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Antibacterial Effect of Bee Venom on *Panibacellus larvae* causing American Foul Brood (AFB) in Honey Bee: Invitro st.

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Abstract: ***The antibacterial activity of bee venom and tylosin was evaluated against of Paenibacillus larvae, the causal agent of American Foulbrood (AFB) disease in honey bee colonies Apis mellifera L which was identified by using biolog assay, and characterized chemically and microscopically. Bee venom showed more antibacterial effect against AFB with Minimal inhibitory concentration (MIC), and MBC in BHIT which was 0.68±0.011mg/ml, and 0.82±0.009 consequently. While, mean MIC and MBC values of tylosin was 2.52±0.16mg/, and 3.5±0.18 consequently***

1. Introduction

The bacterial pathogen *Paenibacillus larvae* (White), is the etiological agent of American foulbrood (AFB) disease, an extremely contagious disease of honey bee brood (Genersch *et al.*, 2006). AFB preventive and curative treatments usually consist in the application of antibiotics, such as tetracycline hydrochloride, but their extensive use have led to the accumulation of residues (Bogdanov, 2006) in honey and other beehive products, decreasing their quality and making their marketing more difficult. Besides residue accumulation, antibiotic-resistant isolates of *P. larvae* have been detected in many countries (Alippi, 1996; Miyagi *et al*., 2000; Evans, 2003). The concern for problems arising from microbial resistance is growing and the outlook for the future use of antimicrobial drugs is still uncertain. Therefore, actions must be taken to reduce this problem, for example optimizing the use of antibiotics when legally permitted and/or developing new drugs, either synthetic or natural (Nascimento *et al*., 2000), potentially efficient in the control of this very serious honeybee diseases (Liesel *et al* 2010). The use of bee venom, The most important active and prevalent substance which constitutes 50% of dry venom is melittin, a type of amphipathic, water soluble, linear peptide consisting of 26 amino acids with 6 positive charges, with a molecular weight 2849 Dalton. This substance causes local pain via histamine release and enhances phospholipase A2 (PLA2) induced intravascular hemolysis as well as catecholamine release. It has been reported that melittin has multiple effects, including antibacterial, antivirus, and anti-inflammation, in various cell types. The aim of this study was the *in vitro* evaluation and comparing the antimicrobial activity of bee venom and tylosin against AFB.

2. Materials and Methods

1) Isolation of AFB causative bacteria

Infected larvae from diseased brood were collected and crushed in 0.5% peptone, then put in shaker for 24h until see any growth to the bacteria then divided it in two parts one heated at 100°C for 15 min and centrifuged at 6,000 g for 45 min. The pellet containing the bacterial spores was inoculated and streaking on Brain Heart Infusion Agar media fortified with 0.1 g thiamine hydrochloride (sterilized by Millipore filter paper) per liter of sterilized medium (BHIT) and pH adjusted to 6.6 with HCl. Then, autoclaved at 10 lb/sq. in and 116°C for 20 min. media was poured into 90-mm diameter Petri dishes, 20 ml/plate, then incubated at 35°C for 72 hr (**Shimanuki and Knox, 1991**). Emerged colonies were purified by repeated streaking; and the other part streaking without heated on (BHIT) too and Gram-stained.

The bacterial colonies grown on culturing media were recultured separately in new plates and incubated at the same conditions(37oC for 72 hr). Pure bacterial colonies were inoculated in a liquid medium (agar-free) and prepared for about 5 %inoculums. Slants for different isolates were also prepared for subsequent identification.

2) Identification Methods

a) Gram stain

Bacteria isolated from diseased honey bee classified according to genus and species according to morphological, cultural and biochemical studies according to[7].for primary isolated of the bacteria the samples were dissolve in sterile saline and seeded into plates of nutrient, USA) and incubated at 36◦ for 24 hours, in the Microbiology Laboratory, R and sector, VACSERA. Secondary, purification of isolated bacteria by picked up isolated growing colonies and subculture it (The primary isolation) on the same seed media for more purification. The bacteria obtained isolates were incubated in semisolid agar for preservation and motility test. Also, the obtained bacterial isolates were subculture on slope agar and use as a stock culure for further inoculated in semisolid agar for Identification. Identification of recovered isolated: including, morphological examination using dry heat fixed smears which prepared and stained with gram's stain and

examined for morphological characteristic and stain reaction under the microscope used oil immersion lens. Culture characteristic: Including morphological characterization of the growth colony (shape, color, texture, appearance, pigmentation and haemolysis). Biochemical identification: The isolates were subjected for the following biochemical examination (Indole, Oxidase, Urease, and sugar fermentation test).

b) The Biolog® System for Bacterial Identification

Samples for the Biolog® identification method were prepared according to the manual guide provided by Biolog Inc. (California, USA).21 A Microlog TM 4.2 database was used to interpret the data obtained on the Biolog® microplates. The Biolog® system was developed by Biolog Inc., and is used as an easier option of phenotypic identification.22 The Biolog® technique of microbial identification is based on carbohydrate utilisation by microorganisms.22, 23 The Biolog® microplates consist of 96 wells that contain water and 95 various media of specific carbohydrates, and a redox indicator. The redox dye, tetrazolium, changes into a purple colour if microbial growth occurs in a particular well representing catabolism of the substrate 23. The colour transformation of the dye is considered as a positive reaction. The well containing water is used as a control for the tests. Different microorganisms use different carbon sources depending on their nutritional requirement; therefore, based on the positive and negative reaction, aspecies-specific signature can be produced.21 Figure 2 shows an image of a Biolog® microplate indicating the positive results by the colour transformation.

c) Total protein Total protein assay:-

The total protein concentration was determined by analytical method described by **Warburg and christian (1942**).

d) Determination of bee venom LD50 (*British pharmacopeia'2000*)

The LD50 of venoms were determined according to the method of *British pharmacopeia'2000,* using male albino Swiss mice 16-18 gm B.W. Ascending concentrations of 5 dose levels of the freshly prepared venom solutions in normal saline were arranged in a geometric progression starting by a dose which kills approximately 0-20 % of the animals and ending by a dose which kills approximately 80-100 % of the injected animals. Each dose level was tested in 4 mice, and all injections were given intravenously, and deaths and survivals of injected animals were recorded after 24 hrs from the time of injection. The uncorrected % lethality at each dose level was calculated from the numbers of survivals and deaths directly obtained at that dose level. However, in order to avoid the interference of accidental survivals or deaths resulting from abnormal resistance or susceptibility of tested animals, i.e. by the data were recalculated after correction of the numbers of survivals at each dose level by adding consideration of the number of survivals at higher dose level , and correction of numbers of deaths by adding the number of lower deaths at deaths at each dose level.It is assumed that mice surviving at a given dose level would have survived at a lower dose level, and conversely, mice which died at a given dose level would have died at any higher dose level. Therefore at each dose level, the number of survivals at higher dose levels was added to the uncorrected number of survivals obtained at that dose level; and the number of deaths at lower dose levels was added to the uncorrected number of deaths obtained at that dose level. The accumulated corrected % lethality at each dose was determined from the accumulated corrected deaths and accumulated corrected survivals at that dose level (***Bradford Hill, 1977***). Determination of LD50 represents Lethal activity of bee venom which depends on its main components, melittin and phospholipase A2, that combination of the two at their natural 3:1 mixture in bee venom revealed that the lethal activity of the mixture was about the same as crude bee venom (**Schmidt, 1995**).

e) Determination of the Minimum Inhibitory Concentration (MIC) of bee venom against the isolated bacteria (Brackman *et al.*, 2009)

In brief, a microdilution assay in flat bottomed 96-well microtiter plates (Costar, USA), using (BHIT) as a medium was used. The plates were inoculated by 100 µl of bacteria isolated from diseased honey bee larvae in BHIT media and bee venom in serial dilutions. The plates were incubated for 24 hr at 37°C and the absorption at 600 nm was measured using **Kinetic microplate reader**. In addition, MIC values were also determined for bee venom in serial dilution against bacteria isolated from diseased honey bee larvae as previously mentioned method.

The MIC was determined as the lowest concentration of antibiotics or pure compounds that induce no germination of bacterial spores or inhibited 90% or more of spore germination.

f) Determination of the Minimum Bactericidal Concentration (MBC) (Jobran and Finegold, 1994)

Serial fold dilutions i.e. …….625, 1250, 2500, 5000 & 10000 µg/ml for venom. Each well was inoculated with 100µl (0.5 McFarland) of standardized suspension of tested bacterial species containing about 1.5x108 cell/ml, and then incubated at 37 ׳IC for 24h, 48h, and 72hr for the isolated microorganism. After 24 h incubation, 0.1 ml from each well was sub cultured in TSB agar plates and incubated for 24 h at 37 ׳IC. The lowest dilution of the tested well which gave a viable count less than 0.1% of the original inoculums (1.5x108 cell/ml) was assumed as the minimal bactericidal concentration (MBC) **Attalla et al. (2007).**

3. Results and discussion

1) Gram Stain

The stain was gram positive bacilli (violet) fig(1) .This may be *Paenibacillus larvae*

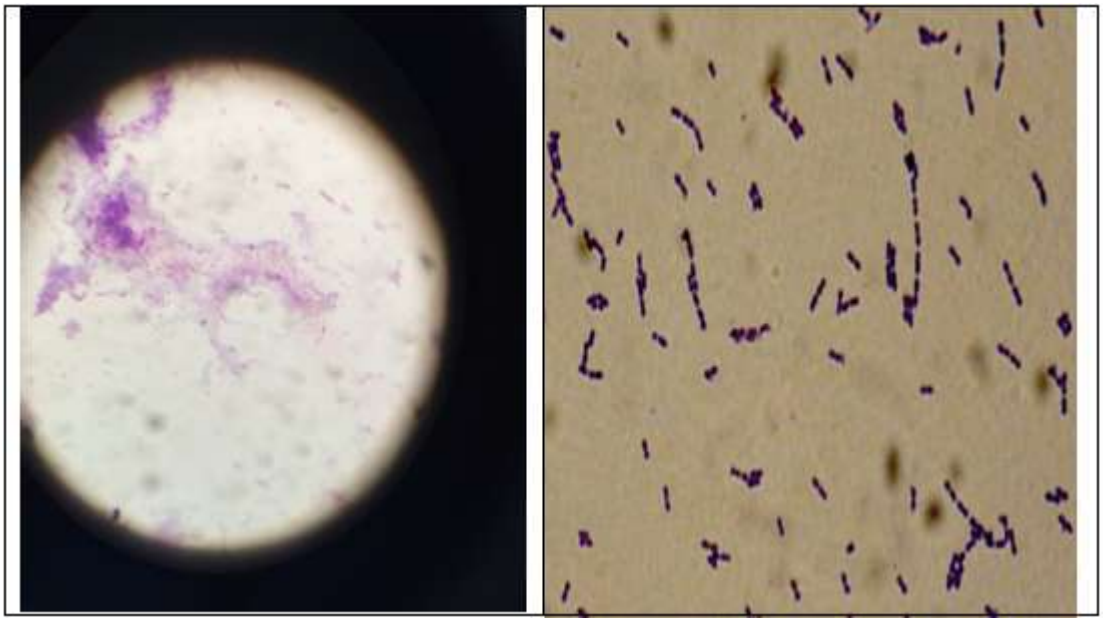

Figure 1: Microscopic examination of the isolated Gram-positive (violet) spore forming rods, (Shows spores and strain of *P. larvae*).

2) The Biolog® System for Bacterial Identification

Figure 2: Biochemical identification: Paenibacillus larvae

Table 1: biochemical identification : Paenibacillus larvae

A1 Negative Control -ve	A2 Dextrin ±ve	A3 D-Maltose ±ve	A4 D-Trehalose ±ve	A5 D-Callobiose -ve	A6 Gentiobiose ±ve	A7 Sucrose ±ve	A8 D-Turanose ±ve	A9 Stachyose ±ve	A10 Positive Control +ve	A11 PH6 +ve	A12 PH 5 -ve
B1 D-Raffinose ±ve	B2 α-D-Lactose ±ve	B3 D-Melibiose ±ve	B4 B-Methyl-D-Glucoside ±ve	B5 D-Salicin ±ve	B6 N-Acetyl-D-Glucosamine ±ve	B7 N-Acetyle-β-D-Mannosamine ±ve	B8 N-Acetyl-D-Galactosamine ±ve	B9 N-Acetyle Neuraminic Acid ±ve	B10 1%Nacl +ve	B11 4%Nacl ±ve	B12 8%Nacl -ve
C1 α-D-Gluctose ±ve	C2 D-Mannose ±ve	C3 D-Fructose ±ve	C4 D-Galactose ±ve	C5 3-Methyl Gluctose -ve	C6 D-Fucose -ve	C7 L-Fucose ±ve	C8 L-Rhamnose -ve	C9 Inosine ±ve	C10 1%Sodium Lactate +ve	C11 Fusidic Acid -ve	C12 D-Serine ±ve
D1 D-Sorbitol -ve	D2 D-Mannitol -ve	D3 D-Arabitol -ve	D4 Myo-lnositol -ve	D5 Glycerol ±ve	D6 D-Glucose -ve	D7 D-Fructose-6-PO4 -ve	D8 D-Aspartic Acid -ve	D9 D-Serine ±ve	D10 Troleandomycin -ve	D11 Rifamycin SV -ve	D12 Minocycline -ve
E1 Gelatine -ve	E2 Glycyl-L-Proline ±ve	E3 L-Alanine ±ve	E4 L-Arginine ±ve	E5 L-Aspartic Acid -ve	E6 L-Glutamic Acid ±ve	E7 L-Histidine ±ve	E8 L-Pyroglutamic Acid -ve	E9 L-Serine ±ve	E10 Lincomycin -ve	E11 Guanidine HCl ±ve	E12 Niaproof 4 -ve
F1 Pectin ±ve	F2 D-Galacturonic Acid -ve	F3 L-Galactonic Acid Lactone -ve	F4 D-Gluconic Acid ±ve	F5 D-Glucouronic Acid ±ve	F6 Glucuronamide ±ve	F7 Mucic Acid -ve	F8 Quinin Acid -ve	F9 D-Saccharic Acid -ve	F10 Vancomycin -ve	F11 Tetrazolium Violet ±ve	F12 Tetrazollum Blue -ve
G1 P-Hydroxy-Phenylacetic Acid -ve	G2 Methyl Pyruvate ±ve	G3 D-Lactic Acid -ve	G4 L-Lactic Acid +ve	G5 Citric Acid -ve	G6 α-Keto-Glutaric Acid -ve	G7 D-Malic Acid -ve	G8 L-Malic Acid ±ve	G9 Bromo-Succinic -ve	G10 Naiddixic Acid +ve	G11 Lithium Coloride +ve	G12 Potassium Tellurite +ve
H1 Tween 40 ±ve	H2 γ-Amino-Butryric -ve	H3 α-Hydroxy Butyric Acid -ve	H4 β-Hydroxy-D,L-Butyric Acid ±ve	H5 α-Keto-Butyric Acid -ve	H6 Acetoacetic Acid -ve	H7 Propionic Acid ±ve	H8 Acetic Acid ±ve	H9 Formic Acid -ve	H10 Aztreonam +ve	H11 Sodium Butyrate +ve	H12 Sodium Bromate ±ve

Table 2: Characterization of P*aenibacillus larvae*

Biochemical test	Results
Bulging sporangium	+ve
Gram reaction	+ve
Holst milk test	+ve
Catalase activity	-ve
Starch hydrolysis	-ve
Casein hydrolysis	+ve
Gelatin liquefaction	+ve
Nitrate reduction	+ve
Voges -Proskauer	-ve
Blood Agar	+ve
Growth in nutrient agar	-ve

P. larvae, Motile spore-forming aerobic bacteria isolated from infected locations were purified and their cultural, morphological as well as biochemical traits were examined. It was found that P. larvae strain examined was Gram-positive rods, small approximately 0.6 µm in diameter and 1.3 µm in length, regular, buttery, and greyish. Bacteria appeared as single cells or pairs and sometimes as short chains and unable to withstand serial transfer in nutrient agar, catalase -negative. Isolate were positive for casein, negative for Voges–Proskauer, positive for gelatin liquefaction, displayed high proteolytic activities in milk and reduced nitrate to nitrite and did not hydrolyze starch at 37°C (Table1,2). These results were in agreement with the phenotypic features that are characteristics of the species P. l. subsp. **Gordon *et al*. (1973), Jelinski (1985)** distinguished seven biochemical types of P. larvae populations according to seven possible combinations of three variable properties between strains that are reduction of nitrate to nitrite, hydrolysis of mannitol, and acid production from salicin. A preliminary investigation by **Owayss (2007)** recorded some infections signs of AFB in Fayoum apiaries on the diseased brood combs fit with those described by **Shimanuki and Knox (1988)** for AFB type. **Gende *et al*. (2011)** also found that most samples collected from Sardinian bee hives showed typical P. larvae colonies small, regular with a rough surface, flat, and pale beige color. All isolates were Gram positive and catalase negative showed a typical carbohydrate acidification profile with acid from glucose and trehalose. Some isolates showed variability on OrthoNitro-Phenil-β-D-GalactoPyranoside, on gelatin hydrolysis and utilization of

rhamnose **Kilwinski *et al.* (2004**) and **Alippi A. M. (1999) Dina Tak. (2015).**

3) Total protein Total protein assay:-

Determination of bee venom total protein content:.

The total protein concentration was 1.280 mg/ml as seen in table1. This result was in difference with TP determined by **Kim, 1997**, this difference may be due to geographical distribution of bee venom as we determine bee venom craniolan species in Egypt while Kim determine bee venom from Kanada.

Table 3: total protein of craniolian bee venom in Egypt

Type	Protein content
Carniolan spec	1.280 mg/ml

4) Determination of bee venom LD50 (*British pharmacopeia*'2000)

Figure 3: I.V injection of bee venom into mice to determine LD50

- 1. Determination of Bee venom lethality:
- 3.1. carniolan Bee Venom Potency in albino swiss Mice

Table 4

Dose	Directly observed		Corrected		Lethality%
µg/mouse	Death	Survival	Death	Survival	
216	0	5	0	15	00.0
260	1	4	1	10	09.0
312	2	3	3	6	33.0
374	3	2	6	3	66.6
449	4	1	10	1	90.0

- Dose increasing factor = 1.2
- Proportionate distance = (%mortality next above - % mortality next below) / (% mortality next above + % mortality next below 50%)
- $= \frac{66.6 - 33.3}{66.6 + 33.3} = \frac{33.3}{99.9} = 0.333$
- Log.LD50 = Log. LD next below 50% + (proportionate distance× Log. dose increasing factor)
- Log.LD50 = Log.312 + (log 1.2 × 0.333)
  = 2.49 + (0.079 × 0.333) = 2.516
- LD50 = 330 µg/mouse. LD50 =16.5 µg/gm
  = 0.0165 ±0.01 mg/gm

The result showed that LD50 equal 330 µg/mouse, that was mean LD50=16.5 µg/gm, and Total protein of bee venom was 1.280 mg/ml. This results were disagree with **Hossein Zolfagharian (2016), H. Costa (1999) Hossein Zolfagharian (2015)** the difference was due to the geographical difference between bee venom species. Mellitine, a major component of bee venom, is more active against gram positive than gram negative bacteria. Moreover, Bee venom has been reported to have multiple effects, including antibacterial, antivirus, and anti-inflammation effects, in various types of cells. This investigation was set up to evaluate the antibacterial activity of bee venom against bacterial diseases of honey bee. This study indicate that bee venom inhibits the growth and survival of bacterial strains and that bee venom can be used as a complementary antimicrobial agent against pathogenic bacteria. Bee venom lacked the effective proteins necessary for it to exhibit antibacterial activity for some specific strains while being very effective against other specific strains. Thus, one may conclude, that *Apis mellifera carneca* venom may have a specific mechanism that allows it to have an antibacterial effect on certain susceptible bacteria. Bee venom is a complex mixture of proteins and contains proteins such as phospholipase and melittin.

5) Determination of the Minimum Inhibitory Concentration (MIC) of bee venom against the isolated bacteria (Brackman et al., 2009)

Result in Table (5) showed that MIC for bee venom was the most inhibitory effect on radial growth of *Paenibacillus larvae* giving (0.68) reduction in mycelium growth at all concentrations of 10 mg/ml. but in case of tylosin MIC give 2.53 at concentration 10 mg/ml. This tests showed that bee venom effective on controlling the bacterial growth zone Pict (12). This results showed the MIC of bee venom 10mg/ml and tylosin were 0.68,2.52.This result agree with **Saurendra (2011), Sang (2016).** And disagree with **Soon Tae Kim (2006),Omran Alia (2013).** This difference was due to the geographical deference between bee venom species.

6) Determination of the Minimum Bactericidal Concentration (MBC) (Jobran and Finegold, 1994)

Data in table (5) showed that bee venom had the highest level of inhabitation to germination of AFB when used at concentration of 5 mg/ml and the mean ± stander deviation of MBC was **0.82±0.009**of AFB germination at concentration of 5 mg/ml. But on the other hand in case of tylosin showed that it had the lowest level of the inhibition to the growth of the bacteria at concentration of 5 mg/ml and the mean ± standard deviation of MBC was **3.5±0.18.** These values indicate that very low concentrations of tylosin are required to inhibit the growth of *P .l.*. These results are in accordance with previous studies on strains from Japan, where MIC values were between 0.025 and 0.1 mg/ml **Okayama *et al.* (1996).** The National Committee for Clinical Laboratory Standards do not provide a standard method for determining MIC values for *P .l.* larvae **NCCLS (1999**), and have no breakpoints for antibiotic resistance.

Disadvantages of using tylosin and oxytetracycline in treatment of AFB and EFB diseases are residuals of both antibiotics in honey bee products (honey-pollen-royal jelly-propolis) that after ingestion by human will be accumulate in cells of different organs as liver and kidneys that will be

harmful to these important organs (liver cell failure and renal failure).

Regular ingestion of tylosin and oxytetracycline in honey bee products lead to increase the risk that antibiotic-resistant strains of bacteria will be develop and spread. That cause it difficult to treat diseases by these antibiotics. So using of bee venom carrying no harmful effect on human being. So bee venom was the best in The treatment of bacterial diseases than others antibiotics.

Table 5: Determination of the Minimum Bactericidal Concentration (MBC) and the Minimum Inhibitory Concentration (MIC) for (AFB)

AFB	Tylosin	Bv	control +	Control-
MIC	10 mg/ml	10mg/ml	0.5 Mcf	
Inhibition value	2.52±0.16	0.68±0.011	1.12±0.045	0.37±0.035
MBC	5mg/ml	5mg/ml		
Inhibition value	3.5±0.18	0.82±0.009		

MIC values obtained for tylosin against *P. larvae* isolates was 2.52±0.16mg/ml. The MIC values of susceptible strains obtained were comparable to those reported by **Piccini and Zunino (2001)**. On the other hand, **Alippi *et al.* (2007**) found that the tylosin susceptible **et al., (1972); leluk et al., (1989); El- Ashhab (2001); Mohanny (2005); El-Shaarawy et al., (2007) (Han et al., (2007). and Hegazi et al. (2014).**strains showed MIC values lower than 4 µg/ml, considering that some of the isolates analyzed were intermediate to the antibiotic. Results of bee venom antimicrobial activity were similar to those found by **Gende *et al.* (2008b**). In the attempt to enhance the antimicrobial activity and reduce the development of resistance, the *in vitro* ad-ministration of antibiotic in combination with another active ingredient was conceived. In this particular case, trials aimed to determine whether it is possible to main-tain efficacy decreasing the dose of OTC when administered in a mixture with CEO. The results indicate that it is not possible to generalize the combined use of these antimicrobials in apiary, since synergism was observed only in 50% of the cases, while effects were antagonistic for 33% of the isolates studied. In practice, this variability suggests to conduct laboratory tests before adopting antibiotic cinnamon essential oil mixture application against *P. larvae* as an effective alternative to control the AFB in apiary. It was noticed that the gram positive were more affected by tested venoms. These findings confirmed by many authors as Bachmayer.

Data in table (4) showed that bee venom had the highest level of inhabitation to spore germination of AFB when used at concentration of 10 mg/ml and inhibited 0.68% of AFB spore germination at concentration of 10 mg/ml in the determination of MIC.

References

[1] **ALIPPI A. M.,** 1996.- Caracterización de aislamientos de Paenibacillus larvae mediante tipo bioquímico y resistencia a oxitetraciclina.- Revista Argentina Microbiologia, 28: 197-203.

[2] **ALIPPI A. M., RINGUELET J. A., CERIMELE E. L., RE M. S., HENNING C. P.,** 1996.- Antimicrobial activity of some essential oils against Paenibacillus larvae, the causal agent of American foulbrood disease.- Journal Herbs Spices Medicinal Plants, 4 (2): 9-16.

[3] **Alippi A.M. Bacterial diseases. In : Colin M.E. (ed.), Ball B.V. (ed.), Kilani M. (ed.).** *Bee disease diagnosis.* Zaragoza : CIHEAM, 1999. p. 31 (Options Méditerranéennes : Série B. Etudes et Recherches; n. 25)

[4] **Bachmayer, H.; Krell, G.; Suchamec, E.** ,1972. Synthesis of promelittin and melittin in the venom gland of queen and worker bees: patterns observed during maturation. J. Insect. Physiol., 18: 1515-1521.

[5] **BAZZONI E., FLORIS I.,** 1999.- Azione in vitro di diversi oli essenziali contro Paenibacillus larvae e Ascosphaera apis, pp. 191-197. In: Atti del Convegno Apilombardia 98, Giornate di studio sull'apicoltura (SABATINI A. G., COLOMBO M., SPREAFICO M., Eds), 25-27 September 1998, Minoprio, Italy.

[6] **BOGDANOV S.,** 2006.- Contaminants of bee products.- Apidologie, 37: 1-18

[7] **Bradford,M. M.** (1976). A rabid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72,248-254.

[8] **British pharmacopoeia NA** 2000. British pharmacopoeia NA,London stationary office, Medical sciences, ISBN NA. 2 Vols. +CD-ROM

[9] **CARTA C., FLORIS I., 1989**. Prospettive di controllo della peste americana delle api con oli essenziali. Prove preliminari.- Atti Società Italiana di Ecologia, 8: 183-187.

[10] **Dina Mohammed Ali El-saied Taksira**; Studies on treatment of some honeybees diseases using some botanical extracts; Doctor of Philosophy in science (Zoology – Entomology); Faculty of Science, Mansoura University 2015.

[11] **El-Shaarawy, K. O.; Zakaria, M. E.; Azza Taufik, A.; El-Shemy, a. a. m.**, 2007. Effect of different bee venom collectin periods using electrical shock device on some venom characteristics and honeybee colonies activites. J. Agric. Sci. Mansoura Univ., 32(6); 50775083.

[12] **El-Shahab, K.** , 2001. Studies on bee venom in honey bee venom colony. M. Sc. Thesis, Faculity of agriculture, Moshtohr, Zagazig univ., pp125.

[13] **EVANS E.,** 2003.- Diverse origins of tetracycline resistance in the honey bee bacterial pathogen Paenibacillus larvae.- Journal of Invertebrate Pathology, 83: 56-50.

[14] **FLORIS I., CARTA C.,** 1990.- In vivo activity of Cinnamomum zeylanicum Nees essential oil against Bacillus larvae White.- Apicoltura, 6: 57-61.

[15] **FLORIS I., CARTA C., MORETTI M. D. L.,** 1996.- Activité in vitro sur Bacillus larvae White de plusieurs huiles essentielles et essai d'utilisation au le rucher.- Apidologie, 27: 111-119.

[16] **FUSELLI S. R., GARCIA DE LA ROSA S. B., EGUARAS M. J., FRITZ R.,** 2008.- Chemical composition and antimicrobial activity of Citrus essences on honeybee bacterial pathogen Paenibacillus larvae, the causal agent of American foulbrood.- World Journal of Microbiology & Biotechnology, 24: 2067-2072.

[17] **GENDE L. B.,** 2009.- Principales componentes de aceites esenciales con actividad antimicrobiana frente a Paenibacillus larvae. 233 pp. Doctoral thesis, Buenos Aires University, Argentina. (In Spanish).

[18] **GENDE L. B., BAILAC P., MAGGI M. D., PONZI M., FRITZ R., EGUARAS M. J.,** 2009a.- Antimicrobial activity of Pimpinella anisum and Foeniculum vulgare essential oils against Paenibacillus larvae.- Journal of Essential Oil Research, 21: 91-93.

[19] **GENDE L. B., EGUARAS M. J., FRITZ R.,** 2008c.- Evaluation of culture media to support growth of Paenibacillus larvae subsp. larvae applied to studies of antimicrobial activity.- Revista Argentina de Microbiología, 40: 147-150.

[20] **GENDE L. B., FLORIS I., FRITZ R., EGUARAS M. J.**, 2008b.- Antimicrobial activity of cinnamon (Cinnamomum zeylanicum) essential oil and its main components against Paenibacillus larvae from Argentine.- Bulletin of insectology, 61 (1): 1-4.

[21] **GENDE L. B., MAGGI M. D., DAMIANI N., FRITZ R., EGUARAS M., FLORIS I**., 2009b.- Advances in the apiary control of the honeybee American Foulbrood with Cinnamon (Cinnamomum zeylanicum) essential oil.- Bulletin of Insectology, 62 (1): 93-97.

[22] **GENDE L. B., PRINCIPAL J, MAGGI M. D., PALACIOS S. M., FRITZ R., EGUARAS M. J.,** 2008a.- Extracto de paraíso (M. azedarach) y aceites esenciales de C. zeylanicum, M. piperita, L. officinalis, como control de Paenibacillus larvae.- Zootecnia Tropical, 26 (2): 151-156.

[23] **Gende, L. B.; Fernandez, N.; Buffa, F.; Ruiu, L.; Satta, A.; Fritz, R.; Eguaras, M. J. and Floris, I.(2011).** Susceptibility of (Paenibacillus larvae) isolates to a tetracycline hydrochloride and cinnamon (Cinnamomum zeylanicum) essential oil mixture. Bull. Insectol. 64(2): 247-250.

[24] **GENERSCH E., FORSGREN E., PENTIKAINEN J., ASHIRALIEVA A., RAUCH S., KILWINSKI J., FRIES I.,** 2006.- Reclassification of Paenibacillus larvae subsp. pulvifaciens and Paenibacillus larvae subsp. larvae as Paenibacillus larvae without subspecies differentiation.- International Journal of Systematic and Evolutionary Microbiology, 56: 501-511.

[25] **GORDON R. E., HAYNES W. C., PANG H. N.,** 1973.- The genus Bacillus.- United States Department of Agriculture, Agricultural Handbook No.427, Washington, DC, USA.

[26] **Gordon, R. E., Haynes, W.C. and Pang, H.N.C. (1973**). The genus

[27] **H. Costa, M. Boni-Mitake; C. F. Souza; J. R. Rogero (1999)**,EFFECTS OF GAMMA RADIATION ON BEE VENOM: PRELIMINARY STUDIES Energy and Nuclear Research Institute (IPEN/CNEN/SP) Brazil Radiobiology Division Travessa R , 400 - Cidade Universitária - 05508-900 - Caixa Postal 11049 –SP

[28] **Han S., Lee K., Yeo j., Kweon H., KimB., Kim J., Baek H and Kim S.T.** 2007: Antibacterial Activity of the Honey Bee Venom against Bacterial Mastitis Pathogens Infecting Dairy Cows. Int. J. Indust. Entomol. 14, (2), 137-142.

[29] **Hegazi A. G. and Fyrouz M. Abd Allah**, 2012. Antimicrobial Activity of Different Saudi Arabia Honeys. Global Veterinaria, 9 (1), 53-59, 2012

[30] **Hegazi A.G, Abdou A. M. and Abd Allah F.,** 2014: Evaluation of the antibacterial activity of bee venom from different sources. World Applied Sciences Journal 30 (3): 266-270.

[31] **Hegazi A.G**. , 2012: Medical importance of bee products. ARI B L M / BEE SCIENCE 12(4): 136-146.

[32] **Hegazi, A. G.; Moharram, N. Z.; Abd-Allah, F. A.; Nour, M. S. and Khair, A. M.**, 2002 : Antibacterial activity of different Egyptian honeys in relation to some bee products. Egypt. J. Vet. Sci., 36: 31-42.

[33] **Hossein Zolfagharian,1,* Mohammad Mohajeri,2 and Mahdi Babaie(2015),** Honey Bee Venom (Apis mellifera) Contains Anticoagulation Factors and Increases the Blood-clotting, Time. J Pharmacopuncture. 2015 Dec; 18(4): 7–11.

[34] **Jelinski, M. (1985)**. Some biochemical properties of Bacillus larvae White. Apidologie 16, 69–76.

[35] **Jobran Ellen, L. R. and Finegold, S. M.** 1994. Diagonative Microbiology. 9th ed. Part 2 pp: 168-188. Mosby Saint Louis, USA.

[36] **Khaled M. Attalla[1] A. Owayss[2] and Karem M. Mohanny**; Antibacterial activities of bee venom, propolis, and royal jelly produced by three honey bee, *Apis mellifera* L., hybrids reared in the same environmental conditions; *Annals of Agric Sci., Moshtohor, Vol. 45, No. 2: 895-902 pp.*, 2007.

[37] **Kilwinski J.; Peters M.; Ashiralieva A. and Genersch E. (2004).** Proposal to reclassify Paenibacillus larvae subsp. pulvifaciens DSM 3615 (ATCC 49843) as Paenibacillus larvae subsp. larvae. Results of a comparative biochemical and genetic study. Vet. Microbiol. 104: 31-42.

[38] **Leluk, J.; Schmidt, J.; Jones, D**.,1989. Comparative studies of the protein coposition of hymenoptera venom reservoirs. Toxicon. 27(1): 105-114.

[39] **Marian Brackmann, Ana I. Caceres, Donato del Camino,** 2009. A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. University of California, San Francisco, CA,106 (22) 9099-9104.

[40] **MERKER R. L.,** 1998.- Media and reagents, Appendix 3. In: Food and Drug Administration bacteriological analytical manual, 8th ed. (revision A) (CD-ROM version).- AOAC International, Gaithersburg, MD, USA.

[41] **MIYAGI T., PENG C. Y. S., CHUANG R. Y., MUSSEN E. C., SPIVAK M. S., DOI R. H.,** 2000. Verification of oxytetracycline-resistant American Foulbrood pathogen Paenibacillus larvae in the United States.- Journal of Invertebrate Pathology, 75: 95-96. **NASCIMENTO G. G. F., LOCATELLI J., FREITAS P. C., SILVA G. L.,** 2000. Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria.- Brazilian Journal of Microbiology, 31: 247-256.

[42] **Owayss, A. A. (2007).** Prelimenary investigation on American foulbrood disease. [1. Recording of the infection in the apiaries of Fayoum governorate]. Ann. Agric. Sc., Moshtohor, 45 (2): 903-910.

[43] **PICCINI C., ZUNINO P.,** 2001. American Foulbrood in Uruguay: Isolation of Paenibacillus larvae larvae from larvae with clinical symptons and adult honeybees

and susceptibility to oxytetracycline.- Journal of Invertebrate Pathology, 78: 176-177.

[44] **Richard J. Schmidt,** 1995. Contact Dermatitis. Tb00773.x, 32, 124-125.

[45] ***Sandra Schroeder Ingrid Müller, Rosângela Uhrig Salvatori, Cláudia Majolo and Hans Fröder*;** Detection of *Paenibacillus larvae* Subsp. *larvae* Spores in Honey Samples from Beekeepers of the Taquari Valley, Rio Grande Do Sul State, Brazil; International Journal of Microbiological Research 2 (3): 217-221, 2011; ISSN 2079-2093 © IDOSI Publications, 2011.

[46] **Sang Mi Han [1,*], Joung Min Kim[1], In Pyo Hong[1], Soon Ok Woo[1], Se Gun Kim[1], He Rye Jang[1] and Sok Cheon Pak;** Antibacterial Activity and Antibiotic-Enhancing Effects of Honeybee Venom against Methicillin-Resistant Staphylococcus aureus ;Molecules 2016, 21, 79; doi:10.3390/molecules21010079

[47] **Sedmak JJ, Grossberg SE,.** 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal Biochem. 1;79(1-2):544-52.

[48] **Shimanuki, H. and Knox, D.A. (1988).** Improved method for the detection of Bacillus larvae spores in honey. Am. Bee J., 128: 353-354.

[49] **Shimanuki, H.; Knox, D. A. (1991).** Diagnosis of Honey Bee Diseases. US Department of Agriculture, Agric. Handbook No. AH-690, 53p.

[50] **Soon-Tae Kim[1], Ji-Young Hwang, Myoung-Suk Sung, So-Yang Je,Dong-Rok Bae, Sang-Mi Han[*], and Sung-Hae Lee;** The Minimum Inhibitory Concentration (**MIC**) of Bee Venom Against Bacteria Isolated from Pigs and Chickens; *Korean J Vet Serv (2006) 29(1): 19-26.*

[51] **Warburg,o. and Christian, W.** 1942. Isolation and crystallization of enolase. Biochem. 310:384-421.

[52] **Zolfagharian H[1], Mohajeri M[2], Babaie M[3].(2016)**, Bee Venom (Apis Mellifera) an Effective Potential Alternative to Gentamicin for Specific Bacteria Strains: Bee Venom an Effective Potential for Bacteria. J. Pharmacopuncture. 2016 Sep;19(3):225-230