Egyptian Propolis: 1-Antimicrobial Activity and Chemical Composition of Upper Egypt Propolis

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Propolis, Polyphenols, Antimicrobial Activity

The antimicrobial activity of four propolis samples collected from Upper Egypt against *Staphylococcus aureus; Escherichia coli and Candida albicans* was evaluated. There was a variation in the antimicrobial activity according to the propolis origin. Banisweif propolis showed the highest antimicrobial activity against *Staphylococcus aureus, Escherichia coli and Candida albicans*, but Fayoum propolis had moderate activity against all tested pathogens. Propolis collected from Assiut and Souhag gave lower antimicrobial activity.

Propolis samples were investigated by GC/MS, 71 compounds were identified, 14 being new for propolis. Banisweif propolis is characterized by the presence of 7 caffeate esters and 4 triterpenoids. Fayoum propolis showed the highest amount of lactic acid and the presence of 3 chalcones. But Assiut propolis is characterized by the presence of 4 prenylated coumarates. Souhag propolis is characterized by the presence of 5 aliphatic dicarboxylic acids and some other new compounds to propolis.

Introduction

Egyptian propolis (bee glue) has recently become a subject of increasing interest for chemists and biologists (Hegazi, *et al.*, 1993, 1995, 1996a, 1997 and 1998; Abd El-Hady, 1994; Abd El-Hady and Hegazi, 1994; Hegazi and Abd El Hady, 1994, Bankova *et al.*, 1997 and Christov *et al.*, 1998). Throughout significant differences have been found in the chemical composition as well as the antimicrobial activity (Kujumgiev *et al.*, 1999 and Hegazi, *et al.*, 2000). Therefore, this investigation aimed to determine the antimicrobial activity as well as the chemical composition of propolis collected from different provinces of Upper Egypt.

Materials and Methods

Propolis

Four Propolis samples were collected From Baniswief (A), Fayoum (B), Assiut (C) and Souhag (D) provinces of Upper Egypt.

Extraction and sample preparation

One gram of each sample was cut into small pieces and extracted at room temperature with 50 ml of 70% ethanol (twice after 24 hours). The alcoholic extract was evaporated under vacuum at 50° C until dryness. The percentage of extracted matter was as follows: Baniswief (A) propolis 0.20 gm/dry weight, Fayoum (B) propolis 0.13 gm/dry weight, Assiut (C) propolis 0.24 gm/dry weigh and Souhag (D) propolis 0.10 gm/dry 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 BSTFA and analyzed by GC/MS.

GC/MS analyses

A Finnigan MAT SSQ 7000 mass spectrometer was coupled with a Varian 3400 gas chromatograph. DB-1 column, 30 m × 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. intial 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 amu to 650 amu.

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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.

Antibacterial assay

Two bacterial strains were used: *Staphylococcus aureus* and *Escherichia coli*. The bacterial suspension was prepared and adjusted by comparison against 0.5 Mc-Farland turbidity standard (5×10^7 organisms / ml) tubes. It was further diluted to obtain a final of 5×10^6 organisms / ml. *Staphylococcus aureus* was enriched on polymyxin agar (Finegold, and Sweeny, 1961) as a selective media While *E. coli* was enriched on MacConkey broth. Both bacteria were subculture on nutrient broth for further bacterial propagation (Cruickshank *et al.*, 1979). The broth was inoculated by the 0.20 µl/10 ml broth either with *Staphylococcus aureus* and *E. coli*, then added 40 µl of 20% propolis. The tubes were incubated at 37 °C for 24 hr. The growth of control bacterial strains as well as inhibitions of the bacterial growth due to propolis were measured by turbidity at 420 nm wave length. The mean values of inhibition were calculated from triple reading in each test. The minimal inhibitory concentration (MIC) of propolis was determined by the ten-fold dilution method against bacterial strains in in-vitro (Hegazi *et al.*, 1996). Data were analyzed statistically using student "T" test according to Senedcor (1961).

Antifungal assay

The antifungal activity of propolis was carried out against *Candida albicans* as described in British Pharmacopoeia (1968). Sabouraud's glucose agar and broth inoculated by the spore suspension (0.20 µl/10 ml). Then added 40 µl of 20% propolis. The tubes were incubated at 28 °C for 48h. The growth as well as inhibition were measured as turbidity at 420 nm wavelength .The mean value of inhibition were calculated from triple reading in each test. Data were analyzed statistically using student "T" test according to Senedcor (1961).

Result and Discussion

The antimicrobial activity of propolis collected from four provinces of Upper Egypt against *Staphylococcus aureus; Escherichia coli,* and *Candida albicans* were recorded in Table I. All propolis samples showed an inhibition in the growth of all examined bacteria but the inhibition varied according to the propolis origin. It was obvious that propolis collected from Banisweif showed the highest antimicrobial activity against *Staphylococcus aureus, Escherichia coli* and *Candida albicans*, but propolis collected from Fayoum province had moderate activity against all tested pathogens. Propolis collected from Assiut and Souhag gave lower antimicrobial activity if compared with

Table I. Antimicrobial activity of different Egyptian propolis.

Treatment	*Staphylococcus aureus*		*Escherichia coli*		*Candida albicans*	
	Growth inhibition	MIC [µg/ml]	Growth inhibition	MIC [µg/ml]	Growth inhibition	MIC [µg/ml]
Pathogen normal growth	1.361 ± 0.0005*	–	1.315 ± 0.007	–	1.205 ± 0.002	–
Banisowief Propolis (A)	0.137 ± 0.006	2600**	0.262 ± 0.003	1800	0.358 ± 0.004	1400
Fayoum Propolis (B)	0.379 ± 0.004	3400	0.538 ± 0.002	2300	0.506 ± 0.003	2600
Assiut Propolis (C)	0.697 ± 0.003	5400	0.592 ± 0.029	2600	0.547 ± 0.003	2800
Souhag Propolis (D)	0.549 ± 0.003	4200	0.853 ± 0.006	3200	0.635 ± 0.002	3200
Tetracycline (50 µg)	0.095 ± 0.0001	1000	0.469 ± 0.0003	1400	1.700 ± 0.002	6400
Cefotaxime (50 µg)	0.693 ± 0.0006	1600	0.532 ± 0.0005	2400	1.654 ± 0.002	6200
Ketoconazole (50 µg)	1.233 ± 0.004	8400	1.270 ± 0.0011	5600	0.638 ± 0.003	2400
Clotrimazole (50 µg)	1.694 ± 0.004	7800	1.435 ± 0.0032	6400	0.753 ± 0.008	2800

* Growth Inhibition = Inhibition of the growth measured by turbidity at 420 nm.
** MIC: Minimal inhibition concentration).

propolis collected from Banisweif and Fayoum provinces. The variation in antimicrobial activity seems to be due to the differences in the chemical composition of different propolis samples. The higher antimicrobial activity of Banisweif propolis probably attributed to the presence of some esters as (isopentenyl caffeate, dimethyl allyl caffeate, benzyl caffeate, dodecyl caffeate, tetradecyl caffeate and tetradecenyl caffeate); triterpenes as (cycloartinol, lanosterol, β-amyrin and triterpene of β-amyrin type) and flavonoids as (pinostrobin, pinocembrin, pinobankasin, pinobankasin-3-acetate, chrysin and galangin).

The results of the antimicrobial activity of such propolis samples are in agreement with the findings of Mertzner *et al.* (1979) who found that the antimicrobial activity of propolis can be attributed to its component as pinocembrin, galangin, pinobanksin, pinobanksin-3-acetate, p-coumaric acid benzyl ester and caffeic acid esters. Relatively good antimycotic activity was previously identified in the Egyptian propolis by Hegazi and Abd El Hady (2000). Also Kujumgiev *et al.* (1999) found that all investigated propolis samples were active against the fungal and Gram-positive bacterial strains, and most of them showed antiviral activity. Propolis from the Temperate Zone contains flavonoids and esters of phenolic acids are known to be responsible for antimicrobial activity. Tropical samples did not contain such substances but showed similar activities.

The comparison between the activity of different therapeutic agents (against bacteria and fungi) as Tetracycline, Cefotaxime, Ketoconazole, and Clotrimazole in relation to different propolis samples revealed that the propolis samples were effectively acting to inhibit the pathogens growth. The minimal inhibitory concentration (MIC) of propolis samples was determined by ten-fold dilution in-vitro against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. The results of MIC are illustrated in Table I. There were differences in their minimal inhibitory concentration. The MIC ranged from 2600 to 5400 μg /ml for *Staphylococcus aureus* while it was 1800 to 3200 μg /ml for *Escherichia coli*. But it was ranged from 1400 to 3200 μg /ml in case of *Candida albicans*. The variation of the antibacterial activity of propolis from area to area referred to the chemical composition of propolis, which had a synergistic effect of various phenolic compounds. Also geographic areas differ due to plant flora which reflected in the propolis constituents as observed by Shub *et al.* (1978) in USSR, Meresta and Meresta (1983) in Poland; Pepeljnjak *et al.* (1985) in Croatia, Yugoslavia; Petri *et al.* (1988) in Hungary and Serra and Escola (1995) from Brazil, Uruguay and China. Abd El Fattah *et al.* (1993), Hegazi *et al.* (1996) from Egypt and Hegazi *et al.* (2000) from Europe. It seems that propolis has general pharmacological values as a natural mixture (Kujumgiev *et al.*, 1999).

Propolis samples were collected from different provinces of Upper Egypt, each of them characterized by some types of predominant trees or shrubs. They were extracted at room temperature with 70% ethanol, the extracts were silylated and subjected to GC/MS analysis. The results obtained are summarized in Table II. It is clear that the four propolis samples showed qualitative similarities in 4 compounds: lactic acid, 3,4-dimethoxycinnamic acid, caffeic acid and phosphoric acid, the concentration of lactic acid was significantly higher in sample B. According to the difference in plant source, each propolis sample characterized by certain specific compounds. Sample A is the only sample contained benzoic acid, trans-p-coumaric acid, ferulic acid, dimethylallyl caffeate, benzyl caffeate and caffeate esters with long chain fatty alcohols and typical flavonoids from poplar origin (*Populus nigra*). It also contained high amounts of triterpenic alcohols including β-amyrin, unidentified triterpene of β-amyrin type, lanosterol and cycloartenol. Sample B was characterized by the presence of the following new compounds to propolis: 2,3,4,5 tetrahydroxypentanoic acid-1,4-lactone, 2-propenoic acid-3-methoxy-3-hydroxy methyl ester, monoethyl succinate and benzylmethylketone, the last compound present with high significant amount. It was also characterized by the presence of pinostrobin chalcone, pinocimbrin chalcone and sakauranetin chalcone. Sample C was characterized by the presence of 4 prenylated coumarate esters, 4-methoxycinnamic acid, 3-methyl-2-butenylcaffeate and benzyl-2-methylpropyl which is new to propolis. Sample B and C did not contain any triterpenic alcohols. Sample D was characterized by the presence of 15 aliphatic acids, from which 2-hydroxy-cyclohexane-1-carboxylic acid, 3-methyl-3-hydroxy-pentanedioic

Table II. Chemical composition assessed by GC/MS of alcohol extracts of Upper Egypt propolis samples.

Compound	Baniswief (A)[C]	Fayoum (B)	Assiut (C)	Shouhag (D)
	% TIC [a]			
Aliphatic Acids				
Lactic acid	1.3	18.5	4.54	0.31
Hydroxyacetic acid		4.3	1.86	0.05
3-Hydroxypropanoic acid				0.01
2,3-Dihydroxypropanoic acid		0.29		0.06
Nonanoic acid		0.01		
Malic acid		0.68		0.32
Succinic acid	0.30	8.00		0.16
2-Butenedioic acid (E)		0.11		0.01
2-Hydroxy-cyclohexane-1-carboxylic acid [b]				0.10
Pentanedioic acid-3-methyl-3-hydroxy [b]				0.02
2-Hydroxy-cyclohexane-1-carboxylic acid [b]				0.10
2,3-Dihydroxypentanedioic acid [b]				0.05
2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone [b]		016		
Palmitic acid	3.00		0.36	2.31
Heptadecanoic acid				0.02
Linoleic acid				0.10
Oleic acid	4.00			2.13
Stearic acid	0.90			0.44
Tetracosanoic acid	1.60			
Aromatic acids				
Benzoic acid	0.20			
4-Hydroxy-benzoic acid				0.10
4-Methoxy-cinnamic acid			1.81	
***Trans-p*-Coumaric acid**	0.50			
3,4-di-Methoxy-cinnamic acid	0.40	0.13	1.57	0.01
Isoferulic acid			0.70	
Ferulic acid	0.20			
Caffeic acid	0.30	0.29	0.86	0.05
Esters				
2-Propenoic acid-3-methoxy-3-hydroxy methyl ester [b]		0.73		
Monoethyl succinate [b]		0.43		
Ethyl palmitate	0.50			0.01
Ethyl oleate	1.20			0.40
3-Methyl-2-butenyl-*cis*-4-coumarate			0.68	
3-Methyl-3-butenyl-*trans*-4-coumarate			0.35	
2-Methyl-2-butenyl-*trans*-4-coumarate			0.13	
3-Methyl-2-butenyl-*trans*-4-coumarate			0.35	
Isopentenyl caffeate	0.90	0.53	0.82	
Dimethylallyl caffeate	1.30			
2-Methyl-2-butenyl caffeate		1.01	0.84	
3-Methyl-2-butenyl caffeate			1.36	
Benzyl caffeate	0.60			
Dodecyl caffeate	1.10			
Tetradecyl caffeate	3.10			
Tetradecenyl caffeate	0.30			
Hexadecyl caffeate	4.70			
Di and Triterpenes				
Dehydroabietic acid				0.40
Cycloartinol	7.10			
Lanosterol	1.20			
β-Amyrin	4.70			0.17
Triterpene of β-amyrin type	4.80			0.08

Table II. (continued).

Compound	Baniswief (A)[C]	Fayoum (B)	Assiut (C)	Shouhag (D)
		% TIC [a]		
	Flavonoids			
2′,6′-Dihydroxy-4′-methoxychalcone (Pinostrobin chalcone)		0.90	3.67	0.04
2′,4′,6′-Trihydroxy chalcone (Pinocembrin chalcone)		0.78		
2′,4′,6′-Trihydroxy 4′-methoxy chalcone (Sakauranetin chalcone)		0.28		
Hexamethoxyflavone				0.15
Pinostrobin	0.60			
Pinocembrin	1.10		1.35	
Pinobankasin	0.30	0.50	0.37	
Pinobankasin-3-acetate	1.10	0.42		
Chrysin	0.80	0.16	0.68	
Galangin	0.70	0.26		
	Others			
Glycerol		22.03	11.61	4.50
Phosphoric acid	2.70	0.30	1.67	0.06
Glycerol octadecyl ether (unidentified)	1.80			
2,3-Dihydroxy butane		0.68		
Benzyl-methyl ketone [b] (Insect repellent)		10.7		
Benzene, (2-methylpropyl)[b]			0.98	
3-Hydroxy pyridine [b]		0.19	1.87	0.01
1,2-Dihydroxy cyclohexene [b]				0.01
Eugenol				0.03
1,2,4-Trihydroxy butane [b]				0.02
1,2,3-Trihydroxy butanal [b]		0.54	1.41	0.36
2,4-Bis(dimethylbenzyl)-6-t-butyl-phenol [b]				0.10
1,8-Dihydroxy-3-methyl-anthraquinone [b]				0.10

[a] 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] has been identified before (Christov *et al.*, 1998).

acid and 2,3-dihydroxy pentanedioic acid are new to propolis , and also other new compounds like: 1,2-dihydroxy cyclohexene, 1,2,3-trihydroxy butane, 1,2,4-trihydroxybutanal, 2,4-bis(dimethylbenzyl)-6-t-butylphenol and 1,8-dihydroxy-3-methyl anthraquinone. Sample D did not contain any aromatic acid esters, any flavonoids except pinostrobin chalcone and a hexamethoxy flavone.

On the basis of the results obtained, some conclusions could be drawn concerning the plant origin of the investigated samples. One of the plant source appeared to be common for the first 3 samples is poplar, but with different species. Sample A appeared to be from the origin of *Populus nigra*. The high concentration of esters of phenolic acids, the presence of pentenyl caffeates and typical flavanones indicate this. Samples B and C appeared to be from other poplar species as sample B was rich in chalcones, flavanones and flavones, while sample C was characterized by the presence of prenylated coumarates. The primary source of the plant exudate incorporated into propolis samples A, B and C is bud exudate of poplar trees. The composition of propolis is therefore directly related to the composition of the poplar bud exudate collected by the bees (Greenaway *et al.*, 1987, 1989, 1990; Papay *et al.*, 1985, 1987; Bankova *et al.*, 1989, 1994; Wollenweber *et al.*, 1987). Bud extracts of *P. balsamifera* and *P. candicans* are very high in dihydrochalcones and rich in cinnamic and coumaric acids plus their esters, *P. balsamifera* appeared to be higher in flavones. *P.nigra* and *P.* × *euramericana* bud exudates are very low in dihydrochalcones and low in cinnamic and coumaric acids plus their esters (Whatley *et al.*, 1989). Also each poplar species has its own characteristic mixture of

compounds in its bud exudate (Wollenweber *et al.*, 1975; Greenaway *et al.*, 1989) and there can be considerable difference in bud exudate composition between different poplar species (Greenaway *et al.*, 1989, 1990, 1990a, 1990b).

So the original source of sample B and C needs more investigation to know the other poplar species. The presence of substances unusual for poplar buds such as sterol precursors in sample A and amyrins in samples A and D rather than B and C is an indication that there could be another plant source for propolis which needs more investigation.

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