http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (19): 3295-3305, 2007
ISSN 1028-8880
© 2007 Asian Network for Scientific Information

Inhibitory Effect of Egyptian Propolis on *Fasciola gigantica* Eggs with Reference to its Effect on *Clostridium oedematiens* and Correlation to Chemical Composition

[1]Ahmed G. Hegazi, [2]Faten K. Abd El Hady and [3]Hatem A. Shalaby
[1]Department of Zoonotic Diseases,
[2]Department of Chemistry of Natural Products,
[3]Department of Parasitology, National Research Center, Giza, Egypt

Abstract: The chemical composition of the propolis samples was investigated by GC/MS and HPLC. 91 compounds have been identified, 26 compounds are being new to propolis. Siwa oasis propolis was characterized by the presence of diprenyl-dihydrocoumaric acids (4.15%), coumarate esters (10.93%), benzofuran lignans (13.47%) and valeric acids derivatives (11.42%). Matrooh sample was characterized by the presence of new ferulate esters, hydroxy acetophenones and furanon derivatives, furofuran lignans and sterol from pregnane type. Dakahlia propolis was a typical poplar propolis. The present study proved evidence of inhibitory activity of propolis on the vitality and hatchability of immature *F. gigantica* eggs, where three different localities in Egypt were tested for this purpose. The Siwa propolis showed the highest inhibitory effect than the other sources. The complete failure of development and death of all immature eggs were recorded at concentration of 200 μg mL^{-1} of Siwa, 400 μg mL^{-1} of Matrooh and 800 μg mL^{-1} of Dakahlia propolis. The exposed *F. gigantica* egg shell revealed the same features as the non exposed egg shell when scanning with the electron microscopy. The three propolis samples showed inhibition in the growth of associated bacterium *Clostridium oedematiens* (*Clostridium novyi*) type B to fascioliasis. The inhibition varied according to the propolis origin.

Key words: Propolis composition, *F. gigantica*, *Clostridium oedematiens*

INTRODUCTION

Propolis (bee glue) is a resinous hive product. Propolis possesses variable biological activities: antiviral activity (Hegazi *et al*., 1993), antibacterial (Hegazi and Abd El Hady, 2002), fungicidal (Hegazi *et al*., 2000), antiulcer and anti-tumor etc. (Marcucci, 1995; Hegazi *et al*., 1998). The major constituents of propolis are: phenolic acids, phenolic acid esters, aliphatic acids, sugars, flavones, flavanones, flavonols and terpenoids (Hegazi and Abd El-Hady, 2001, 2002; Abd El-Hady and Hegazi, 2002). Phenolic acids, phenolic acid esters, flavanones, flavonols and flavones were responsible for antibacterial, anti-inflammatory and antioxidant activities. Also caffeic acid phenethyl ester, prenylated-p-coumaric acids, clerodane diterpenes, benzofurans, prenylated (benzophenones and flavanones) had antitumor and hepatoprotective activities (Bankova, 2005). It had been proven to be 100% effective against some lethal protozoa and would also decrease inflammation associated with parasite infection (Higashi and De Castro, 1994).

In the last decade, fascioliasis had imposed itself as an important zoonotic disease in Egypt posing a clinical and epidemiological health problem (Haseeb *et al*., 2002). Economical losses were determined at over $2 billions in USA. According to some estimates every year about 600 billions of domestic animals became infected world wide (2.4 to 17 millions people were infected) (Dalton, 1999).

Besides, repeated and continuous use of synthetic drugs led to development of resistant strains to these chemical compounds. Drug resistance to fasciolicidal has been identified in the field and in the laboratory (Boray, 1997). Thus natural safe effective fasciolicidal drugs are urgently needed. So the aim of the present investigation was to study-for the first time whether propolis had any effect on the vitality and hatchability of immature *F. gigantica* eggs this could be used as a preliminary marker for further studies on the fluke. Also the aim was directed to assess the chemical composition of propolis from different localities in Egypt.

MATERIALS AND METHODS

Propolis: Three Egyptian propolis samples were collected from different localities. The first sample was from Siwa oasis (S), the second sample from Matrooh beach (M) and the third sample from Dakahlia (D).

Corresponding Author: Ahmed G. Hegazi, Department of Zoonotic Diseases, National Research Center, Giza, Egypt

Extraction and Sample preparation of propolis: One gram of each propolis sample was cut into small pieces and extracted at room temperature with 50 mL of 70% ethanol (twice after 24 h). The alcoholic extract was evaporated under vacuum at 50°C until dryness. The percentage of extracted matter was as follows: Siwa propolis 0.37 g dry^{-1} weight, Matrooh propolis 0.38 g dry^{-1} weight and Dakahlia propolis 0.80 g dry^{-1} weight.

2.5 mg of the dried matter was prepared for chromatography by derivatization for 30 min at 100°C with 50 µL pyridine+100 µL N,O, bis-(Trimethylsilyl) Trifluoroacetamide (BSTFA) and analyzed by GC/MS (Greenaway *et al*., 1991).

GC/MS analyses: A Finnigan MAT SSQ 7000 mass spectrometer was coupled with a Varian 3400 gas chromatograph. DB-1 column, 30×0.32 mm (internal diameter), was employed with helium as carrier gas (He pressure, 20 Mpa cm^{-2}), injector temperature, 310°C; GC temperature program, 85-310°C at 3°C/min (10 min initial hold). The mass spectra were recorded in electron ionization (EI) mode at 70 eV. The scan repetition rate was 0.5 s over a mass range of 39-650 atomic mass units (amu).

Identification of compounds: The identification was accomplished using computer search user-generated reference libraries, incorporating mass spectra. Peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the bases of its mass spectral fragmentation. Reference compounds were co-chromatographed where possible to confirm GC retention times.

HPLC analysis of propolis: The dry extract was dissolved in methanol and filtered through 0.45 µm filter before direct HPLC analysis. The HPLC analysis was achieved with Agilent 1100 series liquid chromatograph with UV detector and an auto-sampler. The column used was a Lichrochart RP-18 (Merck, Darmstadt, Germany; 25×0.4 cm, 5 µm particle size). Elution was with water: formic acid (19:1 v:v; solvent A) and acetonitrile (solvent B) and the flow rate was 1 mL min^{-1}. Gradient elution started with 20% B, reaches 25% B at 25 min and 30% B at 35 min and then the system became isocratic until 50 min, reaches 50% B at 60 min and 70% B at 67 min. The flavonoids were detected with UV detector and the chromatograms were recorded at 340 and 290 nm.

Flavonoid identification and quantification: The different flavonoids were identified by chromatographic comparisons with authentic markers, some of them are commercial and the others were kindly provided by Prof. Wollenweber, (Institut of. Botanik Schittspahnstr. TU Darmstadt, Germany). The flavanones were detected at 290 nm and the flavones at 340 nm. Also caffeic acid esters, such as dimethylallyl caffeate and phenylethyl caffeate, which are characteristic of propolis, were detected as reported (Tomas-Barberan *et al*., 1993; Gil *et al*., 1995). Flavonoid identification was carried out by direct HPLC comparison of authentic markers (Table 1) and was based on co-chromatography in 290 and 340 nm. Response factors for the authentic markers and the concentration of flavonoids in each propolis sample were calculated according to Ogan and Katz (1981) and Annual book of ASTM Standards (1983).

***Fasciola gigantica* eggs:** *F. gigantica* eggs were obtained from the gall bladders of naturally infected buffaloes slaughtered in El Warak abattoir, Giza, Egypt known by the presence of adult *F. gigantica* worms in the bile passages of the livers of these buffaloes.

Collection of *F. gigantica* eggs: The obtained gall bladders were transferred immediately to the laboratory, washed and examined individually for the presence of adult *F. gigantica* worms. Each gall bladder was evacuated separately in one-liter cylinder, mixed with tap water, left to sediment and then the supernatant was decanted without disturbance of the sediment. This process was repeated 3-5 times. The sediment of the last decant was sieved through two sieve system 400 and 100 microns, respectively, called Fluke finder technique according to Welch *et al.* (1987). The clean *F. gigantica* eggs were collected after sedimentation and sieving and stored in distilled water in the refrigerator to be used as fresh as possible.

Embryonation of *F. gigantica* eggs and production of miracidia: Eggs embryonation was carried out according to Boray (1963). One layer thickness of fresh collected eggs was transferred to petri dishes of 6 cm diameter half filled with distilled water (pH 6.8-7.2). Each dish was placed inside another one of 8cm diameter covered from outside with aluminum foil. The dishes were covered and incubated at 26°C. The cultured eggs were examined every 3 days until the eggs approached hatching, as evidenced by movement of the miracidia within the eggs (Roweliffe and Ollerenshaw, 1959). The day of hatching was the day in which most of the eggs with developed active miracidia hatch after exposure to artificial light for 15 min.

Table 1: Chemical composition of Egyptian propolis assessed by GC/MS analysis

	Siwa	Matrooh	Dakahlia*
Compounds	----------	TIC (%)	----------
Aliphatic acids			
Lactic acid	----	----	0.25
Hydroxyacetic acid	0.17	----	---
2- Hydroxypropanoic acid	0.51	----	---
Propanoic acid-3- hydroxyl-2-oxo	0.17	----	---
5-Hydroxy-n-valeric acid	----	----	0.28
Malic acid	----	----	0.7
Octanoic acid	0.08	----	---
Methyl-propanedioic acid	0.12	----	---
2,3-Dihydroxypropanoic acid	0.15	----	---
2,3,4-Trihydroxybutyric acid	0.14	----	---
2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone	0.21	----	---
Nonanoic acid	---	----	0.20
Decanoic acid	---	----	0.20
Dodecanoic acid	---	----	0.50
Tetradecanoic acid	---	----	0.50
Palmitic acid	11.5	0.1	13.30
8-Methoxy hexadecanoic acid-	-	0.21	-
γ-Dodecalactone	----	0.09	----
Linoleic acid	----	----	1.5
Oleic acid	----	0.3	12.30
Hydroxyl-octadecenoic acid	----	0.08	----
3,5-Dihydroxy-3-heptenylvaleric acid[b]	1.8	1.12	----
3,5-dihydroxy-3-octenyl valeric acid[b]	2.0		
3,5-dihydroxy-3-octenyl valeric acid isomer[b]	1.32		
3,5-dihydroxy-3-octenyl valeric acid isomer[b]	6.3		
Eintriacontenoic acid	4.62	0.03	----
Aromatic acids			
Benzoic acid	----	----	2.70
4-Hydroxy benzoic acid	----	----	0.60
2,4- Dihydroxybenzoic acid	----	0.03	----
2,6- Dihydroxybenzoic acid	----	0.03	----
2,3,4- Trihydroxybenzoic acid	----	0.08	----
2,4,6- Trihydroxybenzoic acid	----	0.13	----
3,4,5- Trihydroxybenzoic acid	----	0.03	----
2-Phenyl- 2-hydroxy acrylic acid	----	----	0.40
Dihydrocinnamic acid	----	----	0.30
Cinnamic acid	----	----	2.80
4- Methoxy-cinnamic acid	----	----	0.80
cis-p-Coumaric acid	----	----	0.40
trans- p-Coumaric acid	----	----	2.30
3,5-diprenyl coumaric acid			
3,4-Dimethoxy-cinnamic acid	----	----	2.90
Isoferulic acid	----	----	1.10
Ferulic acid	----	----	2.40
Caffeic acid	----	----	4.40
3,5-dimethoxy-4-pentanyl cinnamic acid[b]	0.46		
3,5-diprenyldihydrocoumaric acid[b]	2.87		
3,5-diprenyldihydrocoumaric acid isomer[b]	1.28		
Esters			
Ethyloleate	----	0.17	----
Octadecenoic acid methyl ester	----	0.22	----
Phthalate ester	----	0.2	----
Hydroxyl-pentacosatrienoic acid methyl ester	----	0.6	----
Hydroxyl-pentacosatrienoic acid methyl ester (isomer)	---	0.28	----
Methyl palmitate	----	----	0.27
Ethyl palmitate	----	----	0.13
Stearic acid methyl ester	----	----	0.20
2- Hydroxy-hexandioic acid monodecenyl ester	-	0.17	-
Benzyl benzoate	----	----	0.40
Benzyl –trans-4- coumarate	----	----	0.03
Cinnamyl –trans-4- coumarate	----	----	0.09
3,5-diisopropoxy-nonanyl coumarate[b]	1.93	-	
3,5-diisopropoxy-nonanyl coumarate isomer[b]	2.5	-	
3,5-diisopropoxy-nonanyl coumarate isomer[b]	3.08	-	
3,5-diisopropoxy-nonanyl coumarate isomer[b]	2.55	-	
3,5-dimethoxy-tetradecenyl coumarate[b]	0.87	-	
3,5-dimethoxy-tridecenyl dihydrocoumarate[b]	-	0.6	
3-Methyl-3-butenyl isoferulate	----	----	0.07
3-Methyl-2-butenyl isoferulate	----	----	0.14
3-Pentanyl-octyldihydroferulate[b]	-	1.2	-
3-Hexanyl- octyldihydroferulate[b]	-	0.38	-
3-Hexanyl- octyldihydroferulate isomer[b]	-	0.71	-
3-Methyl-3-butenyl caffeate	----	----	0.64
2-Methyl-2-butenyl caffeate	----	----	0.18
3-Methyl-2-butenyl caffeate	----	----	0.90
Benzyl caffeate	----	----	0.32
Phenylethyl caffeate	----	----	0.30
Cinnamyl caffeate	----	----	0.10
Tetradecyl caffeate	----	----	0.18
Tetradecenyl caffeate	----	----	0.05
Tetradecenyl caffeate (isomer)	----	----	0.13
Tetradecanyl caffeate	----	----	0.05
Hexadecyl caffeate	----	----	0.15
Di and Triterpenes			
Dehydroabietic acid	----	----	0.14
Lupeol	----	----	0.42
Cycloartinol	----	----	0.58
α -Amyrin	----	----	0.30
β-Amyrin	----	----	0.21
Triterpene of β-amyrin type $[M^+]$ m/z = 498	----	----	0.44
Sterols			
Sterol (pregnane type)$[M^+]$ m/z = 372	----	0.45	----
Sterol (pregnane type) $[M^+]$ m/z = 460	----	0.15	----
Sterol (pregnane type) $[M^+]$ m/z = 372	----	0.2	----
Sterol (pregnane type) $[M^+]$ m/z = 386	----	0.6	----
Sterol (pregnane type) $[M^+]$ m/z = 386	----	0.7	----
Sterol (cholest- type) $[M^+]$ m/z = 458	-	0.17	----
Flavonoids			
Pinostrobin	----	----	0.04
Pinocembrin	----	----	6.06
Pinobankasin	----	----	0.30
Pinobankasin-3-acetate	----	----	1.16
Chrysin	----	----	0.35
Galangin	----	----	0.40
5,7- Dihydroxy-3-butanoyloxyflavanone	----	----	0.30
Lignans			
Furofuran lignans:			
Sesamin		0.13	
Iso sesamin		0.01	
Dihydroxy-trimethoxy furofuran lignans	0.4	0.2	
Benzofuran lignans:			
Benzofuran dodecenyl dihydrocaffeate[b, c]	0.76	0.1	
Benzofuran dodecenyl dihydrocaffeate isomer[b, c]	4.11	-	
Benzofuran dodecenyl dihydrocaffeate isomer[b, c]	3.17	-	
Benzofuran-propyl ferulate[b, c]	0.81	-	
Benzofuran-diprenyl dihydrocoumaric acid[b, c]	4.62	0.03	
Other			
2(3H)-Furanone-5-butyldihydroxy[b, c]	----	0.02	----
2(3H)-Furanone-5-hexyldihydroxy[b, c]	----	0.04	----
2(3H)-Furanone-5-heptyldihydroxy[b, c]	---	0.02	----
Glycerol	0.36	----	1.4
Tetrahydrodibenzopyran-6,6, 9-trimethyl-3-prenyl[b]	-	0.4	-
Phosphoric acid	0.73	----	0.4
Acetophenone-4'-hydroxy	----	0.03	
Acetophenone-2'-hydroxy	----	0.02	0.76
Acetophenone-3'-hydroxy	----	0.09	4.11
Glucitole	0.34	1.02	3.17

Table 1: Continued

Compounds	Siwa	Matrooh	Dakahlia*
	TIC (%)		
1,4-Naphthalenedione2,3,5,8-tetrahydroxy-6, 7-dimethoxy	----	0.22	----
Phenanthrenone derivative	----	0.39	----
1,4-Dihydroxy benzene	----	----	0.06
4 –Hydroxy-benzaldehyde	----	----	0.04
Vanillin	----	----	0.50
Arabinonic acid	----	0.02	----
Ribonic acid	0.16	----	----
Gluconic acid	----	0.22	----
Glycerol derivative	0.4	0.50	0.07
1,2,3-Trihydroxy butanal	0.74	----	----

[a]TIC = The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation, [b] For the first time in propolis, c Tentatively identified by analysis of mass spectrum, *- Abd El Hady F.K. and A.G. Hegazi (2002): Egyptian propolis: 2-Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis. Z. Naturforsch 57c, 386-394

Effect of propolis on *F. gigantica* eggs: Three propolis samples were used in this investigation. Each propolis sample was tested separately against immature *F. gigantica* eggs in different concentrations starting from 25 up to 800 μg mL^{-1}. Another group of eggs was incubated in distilled water as a control group, while solvent control group was also used. All groups were incubated at 26°C for embryonation.

Calculation of hatching percentage: Calculation of hatching percentage of eggs in the tested and control egg cultures were preformed according to El-Badry (1996), the eggs were homogenously mixed in the culture using a fine brush. The number of hatching eggs in 100 eggs existed in five separate microscopic fields was counted. Hatching mean percentage was calculated for the whole dish (El-Badry, 1996).

Scanning electron microscopy (SEM) of *F. gigantica* eggs: After incubation, immature *F. gigantica* eggs were fixed for 12 h in a 3:1 mixture of 4% (w/v) glutaraldehyde in 0.12 M-Millonig's buffer, pH 7.4 and 1% aqueous osmium tetroxide. The eggs were washed repeatedly in double-distilled water, dehydrated through acetone, critical point dried in carbon dioxide, fixed to aluminum stubs and coated with gold-palladium. The specimens were viewed in a Jeol scanning electron microscope operated at 15 Kv.

Antibacterial assay: *Clostridium oedematiens* (*Clostridium novyi*) type B strain was used: The bacterial suspension was prepared and adjusted by comparison against 0.5 Mc-Farland turbidity standard (5×10^7 cells mL^{-1}) tubes. It was further diluted to obtain a final of 5×10^6 cells mL^{-1}. *Clostridium oedematiens* was enriched on Cystein Dithiothreitol blood agar as a selective media (Moor, 1968). Bacteria were subculture on cooked meet broth for further bacterial propagation (Cruickshank *et al*., 1979). The broth was inoculated by the 0.20 μL 10 mL broth with *Clostridium oedematiens*, then added 40 μL of 20% propolis. The tubes were incubated at 37°C for 24 h in anaerobic jar with Gaspak system. The growth of control bacterial strain as well as inhibition of the bacterial growth due to propolis was measured by turbidity at 420 nm wave length. The mean values of inhibition were calculated from triple reading in each test. The minimum inhibitory concentration (MIC) of propolis was determined by the ten-fold dilution method against bacterial strain *in-vitro* (Hegazi *et al*., 2000).

Data were analyzed statistically using student t-test according to Senedcor (1961).

RESULTS

Propolis samples were collected from completely different three localities. The first one was from Nile Delta region (Dakahlia province), the second from Matrooh beach and the third sample from Siwa Oasis. Each locality was characterized by certain types of trees and shrubs. The preliminary TLC investigation of the 70% alcoholic extracts showed complete difference between the three propolis samples. In order to perform a complete analysis and to compare the results obtained from the three propolis samples, the total alcoholic extracts were silylated and subjected to a GC/MS analysis. The alcoholic extracts were dissolved in methanol and subjected to HPLC investigation. The results were summarized in (Table 3 and 4).

The GC/MS investigation of the three propolis samples showed Table 1 and 2, no any qualitative similarities in any compound, except in palmetic acid. Propolis (S) was characterized by the presence of four new valeric acids (11.42%): 3,5-dihydroxy-3-heptenyl-valeric acid and three isomers of 3,5-dihydroxy-3-octenyl valeric acid; two new isomers of 3,5-diprenyl dihydrocoumaric acid (4.15%); four new isomers of 3,5-diisopropoxy-nonanyl coumarate ester (9.53%); the new 3,5-dimethoxy-tetradecenyl coumarate ester (0.87 %) and five new benzofuran lignans (13.47%). These new compounds to propolis represented about 39.44% (Table 3).

Propolis (Table 1) was characterized by the presence of the new 3,5-dihydroxy-3-heptenyl-valeric acid (1.12%); 3,5-diprenylcoumaric acid (0.08%); three new dihydroferulate esters (2.39%): 3-pentanyl-octyldihydroferulate and two isomers of 3-hexanyl-octyldihydroferulate) and two benzofuran lignans (0.13%). It was characterized by the presence of five steroids (pregnane type, 2.1%) and three hydroxy acetophenones (acetophenone-4′-hydroxy, acetophenone-2′-hydroxy and acetophenone-3′-hydroxy), all these compounds were identified for the first time in

Table 2: Identification of flavonoids in Egyptian propolis by HPLC analysis (conc. µg g^{-1} Propolis)

No.	Name	Structure	Siwa	Matrooh	Dakahlia
1	Luteolin	5,7,3',4'-tetrahydroxyflavone	1584.9	428.6	959
2	Quercetin	3,5,7,3',4'-pentahydroxyflavone		100.1	
3	Pinobankasin	3,5,7-trihydroxyflavanone			1486
4	Quercetin-3-methylether	5,7,3',4'-tetrahydroxy-3-methoxyflavone			1256
5	Hesperetin	5,7,3'-trihydroxy-4'-methoxyflavanone	320.0		
6	Apigenin	5,7,4'-trihydroxyflavone		69.1	371
7	Kaempferol	3,5,7,4'- tetrahydroxyflavone	194.5	394.4	
8	Luteolin-3-methylether	5,7,4'-trihydroxy-3'-methoxyflavone	785.8	375.0	2304
9	Quercetin-3,3'-dimethylether	5,7,4'-trihydroxy-3,3'-dimethoxyflavone		430.8	1000
10	Formonontin	7-hydroxy-4'-methoxyisoflavone	481.2	818.8	3445
11	Quercetin-7-methylether	3,5,3',4'-tetrahydroxy-7-methoxyflavone	130.8	272.9	1183
12	Pinocembrin	5,7-dihydroxyflavanone			892
13	Chrysin	5,7-dihydroxyflavone			1261
14	Pinobankasin-3-acetate	5,7-dihydroxy-3-acetyloxyflavanone			2920
15	7-hydroxy-4'-methoxyflavone	7- hydroxy-4'-methoxyflavone			1153
16	Galangin	3,5,7- trihydroxyflavone			5000
17	Acacetin	5,7- dihydroxy-4'-methoxy flavone	183.5		
18	Biochanin A	5,7-dihydroxy-4'-methoxyflavanone			3152
19	Pinostrobin	5-hydroxy-7-methoxyflavanone			2060
20	Dimethylallylcaffeate	3-methylbut-2-enyl caffeate			5110
21	Phenylethylcaffeate	Phenylethyl-trans-caffeate			1897

Table 3: Effect of propolis samples on vitality and hatchability of immature *Fasciola gigantica* eggs

Conc. (µg mL^{-1})	Percentage of hatched eggs			Percentage of developed non-hatched eggs			Percentage of dead eggs		
Propolis	S	M	D	S	M	D	S	M	D
25	36.45	20. 0	79.47	49.53	69.08	9.82	14.02	10.52	10.71
50	11.82	16.58	62.61	74.55	73.06	26.96	13.63	10.36	10.43
100	3.13	14.42	44.75	84.37	75.96	43.36	12.50	9.62	11.89
200	0.00	12.43	5.56	0.00	68.57	73.61	100.00	19.05	20.83
400	0.00	0.00	0.00	0.00	0.00	58.65	100.00	100.00	41.35
800	0.00	0.00	0.00	0.00	0.00	0.00	100.00	100.00	100.00
Non-exposed control	80.80	8.00	11.20						
Solvent control	79.83	6.72	13.45						

Table 4: Antimicrobial activity of Egyptian propolis against *Clostridium oedematiens* (*clostridium novyi*)

	Clostridium oedematiens (*Clostridium novyi*)	
Treatments	Growth inhibition*	MIC (µg mL^{-1}) **
Pathogen Normal growth	0.560±0.012	---
Sewa propolis	0.051±0.006	2100
Matroh propolis	0.060±0.004	1600
Dakahlia propolis	0.046±0.0001	1000
Drug (Triclabendazole)	0.730±0.004	2400
Tetracycline (50 µg)	0.095±0.0001	1100

*Growth Inhibition = Inhibition of the growth measured by turbidity at 420 nm, **MIC: Minimal inhibition concentration, Results are expressed as mean±SD, **p<0.01, vs control

propolis. It was also characterized by the presence of five hydroxy benzoic acids: 2,4- dihydroxybenzoic acid, 2,6- dihydroxybenzoic acid, 2,3,4- trihydroxybenzoic acid, 2,4,6- trihydroxybenzoic acid and 3,4,5- trihydroxybenzoic acid. Matrooh propolis was characterized by the presence of three furanon derivatives. Also it was the first time to identify furofuran lignan compounds in Egyptian propolis, where sesamin and isosesamin were identified. Both (S) and (M) contained no di, triterpenoids and flavonoids identified by GC/MS.

Propolis (D) was identified before as a typical poplar propolis which had aliphatic, aromatic acids, aromatic acid esters, flavonoids as well as some triterpenoids (Abd El Hady and Hegazi, 2002).

The HPLC analysis revealed the presence of 19 flavonoids (Table 2). As the polyhydroxy flavonoids could only be identified by HPLC, propolis (S) showed the highly significant amount of luteolin. Hesperetin and acacetin were only present in this sample. Quercetin was only present in propolis (M). Propolis (D) was characterized by the presence of two caffeate estres (dimethylallylcaffeate and phenylethylcaffeate) and 15 flavonoids from which pinobankasin, quercetin-3-methylether, pinocembrin, chrysin, pinobankasin-3-acetate, 7-hydroxy-4'-methoxyflavone, galangin, biochanin A and pinostrobin, they were only present in propolis (D).

Effect of propolis on vitality and hatchability of immature *F. gigantica* eggs: Three different propolis samples (S, M and D) were tested against the vitality and hatchability of immature *F. gigantica* eggs. The eggs (Fig. 1) appeared oval, large, thin-shelled, operculated and yellowish to golden yellow in color. The propolis samples were tested at different concentrations ranged from 25-800 µg mL^{-1}, where no inhibitory effect of the solvent on Fasciola eggs was recorded (Fig. 3). The results displayed in Table 3 showed that the inhibitory effect was firstly appeared at concentration of 25 µg mL^{-1} of sample (S) and sample (M) and 50 µg mL^{-1} of sample (D), where

Fig. 1: Immature *F. gigantica* eggs. X100

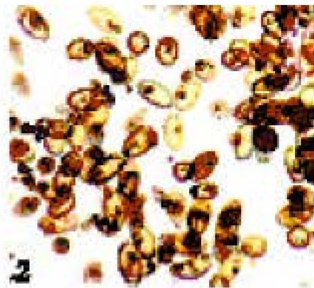

Fig. 2: Rate of hatchability of fresh embryonated *F. gigantica* eggs after 14 days incubation in distilled water at 26°C showing hatched miracidia, remnants of hatching materials and opened operculum. X100

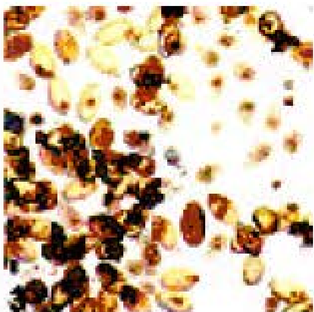

Fig. 3: Rate of hatchability of fresh embryonated *F. gigantica* eggs after 14 days incubation in solvent at 26°C showing hatched miracidia, remnants of hatching materials and opened operculum. X100

the hatching rates were 36.45, 20.4 and 62.6%, respectively, in comparison with the control non-exposed eggs (80.8%) (Fig. 2). The inhibitory effect till complete failure of development and death of all immature eggs was

Fig. 4: Rate of hatchability of *F. gigantica* eggs after 14 days incubation in 200 μg mL^{-1} propolis (S) showing dead eggs containing dark embryoes without any development. Precipitates (arrow) were formed around a few eggs. X100

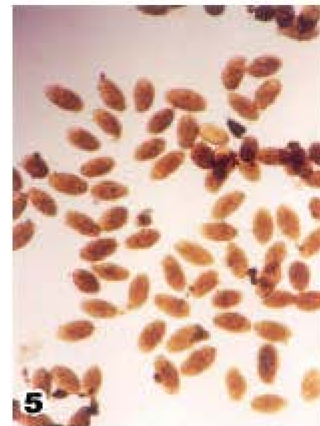

Fig. 5: Rate of hatchability of *F. gigantica* eggs after 14 days incubation in 400 μg mL^{-1} propolis (M) showing dead eggs containing dark embryoes without any development. X100

directly related to the concentration of the propolis, which was 200 μg mL^{-1} of sample (S) (Fig. 4), 400 μg mL^{-1} of sample (M) (Fig. 5) and 800 μgmL^{-1} of sample (D) (Fig. 6).

High rate of developed non-hatched eggs was recorded in exposed eggs at low concentrations of propolis in comparison with control non-exposed eggs. This rate of development of non-hatched eggs was recorded in exposed eggs at low concentrations of propolis in comparison with control non-exposed eggs which ranged from 49.53- 84.37, 69.08-75.96 and 9.82-43.36% in eggs exposed to samples (S), (M) and (D), respectively, when the application concentration increased from 25-100 μg mL^{-1}. In time where the rate of

Fig. 6: Rate of hatchability of *F. gigantica* eggs after 14 days incubation in 800 μg mL^{-1} propolis (D) showing a few dead eggs, dead miracidia within the eggs and large amount of precipitates were formed around all eggs. X100

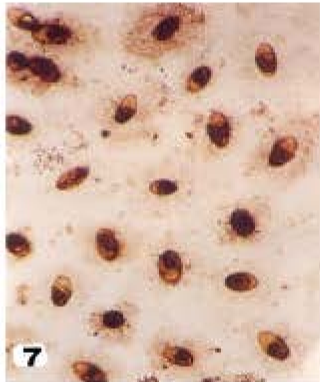

Fig. 7: Rate of hatchability of *F. gigantica* eggs after 14 days incubation in 200 μg mL^{-1} propolis (M) showing dead miracidia and precipitates (arrow) that were formed around all eggs. X100

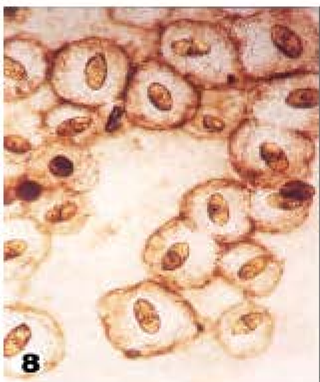

Fig. 8: Rate of hatchability of *F. gigantica* eggs after 14 days incubation in 200 μg mL^{-1} propolis (D) showing exhausted miracidia which fail to hatch and huge amount of precipitates (arrow) were formed around all eggs. X100

Fig. 9: Scanning electron micrograph of immature *F. gigantica* egg

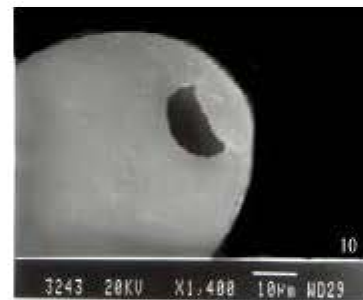

Fig. 10: Scanning electron micrograph of hatched *F. gigantica* egg

Fig. 11: Scanning electron micrograph of propolis exposed *F. gigantica* egg

developed non-hatched eggs was 8.0% in control non-exposed eggs. At low propolis concentration, it was noticed that a precipitate was formed around the exposed egg at late stage of development. In this case, the miracidium still motile till became exhausted and die (Fig. 7 and 8).

The scanning electron microscopy showed that the external surface of non exposed *F. gigantica* egg shell had smooth surface with slightly elevated circle marking the feature of the operculum. The operculum and the aperture had serrated edges. The seam of the operculum was located on a slight elevation (Fig. 9 and 10). In the

meantime the exposed *F. gigantica* egg shell revealed the same features as the non exposed egg shell (Fig. 11).

Antimicrobial activity: The antimicrobial activity of propolis collected from the three localities against *Clostridium oedematiens* (*Clostridium novyi*) type B were recorded in Table 4. The three propolis samples showed inhibition in the growth of the examined pathogen but the inhibition varied according to the propolis origin. It was obvious that the absorbance of the normal bacterial growth reached 0.56, where it was reduced significantly with D sample followed by S and M propolis samples (0.046, 0.05 and 0.060, respectively) if compared with the tested drugs 0.730 and 0.095 (Triclabendazole and Tetracycline), respectively. The MIC μg mL^{-1} of all different propolis samples was ranged from 1000 up 2100 where it was reached 2400 and 1100 in Triclabendazole and Tetracycline, respectively.

DISCUSSION

The present study proved evidence of inhibitory activity of propolis on the vitality and hatchability of immature *F. gigantica eggs*, where three propolis samples from different localities were tested for this purpose. The sample (S) showed the highest inhibitory effect than the others. This inhibitory effect was directly related to the concentration of the propolis. The complete failure of development and death of all immature eggs were recorded at concentration of 200 μg mL^{-1} for sample (S), 400 μg mL^{-1} for sample (M) and 800 μg mL^{-1} for sample (D). In that sense, the inhibitory effect of some chemical compounds increased with increase of its concentration as well as with the prolongation of egg immersion time (Roweliffe and Ollerenshaw, 1959; El-Badry, 1996).

It was very interesting to notice that during the period of egg incubation in low concentration of propolis, a precipitate was formed around the exposed egg at late stage of development and the miracidium was still motile till became exhausted and die. This precipitate might be attributed to the protein affinity of propolis to the metabolic products passed through shell pores of *F. gigantica* eggs. Indeed, El-Sayed *et al*. (2000) used the circumoval precipitin reaction for detection of shell pores of *F. gigantica* eggs by scanning electron microscopy. They reported that precipitin reaction was detected around Fasciola eggs containing mature miracidia when they were incubated for 20 min with sera of acute and chronic fasciolosis patients. They added that, scanning electron microscopy illustrated pores in the shell of Fasciola eggs and it was probable that the metabolic antigen passed through these pores led to the positive circumoval precipitin reaction in cases of fasciola infection.

The present study demonstrated that at low concentration of propolis (D) which produced a markedly heavy precipitate around fasciola eggs rather than (S) and (M) samples. These results might be attributed to the presence of large amount of flavonoids in propolis (D) which induced a protein binding affinity of fasciola eggs secretions in the culture media than that of (S) and (M) samples. The flavonoids of all propolis samples were confirmed by the identification of both GC/MS and HPLC. These results appeared to be in line with Comte *et al.* (2001) who found that flavones bind to P-glycoprotein with higher affinity than flavanones, isoflavones and glycosylated derivatives. Also increasing hydrophobicity at either position 6, 8, or 7 increased the affinity of *in vitro* binding to a purified cytosolic C-substitution produced a high maximal quenching of the protein intrinsic fluorescence. Also these results were in agreement with Barron *et al.* (1998) who found that the direct binding of a series of flavonoids to purified C-terminal domain was monitored by quenching of the protein intrinsic fluorescence. Hydroxyl groups at position 5 and 3 on adjacent rings A and C of flavones were important for affinity probably by increasing the likeness to the adenine moiety of ATP. The presence of hydrophobic substitutions such as methoxy group, also increasing the affinity indicating that other interactions are involved, probably at the steroid-interacting region. More hydrophobic, prenylated flavonoids strongly increase binding affinity; the effect was dependent on the type, the length and the position of prenyl group. These findings were in agreement and confirmed with the results illustrated in Table 2.

GC/MS analysis revealed that propolis samples from Siwa oases and Matrooh beach were completely different from that of Dakahlia province, where it was of poplar origin. Siwa sample contained new compounds some of them were tentatively identified by mass fragmentation.

Concerning the highest inhibitory effect of S and M propolis samples, it was contrary to those expectations related to the previous examined eight propolis samples and also those related to poplar origin (Tomas-Barbaran *et al*., 1993; Hegazi and Abd El Hady, 2001, 2002; Abd El Hady and Hegazi, 2002). Prenylated cinnamic acids, their esters and benzofurans turned out to be a salient chemical feature of Siwa Oasis propolis. diprenyl dihydro-coumaric acids, 3,5-diisopropoxy-nonanyl coumarates, benzofuran lignans and 3,5-dihydroxy-3-octenyl valeric acids which represented

about 39.44% as new compounds. It was the first time to identify new prenylated dihydrocoumaric acids and benzofuran lignans from propolis. It was the first time to identify lignan from the furofuran type in Egyptian propolis; it was identified before by GC/MS analysis in Canary Islands propolis (Bankova *et al.*, 1998) and isolated as sasamin (Christov *et al.*, 1999). In this study for Egyptian propolis only three lignan from the furofuran type were identified while in Canary Islands propolis 13 furofuran lagans were identified and also their plant sources still remain unknown.

In this study three hydroxyacetophenones (acetophenone -4′-hydroxy, acetophenone -2′-hydroxy and acetophenone -3′-hydroxy) were identified in Matrooh beach propolis, other acetophenone derivatives were isolated from Barazilian propolis (Banskota *et al.*, 1998) and benzophenones from Venezuelan propolis (Tomas-Barbaran *et al.*, 1993; Trusheva *et al.*, 2004) and from Cuban propolis (Rubio *et al.*, 1999). Diprenyl coumaric acid, 3-prenyl coumaric acid and other isoprenylated compounds were isolated before from Brazilian Propolis (Banskota *et al.*, 2000; Marcucci *et al.*, 2001; Park *et al.*, 2004). Prenylated cinnamic acids turned out to be a salient chemical feature of green propolis, among them artipillin C had attracted great attention not only for its antimicrobial (Aga *et al.*, 1994), but also for its cytotoxic activity (Matsuno *et al.*, 1997). Prenylated cinnamic acids might also present as esters (Salatino *et al.*, 2005). Dihydrobenzofurans were isolated from Chilean propolis had moderate growth inhibitory activity against *Mycobacterium avium*, *M. Tuberculosis* and two strains of *Staph. aureus* (Valcic *et al.*, 1998; Valcic *et al.*, 1999). Benzofuran derivatives had mild cytotoxic activity against liver-metastatic murine colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cells were isolated from Brazilian propolis (Banskota *et al.*, 2000).

It was important to focus on the antimicrobial activity of propolis against *Clostridium oedematiens* (*Clostridium novyi*) type B, where chronic fascioliasis provided the right environment in the liver for the germination of the spores of the bacterium, *Clostridium novyi* type B, which causes Black disease (Bagadi and Sewel, 1973). It was clear that propolis collected from Dakahlia had the highest antimicrobial activity against *Clostridium oedematiens* (*Clostridium novyi*) type B. The variation in the antimicrobial activity seemed to be due to the differences in the chemical composition of the different propolis samples. The higher antimicrobial activity of Dakahlia propolis to *Clostridium oedematiens* (*Clostridium novyi*) type B probably was attributed to the presence of very high significant amount of flavonoids and the synergistic effects of the other compounds. The antimicrobial activity differed according to the differences in the chemical composition (Hegazi and Abd El Hady, 2002; Moor, 1968; Mertzner *et al.*, 1979; Kujumgiev *et al.*, 1999).

ACKNOWLEDGMENTS

The authors are very grateful to Prof. Dr. E. Wollenweber (Darmstadt, Institut f. Botanik Schnittspahnstr, 4D-64287 Darmstadt, Germany) for providing 18 flavonoids and two caffeate esters (10 of these flavonoids are not found here in this experiment). The authors are grateful for the financial support by the National Research Center of Egypt (Contracts 3/23/6 and 1/48/5).

REFERENCES

Abd El Hady, F.K. and A.G. Hegazi, 2002. Egyptian propolis: 2-Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis. Z. Naturforsch, 57: 386-394.

Aga, H., T. Shibuye, T. Sugimoto, M. Kurimoto and S. Nakagima, 1994. Isolation and identification of antimicrobial compounds Brazilian Propolis. Biosci. Biotechnol. Biochem., 58: 945-946.

Annual Book of ASTM Standards, 1983. Part 11.03 Atmospheric analysis. American Scosity for Testing and Materials, Philadelphia, Pennsylvania.

Bagadi, H.O. and M.M. Sewell, 1973. An epidemiological surveys of infectious necrotic hepatitis (Black disease) of sheep in southern Scotland. Res. Vet. Sci., 15: 49-53.

Bankova, V.S., R.S. Chrostov and A.D. Tejera, 1998. Lgnans and other constituent of propolis from the Canary Islands. Phytochemistry, 49: 1411-1415.

Banskota, A.H., Y. Tezuka, J.K. Prasain, K. Matsushige, I. Saika and S.H. Kadota, 1998. Chemical constituent of Brazilian propolis and their cytotoxic activities. J. Nat. Prod., 61: 896-900.

Banskota, A.H., Y. Tezuka, K. Midorikawa, K. Matsushige and S. Kadota, 2000. Two novel cytotoxic benzofuran derivatives from Brazilian Propolis. J. Nat. Prod., 63: 1277-1279.

Barron, D., G. Comte, G. Consil, J.B. Daskiewicz, C. Bayet, J.M. Jault, H. Cortay, G. Dayan, E. Steinfels and A. Dipietro, 1998. Reversing mLutidrug resistance to chemotherapy: Flavonoid as potential byfunctional inhibitors of P-glycoprotein activity. 2nd International Electronic Conference on Synthetic Organic Chemistry (ECSOC-2) Septamper 1-30.

Boray, J.C., 1963. The ecology of F. hepatica with particular reference to its intermediate host in Austraria. Proc. 17th World. Vet. Gonger. Hannover.

Boray, J.C., 1997. Chemotherapy of Infections with Fasciolidae. In: Immunology, Pathobiology and Control of Fascioliasis. Bory, J.C. (Ed.), Msd AGVET, Rahway, New Jersey, pp: 83-97.

Christov, R., V.S. Bankova, I. Tsvetkova, A. Kujumgiev and A.D. Tejera, 1999. Antibacterial furofuran lagnans from Canary Islands propolis. Fitoterapia, 70: 89 -92.

Comte, G., J.B. Daskiewicz, C. Bayet, G. Consil, A. Viornery-Vanbier, C. Dumontet and D. Barron, 2001. C-isoprenylation of flavonoids enhances binding affinity towards P-glycoprotein and modulation of cancer cell chemoresistance. J. Med. Chem., 44: 763-768.

Cruickshank, R., J.P. Duguid, B.P. Masion and R.H. Swain, 1979. Medical Microbiology. 12th Edn. Churchill Livingstone, Edinburgh, London, New York.

Dalton, J.P., 1999. Fasciolosis. CABI Publishing, CAB International, Dalton, J.P. (Ed.), Wallingford, Oxon OX10 8DE, UK.

El-Badry, A.A., 1996. Study of the effect of some factors on the eggs and encysted metacercariae of Fasciola gigantica. M.Sc. Thesis, Fac. Med. Cairo Univ.

El-Sayad, M.H., A.F. Allam and A.Y. Shehab, 2000. The circumoval precipitin reaction in Fasciola. Detection of shell pores by scanning electron microscopy. J. Egypt. Soc. Parasitol., 30: 655-660.

Gil, M.I., F. Ferreres, A. Ortiz, E. Subra and F.A. Tomàs-Barberàn, 1995. Plant phenolic metabolites and floral origin of Rosemary honey. J. Agric. Food Chem., 43: 2833-2838.

Greenaway, W., J. May, T. Scaysbrook and F.R.S. Whatley, 1991. Identification by gas chromatography mass spectrometry of 150 compounds in propolis. Z. Naturforsch, 46: 111-121.

Haseeb, A.N., A.M. El-Shazly, M.A. Arafa and A.T. Morsy, 2002. A review on fascioliasis in Egypt. J. Egypt Soc. Parasitol., 32: 317-354.

Hegazi, A.G., F. El Berdiny, S. El Assily, Khashabah, E.N. Hassan and S. Popov, 1993. Studies on some aspects of antiviral activity. 1-Influence of propolis on NDV. Vet. Med. J. Giza, 41: 53-56.

Hegazi, A.G., N.Z. Moharm, F. Abd Allah, E. El Sheikh, N.A. Ellithy and A.M. Khair, 1998. Influence of ethanolic extract of Egyptian propolis on immune status in mice bearing Ehrlich. J. Union of Arab Biologists, 10: 387-396.

Hegazi, A.G., F.K. Abd El Hady and F. Abd Allah, 2000. Chemical composition and antimicrobial activity of European propolis. Z. Naturforsch, 55:70-75.

Hegazi, A.G. and F.K. Abd El Hady, 2001. Egyptian propolis: 1-Antimicrobial activity and chemical composition of Upper Egypt propolis. Z. Naturforsch, 56: 82-88.

Hegazi, A.G. and F.K. Abd El Hady, 2002. Egyptian propolis: 3-Antioxidant, antimicrobial activity and chemical composition of propolis from reclaimed land. Z. Naturforsch, 57: 395-402.

Higashi, K.O. and S.L. De Castro, 1994. Propolis extracts are effective against *Trypanosoma cruzi* and have an impact on its interaction with host cells. J. Ethnopharmacol., 43: 149-155.

Kujumgiev, A., I. Tsvetkova, Y. Serkedjieva, V. Bankova, R. Christov and S. Popov, 1999. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J. Ethnopharmacol, 64: 235-240.

Marcucci, M.C., 1995. Propolis: Chemical composition, biological properties and therapeutic activity. Apidologie, 26: 83-99.

Marcucci, M.C., F. Ferreres, C. Garca-Viguera, V.S. Bankova, S.L. De Castro Dantas, A.P. Valente and P.H.M.N. Paulino, 2001. Phenolic compounds from Brazilian Propolis with pharmacological activities. J. Ethnopharmacol., 74: 105-112.

Matsuno, T., S.K. Jung, Y. Matsumoto, M. Saito and J. Morikawa, 1997. Preferential cytotoxicity to tumor cells of 3,5-diprenyl-4-hydroxycinnamic acid (artipillin C). Isolated from propolis. Anticancer Res., 17: 3565-3568.

Mertzner, J., H. Bekemeier, M. Paintz and E. Schneidewind, 1979. Antimicrobial activity of propolis and its constituents. Pharmazie, 34: 97-102.

Moor, W.B., 1968. Solidified media suitable for the cultivation of *Clostridium novyi* type B. J. General. Microbiol., 53: 415.

Ogan, K. and E. Katz, 1981. Liquid chromatographic separation of alkyl phenols with fluorescence and ultra violet detection. Anal. Chem., 53: 160-163.

Park, Y.K., J.F. Paredes-Guzman, C.L. Aguiar, S.M. Alencar and F.Y. Fujiwara, 2004. Chemical constituents in *Baccharis dracunculifolia* as the main botanicalorigin of southeastern Brazilian Propolis. J. Agric. Food Chem., 52: 1100-1103.

Roweliffe, S.A. and O.B. Ollerenshaw, 1959. Observations on the bionomics of the eggs of *F. hepatica*. Ann. Trop. Med. Parasit., 54: 172-181.

Rubio, O.C., A.C. Cuellar, N. Rojas, H.V. Castro, L. Rastrelli and R.A. Aquino, 1999. Polyisoprenylated benzophenones from Cuban propolis. J. Nat. Prod., 62: 1013-1015.

Salatino, A., E.W. Teixeira, G. Negri and D. Message, 2005. Origin and chemical variation of brazilian propolis. Evid Based Complement Alternat Med., 2: 33-38.

Senedcor, G.W., 1961. Statistical methods, 5th Edn., Iowa State University Press, Iowa USA.

Tomas-Barbaran, F., C. Garcia-Viguera, P. Vit-Olivier, F. Ferreres and F. Tomas-Lorente, 1993. Phytochermical evidence for the botanical origin of tropical propolis from Venezuela. Phytochemistry, 34: 191- 196.

Trusheva, B., M. Povova, H. Naydenski, I. Tsvetkova, J.G. Rodriguez and V. Bankova, 2004. New polyisoprenylated benzophenones from Venezuela propolis. Fitoterapia, 75: 683-689.

Valcic, S., G. Montenegro and B.N. Timmermann, 1998. Lignans from Chilean propolis. J. Nat. Prod., 61: 771-775.

Valcic, S., G. Montenegro, A.M. Mujica, G. Avila, S. Franzblau, P. Singh Maya, W.M. Maiese and B.N. Timmermann, 1999. Phytochemical, morphological and biological investigations of propolis from Central chile. Z. Naturforsch, 54: 406-416.

Welch, S., J. Malone and H. Geaghan, 1987. Herd evaluation of *Fasciola hepatica* infection in Louisiana cattle by an ELISA. Am. J. Vet. Res., 48: 345-347.