al*., 2018). Moreover, antibacterial activity in bee honey is mainly depended on H_2O_2 which tested in 233 honey samples from different botanical origins (Farkasovska *et al*., 2019). In contrast, the linden honey showed a strong antibacterial effect is attributed to non-peroxide. The major antibacterial factors in honey are H_2O_2, catalase and glucose oxidase levels and non-peroxide factors such as phenolic acids and flavonoids (Weston, 2000)

3. Minimum inhibitory concentration assay (MIC)

The antibacterial activity of eight different bee honey samples against *S. aureus* and *P. aeruginosa* growth were illustrated (Fig. 1).



a) Asyut Clover bee honey against *S. aureus*

b) Asyut Sesame bee honey against *S.aureus* and *P. aeruginosa*

c) Asyut Black cumin bee honey against *S. aureus*

d) El-Sharqia Clover bee honey against *S. aureus*

e) El-Sharqia Citrus bee honey against *S. aureus*

f) El-Faiyum Clover bee honey against *S. aureus*

g) Supermarket Clover bee honey against *S. aureus*

h) Supermarket Citrus bee honey against *S. aureus*

Fig. 1. Growth Inhibition of bacteria *S. aureus and P. aeruginosa* as affected by different bee honey types concentrations.

It was noted that antibacterial activity of *S. aureus* increased with incresing concentrations levels (25, 50, 75 and 100%) of all tested bee honey samples. But for *P. aeruginosa* there is no antibacterial activity was found in all tested bee honey samples exept sesame bee honey that collected from Asyut governorate. Of the natural products, honey was most bacterial inhibitory and was in certain instances. These and other natural products may have the potential to serve as complementary methods of bacterial inhibition to those already in use by traditional medicine (White *et al*., 1963).

The bacteria chosen *S. aureus* and *P. aeruginosa* are common infectious bacterial organisms. These findings are in agreement with Farag (2013) which found that high concentrations of Egyptian honeys give high inhibition zone for *S. aureus.*

Non-peroxide factors include phenolic acids, which might play an important role in antibacterial activities (Wahdan, 1998). Mohapatra *et al*. (2011) showed that honey has an antibacterial effect against both gram positive bacteria (*S. aureus*, *Bacillus subtilis, Bacillus cereus, Enterococcus faecalis*, and *Micrococcus. luteus*) and anti-gram negative bacteria (*E. coli*, *P. aeruginosa* and *Salmonella typhi*). This effect was either bacteriostatic or bactericidal depending on the type of honey tested. In another study, it was reported that the antibacterial effect exhibited by honey was related to the levels of H_2O_2 present in the honey (Alnaimat *et al*., 2012). Liu *et al*. (2013) found that *P. aeruginosa* was not inhibited by antimicrobial activity in diluted honey. This relation among antimicrobial activity, botanical source, and phenolic compounds might be associated with the composition of eucalyptus and blueweed nectars, whose components could also affect the content of H_2O_2 . *P. aeruginosa* seemed to be less susceptible to the antibacterial activity of honey samples compared to *S. aureus* (Stagos, *et al*., 2018 and Bucekova, *et al*., 2019).

CONCLUSION

It concluded that Egyptian bee honey mostly is monofloral source and contain many plant sources, but at low levels. Each plant specie has characteristics that vary according to geographical area. All the tested bee honeys had an inhibitory activity for the growth of *Staphylococcus aureus.* While, in *Pseudomonas aeruginosa,* sesame bee honey sample from Asyut governorate was the most effective of antibacterial activity. Antibacterial activity depends on many factors, the most important one is H_2O_2, There's a correlation between hydrogen-superoxide and antibacterial activity. the most inhibiting of bacterial growth in tested beehoney was without dilution followed by concentrations from the highest to the lowest.

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النشاط المضاد للبكتريا لبعض الاعسال المصريه تجاه *Staphylococcus aureus* و*Pseudomonas aeruginosa*

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عسل النحل من المواد الغذائية الطبيعية الذى يتميز بقيمته الغذائية والطبية العاليه. فى هذه الدراسه تم جمع أنواع مختلفه من الأعسال المصريه مباشرة من بعض مناحل محافظات أسيوط، الشرقيه والفيوم بالاضافة الى عينتين من المحلات التجارية لدراسة فاعليه الأعسال فى النشاط المضاد لبكتريا *Staphylococcus aureus* و*Pseudomonas aeruginosa* . أظهرت النتائج المتحصل عليها أن الأعسال المصرية وحيدة المصدر النباتى بالرغم من تعدد المصادر النباتية بها داخل النوع الواحد. أيضا أوضحت النتائج أن عسل النحل يختلف تبعا للنوع النباتى المجموع منه الرحيق والمنطقة الجغرافيه. نشاط الأعسال ضد الأنواع المختبره من البكتريا مرتبط بالمقام الأول بمادة فوق أوكسيد الهيدروجين والتى تُنتج من إنزيم الجلوكوز أوكسيديز. كل أنواع الأعسال المختبرة أظهرت نشاط مضاد لبكتريا *S. aureus* ولكن بالنسبه لبكتريا *P. aeruginosa* كانت معظم الأعسال غير فعاله معها ماعادا عسل سمسم محافظة أسيوط. كان أعلى نشاط مضـــاد لنمـــو بكتريـــا *S. aureus* فى الأعسال الغير مخففة يليها التركيزات العالية ثم الأقل.