School of Pharmacy[1], Al-Azhar University, Nasr City, Cairo, Egypt, and School of Pharmacy[2], Texas Tech University Health Science Center, Amarillo, TX-USA

Honey, a prospective antibiotic: extraction, formulation, and stability

A. A. Zaghloul[1], H. H. El-Shattawy[1], A. A. Kassem[1], E. A. Ibrahim[1], I. K. Reddy[2] and M. A. Khan[2]

Old and recent reports show that honey has beneficial effects on the skin as antiseptic for wounds, burns and ulcers and as a healing promoter. Many investigators confirmed the usefulness of honey in the treatment of skin infections as well as internal diseases. The factors behind these effects are not completely explained. The aim of this study is: a) to investigate the antimicrobial activity of crude honey, b) to separate the fractions responsible for its activity, c) to formulate the honey extract as semisolid dosage forms, d) to study its release, and e) to determine its stability. The results showed that the ethylacetate honey extract showed antibacterial, anticandida and antifungal effects at low concentration. The release of honey extract from different ointment bases was depending on the constituents of the base, and its stability was found to be temperature and base dependent.

1. Introduction

The definition of honey depends upon who defined it. Most people think of honey as excellent food, but some others consider it an elixir, and still others as a medicine. Honey was mentioned in Holy books and scientific articles as nutrient and medicine [1]. Honey is obtained from the honey in the honeycomb of the Hive bee (*Apis mellifera* and other species of *Apis* family Apidae) [2]. Honey bees gather nectar and pollen from more than fifty five plant sources related to twenty seven families to produce about a hundred grams of honey [3]. Honey is a supersaturated sugar solution containing vitamins, minerals, proteins, amino acids, and nutrients. The largest portion of dry matter in honey consists of sugars (79%), which are responsible for much of its physical nature, viscosity, hygroscopicity and energy value [4, 5]. Scientific reports showed that honey exhibits important biochemical activities, as it cures and prevents bronchitis and bronchial asthma [6], urethritis [7], tonsillitis [8], rhinitis [9], eye diseases [10], diseases of the oral cavity [11], liver and GIT diseases [12, 13] and increases immunity [14]. Many investigators confirmed the usefulness of honey in the treatment of wounds [15, 16], burns [17, 18], ulcers [19], and as a healing promoter [20–22]. Several studies showed the superiority of honey over many of well-known antibiotics [23, 24]. Honey showed powerful antibacterial effects against pathogenic and non-pathogenic microorganisms, yeasts and fungi even against those that developed resistance to many antibiotics [25–29]. The factors responsible for these effects are not yet completely explored. The subject has been discussed repeatedly in scientific forums as well as by various researchers which is demonstrated by the large numbers of publications. Interpretations differ, however, reflecting the fact that this subject obviously cannot be examined in general, but must be studied from various points of view.

In this work trials were made to fractionate honey to isolate the portions which may be responsible for its antimicrobial effects, then to formulate the obtained fraction(s) in suitable semisolid dosage forms. Before the extraction process, the antimicrobial effects of crude honey obtained from different geographical zones in Egypt were investigated.

2. Investigations, results and discussion

Table 1 shows that honey produced in different regions of Egypt has more or less the same antibacterial effects against the studied gram negative and gram positive bacteria. The lowest concentration that showed this effect was 6.25%. The studied concentrations did not show any effect against *Candida albicans* or *Aspergillus niger*. This may be due to the high sugar concentration in honey [30] rather than an antibacterial factor called "inhibine" [31]. It may also be due to the volatile oils as mentioned by White et al. [32], or the hydrogen peroxide liberated as a result of glucose oxidase activity on glucose present in honey [30]. Obaseiki-Ebort et al. [33] succeeded in separating certain fractions from honey by destructive distillation. The separated fractions showed powerful antibacterial and antifungal effects. Using diethyl ether, Mohammed [34] separated fractions with antibacterial activity. In this study, organic solvents having different polarities and miscibility with water, namely n-hexane, diethyl ether, chloroform and ethylacetate were used to extract honey. Table 2 shows the effects of these extracts against gram positive and gram negative bacteria, yeasts and fungi. The results showed that the n-hexane extract was devoid of any effect against the microorganisms studied. The diethyl ether extract showed a slight antibacterial effect against *E. coli, Klebsiella aerogenes, Pseudomonas aeruginosa, Bacillus pumilis*, and *Proteus vulgaris*. It, however, showed no effect against yeast and fungi. The chloroform extract showed no antibacterial, antiyeast or antifungal effect except a slight effect against *E. coli*. The ethylacetate extract showed marked effects against all the microorganisms studied. These observations are in agreement with the earlier report which proved the presence of thermostable substances in honey which are highly polar and have antibacterial activity against *Bacillus subtilis* [35]. The obtained results were promising and paved the way for the next steps which focus on ethylacetate honey extract. Fig. 1 shows the linearity of plotting log% concentration of ethylacetate honey extract versus inhibition zone diameter; the minimum inhibitory concentration (MIC) was 0.5% v/v.

Table 3 shows the release of ethylacetate honey extract from the investigated ointment bases. This release varied from one class of ointment bases to the other and even in between the same class of ointment bases. The PEG containing bases (base No. 11, 12 and 13) showed higher release values. The higher percentage of low molecular weight PEG 400 (60%) in base 11 decreased its consistency and hydrophilicity and thus the release was pronounced. On the other hand, the presence of cetostearyl or

Table 1: Antimicrobial effect of honey produced in different regions of Egypt

Type of microorganism	Mean* of inhibition zone diameter in cm using honey produced in different regions of Egypt																			
	Region 1					Region 2					Region 3					Region 4				
	Conc.					Conc.					Conc.					Conc.				
	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125
Bacteria																				
Bacillus subtilis	3.1	2.6	2.6	2.3	–	2.8	2.5	2.4	2.2	–	2.9	2.7	2.5	2.3	–	2.8	2.6	2.5	2.4	–
Bacillus pumilis	2.8	2.5	2.6	2.2	–	2.9	2.6	2.4	2.1	–	2.8	2.6	2.5	2.1	–	2.7	2.7	2.5	2.1	–
Staph. aureus	2.5	2.4	2.3	2.0	–	2.6	2.5	2.3	2	–	2.7	2.6	2.4	2.1	–	2.6	2.5	2.3	2.1	–
Pseud. aeruginosa	2.8	2.6	2.5	2.4	–	3.1	2.8	2.6	2.6	–	2.9	2.6	2.5	2.4	–	2.8	2.7	2.6	2.5	–
Protus vulgaris	3.4	3.3	2.4	2	–	3.3	3.2	2.5	2	–	3.4	3.3	2.2	2	–	3.6	3.3	2.1	2	–
Klebsiella aerogenes	2.4	2.2	2.1	1.9	–	2.5	2.3	2.3	2.1	–	2.6	2.5	2.4	2.2	–	2.5	2.4	2.3	1.8	–
Escherichia coli	3.2	3	2.7	2.3	1	3.4	3.2	2.9	2.6	0.9	3.3	3.1	2.9	2.6	1	3.3	2.9	2.7	2.5	1
Yeast																				
Candida albicans	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fungus																				
Aspergillus niger	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

* Mean of five readings

Table 2: Antimicrobial effect of different honey extracts

Type of microorganism	Mean* of inhibition zone diameter in cm using the following honey extracts							
	n-Hexane		Diethyl ether		Chloroform		Ethylacetate	
	Extract	Blank	Extract	Blank	Extract	Blank	Extract	Blank
Bacteria								
Bacillus subtilis	–	–	–	–	–	–	4.2	–
Bacillus pumilis	–	–	1	–	–	–	4.2	–
Staph. aureus	–	–	–	–	–	–	4.3	–
Pseud.aeruginosa	–	–	1	–	–	–	4.5	–
Protus vulgaris	–	–	0.9	–	–	–	4.2	–
Klebsiella aerogenes	–	–	1.2	–	–	–	4.6	–
Escherichia coli	–	–	1.3	–	1.2	–	4.6	–
Yeast								
Candida albicans	–	–	–	–	–	–	4.0	–
Fungus								
Aspergillus niger	–	–	–	–	–	–	3.8	–

* Mean of five readings

cetyl alcohol, being fatty alcohols, decreased the release from bases 12 and 13 when compared to base 11 which may be due to the increased consistency of these bases. Bases containing these two alcohols showed higher release than the oleaginous or absorption bases. This may be due to their emulsifying properties. The low percentage of solid PEG 4000, the presence of the hydrophilic agent glycerol, and the anionic surfactant, sodium lauryl sulphatt, in base 13 led to a release better than that from base 12. The emulsion bases 7 and 8 gave better release than any

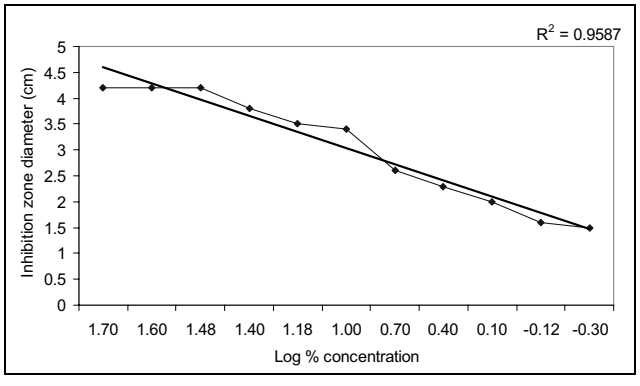

Fig. 1: Relationship between the concentration and inhibition zone diameter of ethylacetate honey extract

Table 3: Release of ethylacetate honey extract from different formulations

Mean* of inhibition zone diameter (cm)		
Base No.	Non medicated base	Medicated base
1	–	1.7
2	–	1.3
3	–	2.0
4	–	2.5
5	–	2.3
6	–	2.1
7	1.0	2.8
8	0.8	2.6
9	–	1
10	–	1.2
11	–	2.9
12	–	2.5
13	–	2.7

* Mean of five experiments

Table 4: Composition of different ointment bases

Ingredients of Different bases	Formulation Number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
	Number of parts of each ingredient												
Yellow bees wax	5	2					1			17			
Yellow soft paraffin	95	90	80	10	85	10		25					
Hard paraffin		3		24	5	24							
Cetostearyl alcohol		5			5								20.8
Liquid paraffin			20	60		45				45			
Wool alcohol				6		6							
Wool fat					5								
Silicon oil						15							
Cetyl alcohol							15					5	
Propylene glycol							10	12					
Sodium lauryl sulphate							2	1					0.6
Purified water							72	37		37			50.4
Methyl paraben								0.025					
Propyl paraben								0.015					
Stearyl alcohol								25					
Stearic acid									20				
Potassium hydroxide									0.5				
Alcohol 90%									5				
Borax									1.5	1			
PEG 4000											40	47.5	11.2
PEG 400											60	47.5	
Glycerol													17

other emulsion, absorption or oleaginous bases. This may be due to the presence of the anionic surfactant sodium lauryl sulphate, which facilitates penetration of the extract to the surrounding media. The absorption bases 4, 5, and 6 gave better release than the oleaginous bases, probably due to the presence of wool alcohol or wool fat. The oleaginous bases 3, 1 and 2 showed the least release values among the investigated ointment bases. This may be due to the oil-like behavior of ethylacetate honey extract, being almost immiscible with water and therefore retained in the oleaginous bases. These results are supported by the finding that the release of drugs will be better from vehicles which have low affinity for the penetrant or in which the agent is less soluble [36]. Silicone oil, being water insoluble and water repellent, affected inversely the release from base 5. From the overall results, the sequence of the release from different bases was as follows: Base No. 11 > 7 > 13 > 8 > 4 & 12 > 5 > 6 > 3 > 1 > 2 > 10 > 9. Fig. 2 and Table 3 show the release of extract from different bases.

The degradation of ethylacetate honey extract was found to follow first order kinetics, since a plot of the logarithms of percentages extract remained versus time yielded an almost straight line. On evaluating the whole set of results obtained in this study, one can observe that ethylacetate honey extract ointments stored at 0 °C have the highest stability while ointments stored at 35 °C have the lowest one. Formulations stored at 25 °C showed intermediate stability. From the kinetic data one can conclude that the emulsion base No.7 is the most stable, while base No. 4 is the worst one.

On the light of chemical and phytochemical analysis, the antibacterial fractions in honey may contain phenols and/or flavenoids beside the probable presence of free acids and bases. From these findings one can deduce the high reactivity of these fractions towards many compounds present in the investigated bases. It was assumed that honey itself can resist storage at normal temperatures for several hundred years as a jar of honey was discovered in one of the Pharaon's tomb just recently and it was in a good condition. However, the stability of the prepared formulae were affected by increasing the temperature of storage as indicated by the increase in (K) values. Further investigations should be done to elucidate the exact chemical composition of these fractions and the mechanism of their degradation.

In conclusion, it can be stated that commercial honey shows remarkable effects against gram negative and gram positive bacteria regardless of the region from which it

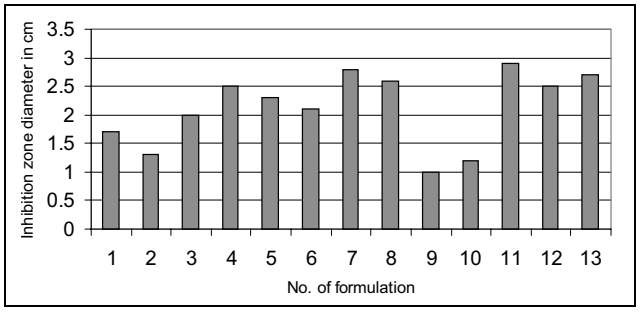

Fig. 2: Release of ethylacetate honey extract from different formulations

Table 5: Kinetic data for honey extract formulations based on accelerated stability testing

Form. No.	K zero (month^{-1})	K 25 (month^{-1})	K 35 (month^{-1})	Ea (cal/mol)	T 10% (month)	T 1/2 (month)
1	0.137	0.107	0.209	12330.52	1.412	9.288
3	0.096	0.126	0.182	6702.06	1.016	6.684
4	0.195	0.286	0.267	−1306.62	0.354	2.330
5	0.185	0.163	0.279	9829.79	0.859	5.651
6	0.225	0.175	0.194	1864.99	0.636	4.184
7	0.068	0.046	0.107	15369.41	3.562	23.436
8	0.138	0.246	0.310	4250.29	0.485	3.190
11	0.032	0.035	0.035	114.24	3.061	20.135
12	0.069	0.067	0.129	11911.60	2.212	14.551
13	0.061	0.046	0.084	11061.81	3.166	20.830

was obtained. The minimum concentration of honey fractions needed for this activity was 6.25%. No fraction showed any effect against yeast or fungi at any concentration. The fractions responsible for these effects could be isolated using organic solvents. The ethylacetate honey extract showed a consistent effect against the studied bacteria, yeast and fungi at low concentrations (0.5%). This extract when formulated in different ointment bases showed different release patterns depending on the constituents of these bases. The formulated bases when stored at different temperatures for six months showed high reactivity reflected by the high decomposition rate constants. The isolated fractions should be chemically investigated to avoid interactions with the constituents of the final formulae.

4. Experimental

4.1. Materials

Samples of pure honey were purchased from different geographical zones in Egypt. 24-hours subcultures of different microorganisms, viz. *Bacillus subtilis*, *Bacillus pumilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella aerogenes* and *Escherichia coli*, 48-hours subcultures of *Candida albicans* and *Aspergilus niger* were supplied by the Dept. of Microbiology, School of Pharmacy, Al-Azhar Univ. Cairo, Egypt. Different media were prepared including nutrient agar, nutrient broth, Sabouraud-glucose agar, and Sabouraud-glucose liquid media (Oxoid, England). n-Hexane, diethyl ether and chloroform (Prolabo, France). Ethylacetate (E. Merck, Germany). Other chemicals were of analytical grade and were used as supplied.

4.2. Tests for adulteration

The following tests were carried out on different samples of purchased honey according to the requirements of the Egyptian Pharmacopoeia: a-Tests for overheated or artificial honey; b-Tests for starch or dextrin; c-Tests for coloring matter; d-Tests for nitrogenous pigments (Azodyes); e-Tests for artificial honey or inverted sugars.

4.3. Microbiological investigation of pure honey

Nutrient agar (Sabouraud-glucose agar for yeast and fungi) medium was prepared, sterilized, cooled to 48 °C and divided into several portions. Each portion was inoculated with one of the studied bacterial suspensions in nutrient broth (yeast and fungi subcultures were prepared in Sabouraud-glucose liquid medium). Aliquots, each 30 ml of inoculated nutrient agar or Sabouraud-glucose agar medium were poured in flat sterile petri dishes (11 cm diameter), and allowed to solidify. Using a sterial cork borer, five equal holes (each 0.7 cm diameter) were made in each plate. Each honey sample was serially diluted with distilled water to produce 50, 25, 12.5, 6.25, and 3.125% v/v solutions. 0.3 ml of each of these solutions was placed in five holes in different petri dishes. All the plates were incubated (Lotus Co., Model 215 incubator, Cairo, Egypt) at 37 °C for 24 h (25 °C for 48 h for yeast and fungi). The diameter of each zone of inhibition was recorded, and the average of five experiments was calculated for each concentration.

4.4. Extraction process

Two kilograms of pure honey were put in a separating funnel, diluted with 3 l of distilled water, shaked well, and extracted with 3 l of one of the organic solvents under investigation (n hexane, diethyl ether, chloroform and ethylacetate). The extractions were performed as three successive extractions using 1 l of the solvent each time. The shaking time for each extraction process was 15 min after which the mixture was allowed to stand for separation, and the organic solvent layer was separated. The three portions were collected and mixed. Water contaminating the organic solvent extract was removed by filtration over anh. sodium sulphate using a Buchner flask. The organic solvent extracts were concentrated under reduced pressure using a rotary evaporator (Hydolph, V.V. 2000, Germany) at 40 °C for n-hexane, 30 °C for diethyl ether, 50 °C for chloroform, and 60 °C for ethylacetate.

4.5. Microbiological investigation of different honey extracts

The effect of honey extracts against bacteria, yeast and fungi were performed by the method reported in the microbiological investigation of honey. The respective pure solvent used for extraction of honey was tested side by side with its extract.

4.6. Determination of the minimum inhibitory concentration (MIC) of ethyl acetate honey extract

For MIC determination of ethylacetate honey extract, *Bacillus subtilis* spores, NCTC 10315 (supplied by Dept. of Microbiology, Alexandria University, Alexandria, Egypt), and nutrient media specific for this microorganism composed of agar, peptone, pancreatic digest of casine, yeast extract, beef extract and dextrose (London Analytical and Bacteriological Media Ltd., Fordlane, England) were used. Different dilutions of ethylacetate honey extract were made using distilled water to give 50, 40, 30, 25, 20, 15, 10, 5, 2.5, 1.25, 0.75, 0.5, and 0.25% v/v solutions. The nutrient agar media was prepared, sterilized, cooled to 48 °C, and divided into portions of 500 ml each. The *Bacillus subtilis* spores were prepared and its concentration was adjusted to give $10^7 : 10^8$ cells/ml and stored at 4 °C. Each portion of the prepared nutrient agar medium was inoculated with 1.5 ml of the spores (suitable to give reasonable growth), mixed well, and then poured in sterile petri dishes. A plot representing the relationship between log percentage concentration and inhibition zone diameter is shown in Fig. 1.

4.7. Formulation of Ethylacetate honey extract in different ointment bases

Ointment bases containing oily and waxy materials were prepared by the fusion method; they were melted together on water bath and allowed to cool with stirring until congealing. For bases containing water soluble ingredient (s), the latter was dissolved in water, warmed at 70 °C, then gradually added to the melted oily mixture with continuous stirring, and allowed to cool with stirring until congealing. All the ointment bases used are reported in Table 4. Five grams of each ointment base was mixed with ethylacetate honey extract to give a 10% V/W concentration.

4.8. Microbiological release study of honey extract formulations

Sterilized nutrient media specific for *Bacillus subtilis* (500 ml) was inoculated with 1.5 ml of the spore suspension, mixed well, then poured in sterile petri dishes of 11 cm diameter and left to solidify. Six holes were made on the periphery of each plate with a sterile cork borer of 0.7 cm diameter. Four holes in different plates were filled with one of the formulated ointments. The non-medicated bases were tested side by side with the medicated ones.

4.9. Stability of ethylacetate honey extract formulations

The ten bases chosen in this study were those that showed the highest release among the thirteen bases tested before. The experiment was performed by packaging stocks of the prepared ethylacetate honey extract formulations in brown glass jars. Each formulation was stored for six months at three different temperatures, viz. 0 °C, 25 °C, and 35 °C. Samples of each were evaluated microbiologically, as previously mentioned, at the beginning of the experiment and at the end of each month of storage. The ratio of the inhibition zone produced at a given time with that produced by the ointment when freshly prepared was converted into percentage stability.

References

1 The Glorious Quran, Sourat Al-Nahl (The Bees), Aya 69, Translated and Commentary by Yousuf Ali, Dar Al-fekr, Beirut, Lebanon, 1980
2 The Pharmaceutical Codex, 11th Ed., Pharmaceutical Press, U. K., 1979
3 Ibrahim, S. H.: Agric. Res. Rev. **54**, 217 (1976)
4 Ibrahim, S. H.; Soliman, N. Z.; Wissa, H.: Agric. Res. Rev. **55**, 125 (1977)
5 Crane, E.: A Comprehensive Survey of Honey, 1st Ed., Heinemann, London, U. K. 1975
6 Bacvarov, V. I.: Ther. Ggw. **109**, 260 (1970)
7 Shkliar, N. P.: Vrach Delo. **3**, 142 (1966)
8 Kachnyi, G. G.: Vestn Otorinolaringol. **2**, 19 (1980)
9 Radchenko, A.; Danilov, L. N.: Zh. Ushn. Nos. Gorl Bolezn. **3**, 64 (1979)
10 Mozherenkov,V. P.; Shubina, L. F.: Feldsher Akush. **47**, 59 (1982)
11 Fasika, O. M.; Arotiba, J. T.; Fasola, A. O.: Afr. J. Med. Sci. **25**, 297 (1996)
12 Kandil, A.; Mounir, A.: The Effect of Honey on Pathologic Liver, 4th International Conference on Islamic Medicine. P. 96, Kuwait, 1986
13 Salem, S. N.; Hassan, M. A.; Jamal El-din, H.: Bul. Islamic Med. (Kuwait) **2**, 411 (1982)
14 Mladenov, S. T.; Mladenov, D.: A New Apitherapeutic Method for the Treatment of Certain Non-specific Acute and Chronic Diseases of the Respiratory Organs, XXXth International Apicultural Congress Abstracts, Japan Beekeeping Association, p. 148, Nagoya, Japan, 1985
15 Tovey, F.: Trop. Doct. **30**, 1 (2000)
16 Ndayisaba, G.; Bazira L.; Habonimana, E.: Presse Med. **21**, 1516 (1992)
17 Hall, M.: Prof. Nurse. **12**, 489 (1997)

18 Subrahmanyam, M.: Br. J. Surg. **78**, 497 (1991)
19 Casey, G.; Rij. A. V.: N. Z. Med. J. **110**, 216 (1997)
20 Oryan, A.; Zaker, S. R.: Zentralbl. Veterinarmed. A, **45**, 181 (1998)
21 Ndayisaba, G.; Bazira, L.; Habonimana, E.; Muteganya, D.: Rev. Chir. Orthop. Reparatrice Appar. Mot. **79**, 111 (1993)
22 Subrahmanyam, M.: Burns **22**, 491 (1996)
23 Al-Waili, N. S.; Saloom, K. Y.: Eur. J. Med. Res. **4**, 126 (1999)
24 Vardi, A.; Barzilay, Z.; Linder, N.; Cohen, H. A.; Paret, G.; Barzilay, A.: Acta Paediatr. **87**, 429 (1998)
25 Cooper, R. A.; Wigley, P.; Burton, N. F.: Lett. Appl. Microbiol. **31**, 20 (2000)
26 Karayil, S.; Deshpande, S. D.; Koppikar, G. V.: J. Postgrad. Med. **44**, 93 (1998)
27 Cooper, R. A.; Molan, P. C.; Harding, K. G.: J. R. Soc. Med. **92**, 283 (1999)
28 Cooper, R.; Molan, J.: Wound Care **8**, 161, (1999)
29 Efem, S. E.; Udoh, K. T.; Iwara, C. I.: Infection **20**, 227 (1992)
30 Willix, D. J.; Molan, P. C.; Harfoot, C. G.: J. Appl. Bacteriol. **73**, 388 (1992)
31 Schuler, R.; Vogel, R.: Arzneimittel Forsch. **6**, 661 (1956)
32 White, J.; Subers, M.: J. Apicult. Res. **2**, 93 (1963)
33 Obaseiki-Ebort, E. E.; Afonya, T. C. A.; Onyekweli, A. O.: J. Pharm. Pharmacol. **35**, 748 (1983)
34 Mohamed, T. R.: Studies on Certain Honey Bee Products, M. Pharm. Thesis, Faculty of Pharmacy, Cairo University, Cairo, Egypt 1980
35 Pierre, M.; Pierre, M.: Compt. Rend. **256**, 1858 (1963)
36 Blank, I. H.; Scheuplein: Progress in the Biological Sciences in Relation to Dermatology, A. Rook and R. H. Champion, Eds. P. 245, University Presss, Cambridge, England 1964

Received December 6, 2000
Accepted January 10, 2001

Abdel-Azime A. Zaghloul, Ph. D.
Assistant Professor of Pharmaceutics
Texas Tech University
Health Sciences Center
School of Pharmacy
Amarillo, TX 79106
USA
abdel@cortex.ama.ttuhsc.edu