Egyptian Propolis: 3. Antioxidant, Antimicrobial Activities and Chemical Composition of Propolis from Reclaimed Lands

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Propolis, Antioxidant, Antimicrobial Activities

The free radical scavenging effect of two propolis samples collected from reclaimed land, Egypt as well as of vitamin C and caffeic acid in 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical system was determined. The antimicrobial (*Staphylococcus aureus; Escherichia coli* and *Candida albicans*) activity was also investigated. The results of the free radical scavenging effect of El-Saff and Ismailia propolis showed a concentration-dependent activity. The antioxidant activity was varied according to the examined material. It was obvious that caffeic acid and vitamin C showed the highest activity if compared with the propolis samples. El- Saff propolis had a higher antioxidant activity than Ismailia propolis, it showed a higher antibacterial activity against *Staphylococcus aureus* and a higher anti-fungal activity against *Candida albicans*. While the Ismailia propolis had a higher antibacterial activity against *Escherichia coli*, than El-Saff propolis.

The chemical composition of propolis samples was investigated by GC/MS, where 75 compounds were identified, 22 being new for propolis. The Ismailia propolis was characterized by the presence of a highly significant amount of aromatic acid esters (47.3%) and triterpenoids (17.3%), while El-Saff propolis contained 3% and 1.9% respectively. The new esters belonged to 4-methoxyhydrocinnamic acid, hydroferulic acid and ferulic acid. El-Saff propolis had a very high significant amount (27%) of 2,6-bis-(pentanyloxy)-4-pentanylphenethanol, which is also a new compound for propolis.

Introduction

Propolis is a sticky resinous hive product. It is used by bees as glue in general-purpose. It is used in folk medicine for long time in different nations as early 3000 BC as in Egypt (Hegazi, 1998). Propolis can cure inflammation, heart diseases, diabetes and cancer (Matsushige *et al.,* 1996). Several biological activities such as anticancer (Matsuno, 1995), antioxidant (Krol *et al.,* 1996; Basnet *et al.,* 1997; Cengarle *et al.,* 1998), anti-inflammatory (Marcucci, 1995), antibacterial (Hegazi *et al.,* 2000a) antifungal (Hegazi and Abd El Hady, 2001) and antiviral (Hegazi *et al.,* 2000b) activities have been reported in propolis and its constituents. Previous studies reported that antioxidants protect cancer patients from the side effects of chemotherapeutic agents (Menegale *et al.*,1970). Antioxidants reverse UV-light cytotoxicity in Chinese hamster embryo cells (Chan and Black, 1997).

Egyptian propolis become a subject of research by biologists and chemists (Hegazi *et al.*, 2000a, 2000b; Hegazi and Abd El Hady, 2001; Abd El Hady and Hegazi, 2001; Bankova *et al.*, 1997; Christov *et al.*, 1998; Kujumgiev *et al.*, 1999). The aim of this study was to evaluate the antioxidant and antimicrobial activities as well as the chemical composition of propolis collected from two different localities of reclaimed land.

Materials and Methods

Propolis

Two Egyptian propolis samples were collected from reclaimed land at El-Saff, near Cairo and in Ismailia province near Saini April, 1999 and stored for 19 months at –20 °C.

Extraction and sample preparation

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: El-Saff propolis 0.45 gm/dry weight, and Ismailia propolis 0.21 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 N,O, bis-(trimethylsilyl)trifluoroacetamide (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 39–650 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 when possible to confirm GC retention times.

Antioxidant assay to determine DPPH scavenging activity

The scavenging effect of propolis samples as well of vitamin C and caffeic acid corresponded to the quenching intensity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) as carried out by Matsushige *et al.* (1996) and Basnet *et al.* (1997). The sample solution of each tested (500 μl) material (Matsushige *et al.*, 1996) was mixed with the same volume of 60 μM DPPH solution and allowed to stand for 30 min at room temperature. The absorbance was then measured at 520 nm. The samples and DPPH were dissolved in ethanol. The percent scavenging effect was determined by comparing the absorbance of solution containing the test sample to that of control solution without the test sample taking the corresponding blanks. The result is the mean of 4 measurements for each sample. Vitamin C and caffeic acid were used as positive control samples.

Antibacterial assay

Two bacterial strains were used: *Staphylococcus aureus* (209) and *Escherichia coli* (H-480). These bacteria were kindly supplied by the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria. The bacterial suspension was prepared and adjusted by comparison against 0.5 McFarland turbidity standard (5×10^7 cells/ml) tubes. It was further diluted to obtain a final of 5×10^6 cells / 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 h. The growth of control bacterial strains as well as inhibitions of the bacterial growth due to propolis were measured by turbidity at 420 nm wavelength. 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 strains in *in-vitro* (Hegazi *et al.*, 2000a). Data were analyzed statistically using student "T" test according to Senedcor (1961).

Antifungal assay

The antifungal activity of propolis was carried out as described by Hegazi *et al.* (2000a). Against *Candida albicans* (562). *Candida albicans* was kindly supplied from Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria. Sabouraud's glucose agar and broth inoculated by the spore suspension (0.20 μl/10 ml). Then 40 μl of 20% propolis was added. The tubes were incubated at 28 °C for 48h. The growth as well as inhibition were measured as turbidity at 420 nm. The mean values of inhibition were calculated from tri-

ple reading in each test. The minimum inhibitory concentration (MIC) of propolis was determined by the ten-fold dilution method against *Candida albicans* in *in-vitro* (Hegazi *et al.*, 2000a). Data were analyzed statistically using student "T" test according to Senedcor (1961).

Results and Discussion

The results of the free radical scavenging effect of the two propolis samples, and positive control (vitamin C and caffeic acid) in DPPH – free radical system were determined (Table I and Fig. 1).

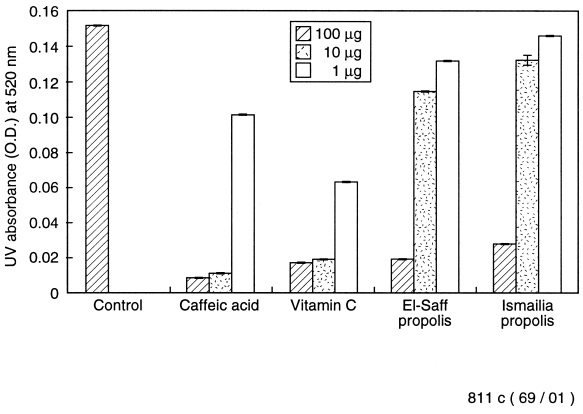

Fig. 1. The DPPH free radical scavenging effect of Egyptian propolis. The DPPH free radical scavenging effect was measured by the absorbance of DPPH radical at 520 nm in a reaction containing the test sample and 6×10^{-5}M DPPH . Results are expressed as mean ± S. D.,**$P < 0.01$, vs control.

The results of the free radical scavenging effect of El-Saff and Ismalia propolis showed a concentration-dependent activity. The free radical scavenging activity of El-Saff and Ismailia propolis was 88.2 and 82.2% respectively at a concentration of 100 μg while it was 25 and 13.2% respectively at a concentration of 10 μg. The results of the free radical scavenging effect of vitamin C and caffeic acid was 94.7 and 89.5% respectively at a concentration of 100 μg but the activity at a concentration of 10 μg was 93.4 and 88.2% respectively.

Regarding to the results of the free radical scavenging effect of two Egyptian propolis samples and the positive control (vitamin C and caffeic acid) in a DPPH free radical system. In the DPPH free radical system, the antioxidant directly reacted with DPPH free radical. The activity of propolis samples in this system was similar in free radical scavenging while the effect of El-Saff and Ismailia propolis showed a concentration-dependent activity with a slight variation. These results were in agreement with the findings of Matsushige *et al.* (1996) and Basnet *et al.* (1997) who studied different fractions of Brazilian propolis.

The antimicrobial activity of propolis collected from two localities of reclaimed lands, (Egypt) against *Staphylococcus aureus; Escherichia coli,* and *Candida albicans* were recorded (Table II and Fig. 2). The two propolis samples showed growth inhibition of the examined pathogens but inhibition varied according to the propolis origin. It was clear that propolis collected from El-Saff had the

Table I. The DPPH free radical scavenging activity of Egyptian propolis. The DPPH free radical scavenging effect was measured by the absorbance of DPPH radical at 520 nm in a reaction containing the test sample and 6×10^{-5} M DPPH . Results are expressed as mean ± S. D. , **$P < 0.01$, vs control.

Treatment	Concentration (μg) X 10^{-3}					
	100μg		10μg		1μg	
	Activity	%	Activity	%	Activity	%
Control	0.152 ± 0.0002	0.00	0.152 ± 0.0003	0.00	0.152 ± 0.0002	0.00
Caffeic acid	0.008 ± 0.0003**	89.47	0.010 ± 0.0002**	88.15	0.101 ± 0.0003**	59.21
Vitamin C	0.016 ± 0.0002**	94.73	0.018 ± 0.0003**	93.42	0.062 ± 0.0003**	33.55
El-Saff propolis	0.018 ± 0.0004**	88.16	0.114 ± 0.0005**	25.00	0.131 ± 0.0005**	13.82
Ismailia propolis	0.027 ± 0.0001**	82.23	0.132 ± 0.0021**	13.15	0.145 ± 0.0002	4.6

Table II. Antimicrobial activity of 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	1.443 ± 0.012*	–	1.398 ± 0.011	–	1.287 ± 0.006	–
El-Saff propolis	0.484 ± 0.006	2200	0.492 ± 0.001	1600	0.303 ± 0.002	1420
Ismailia propolis	0.509 ± 0.004	2600**	0.479 ± 0.006	1460	0.349 ± 0.023	1800
Tetracycline (50 μg)	0.095 ± 0.0001	1000	0.469 ± 0.0003	1400	1.700 ± 0.002	4400
Ketoconazole (50 μg)	1.233 ± 0.004	5400	1.270 ± 0.0011	4600	0.638 ± 0.003	2400

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

highest antimicrobial activity against *Staphylococcus aureus and Candida albicans*, while Ismailia propolis had the highest antibacterial activity against *Escherichia coli*. The variation in the antimicrobial activity seems to be due to the differences in the chemical composition of different propolis samples. The higher antimicrobial activity of El-Saff propolis to *Staphylococcus aureus and Candida albicans* can probably be attributed to the presence of a very high significant amount (27%) of 2,6-bis-(pentanyloxy)-4-pentanylphenethanol and the synergistic effects of the other compounds. These results are in agreement with Mertzner *et al.* (1979); Kujumgiev *et al.* (1999); Hegazi and Abd El Hady (2001) and Abd El Hady and Hegazi (2001) who found that the antimicrobial activity differs according to the differences in the chemical composition.

The comparison between the activity of different therapeutic agents (against bacteria and fungi) as tetracycline and ketoconazole in relation to different propolis samples revealed that the propolis samples were effectively acting to inhibit the pathogens growth. The minimum 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 II. There were differences in their minimum inhibitory concentration. The MIC was 2200 and

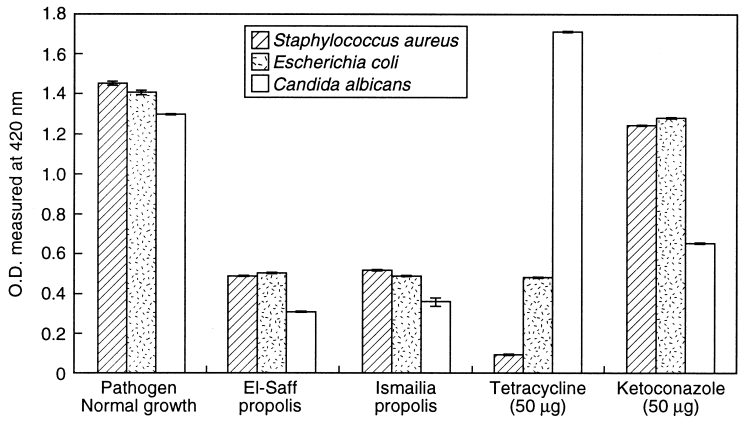

Fig. 2. Antimicrobial activity of Egyptian propolis.

2600 μg/ml for *Staphylococcus aureus* while it was 1460 and 1600 μg/ml for *Escherichia coli*. But it was 1420 and 1800 μg/ml in case of *Candida albicans* for El-Saff and Ismailia propolis, respectively. The variation of the antibacterial activity of propolis referred to the chemical composition of propolis from area to area, which had a synergistic effect of various phenolic compounds (Kujumgiev *et al*., 1999; Hegazi *et al*., 2000 a,b; Hegazi and Abd El Hady, 2001; Abd El Hady and Hegazi, 2001).

The preliminary TLC investigation of the 70% alcoholic extracts showed significant differences between the two propolis samples. In order to perform a complete analysis and to compare the results obtained with the two propolis samples, the total alcoholic extracts were silylated and subjected to a GC / MS investigation. The results are summarized in Table III.

The Ismailia propolis was characterized by high significant amounts of aromatic acid esters (47.3%) and triterpenoids (17.3%), while El-Saff propolis contained 3% and 1.9%, respectively. The Ismailia propolis contained 13.3% (as 5 esters) of 4-methoxyhydrocinnamic acid and 33.8% (as 10 esters) of hydroferulic acid. These esters appeared to be new compounds, they were tentatively identified from there mass spectra as esters with long chain alcohols: heindecenyl, tridecyl, tridecenyl, tetradecyl, tetradecenyl, pentadecyl, pentadecenyl, hexadecyl and hexadecenyl 4-methoxyhydrocinnamate or hydroferulate. The El-Saff propolis appeared to have 3% aromatic acid esters, from which four are new to propolis: two coumarate and two ferulate esters with long chain alcohols. The Ismailia propolis was characterized by the presence of a highly significant amount (17.3%) of the triterpenoids: cycloartinol, cycloartinol isomers, cycloartinol with another double bond and its isomers, α-amyrin, β-amyrin and triterpenes of β-amyrin type. Although the El-Saff propolis contained 1.9% triterpenoids only, it showed the presence of new compound to propolis: lanosterol acetate and triterpene of lupane type. It also contained a triterpene methyl ester (ursane type) and triterpenes of β-amyrin type.

El-Saff propolis contained a very high significant amount (27%) of 2,6-bis-(pentanyloxy)-4-pentanylphenethanol. It also contained 2-pentanyloxy-4-pentenyl phenethanol and tropine alkaloid which are new to propolis. Ismailia propolis showed the presence of 2,6-bis(*t*-butyl)-4-(dimethylbenzyl) phenol and 2,4,6-tri(dimethylbenzyl) phenol which are also new compounds to propolis.

Concerning to the presence of aromatic and aliphatic acids in the investigated propolis, it was clear that the Ismailia propolis had no aromatic acids. While the El-Saff propolis contained 3 aromatic acids: 4-hydroxybenzoic acid, 3,4-dimethoxycinnamic acid and caffeic acid. On the other hand, Ismailia propolis contained one flavone (0.26%) and El-Saff propolis contained 3 flavonoids in very low concentration (0.05%).

The two propolis samples possess different chemical composition. The specificity of the two propolis could be explained by the different geographic locations (it has been show earlier that in Egypt the chemical composition of propolis is changed very much with the location site: Chrsitov *et al.*, 1998; Hegazi and Abd El Hady, 2001; Abd El Hady and Hegazi, 2001). It was clear that the El-Saff propolis was completely different from the Ismalia propolis. In the later, a highly significant amount (47.3%) of 15 new aromatic acid esters were identified. In the El-Saff propolis (3%) from which four new aromatic acid esters were also detected. As the chemical composition differs according to the geographic location or the type of trees and shrub in the same province (Ismailia) as in the previous study the Ismailia propolis contained no aromatic acids and esters (Abd El Hady and Hegazi, 2001), In this study the Ismalia propolis (reclaimed land) collected from the same province showed the presence of 15 new aromatic acid esters (47.3%) and had no aromatic acids. These esters belonged to 4-methoxyhydrocinnamic acid and hydroferulic acid. 4-methoxyhydrocinnamic acid was identified before in European propolis (Greenaway *et al*., 1991; Hegazi *et al*., 2000). Hydroferulic acid was identified in the Brazilian propolis (Marcucci *et al*., 1998; Bankova *et al*., 1998). No esters of long chain alcohols for these two acids have been identified before in propolis. So, that is the first time to identify these new esters.

In this study Ismailia propolis (reclaimed land) contained 17.3% of triterpenoids while in Isamlia propolis from old valley in the previous investigation (Abd El Hady and Hegazi, 2001) contained 2.8% only which they were mainly triterpenic acid

Table III. Chemical composition assessed by GC/MS of alcoholic extracts of propolis

Compounds	El-Saff Propolis	Ismailia Propolis
	% TIC [a]	
Aliphatic Acids		
Hydroxyacetic acid	0.10	–
Lactic acid	0.41	–
3-Hydroxypropanoic acid	0.10	–
2,3-Dihydroxypropanoic acid	0.04	–
Malic acid	0.13	–
Succinic acid	0.18	–
2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone	0.01	–
Palmitic acid	0.58	0.05
Heptadecanoic acid	–	0.01
Oleic acid	0.31	0.03
Octadecenoic acid	–	0.04
Stearic acid	0.12	–
Aromatic acids	–	–
4-Hydroxybenzoic acid	0.01	–
3,4-Dimethoxycinnamic acid	0.04	–
Caffeic acid	0.15	–
Esters	–	–
Ethyloleate	–	0.02
Octadecenoic acid pentyl ester	0.05	0.04
Phthalate ester	0.63	0.16
Tetradecyl-4-methoxyhydrocinnamate[b,c]	–	0.05
Tetradecenyl-4-methoxyhydrocinnamate[b,c]	–	0.29
Hexadecyl-4-methoxyhydrocinnamate[b,c]	–	1.12
Hexadecenyl-4-methoxyhydrocinnamate[bc]	–	0.61
Octadecyl-4-methoxyhydrocinnamate[bc]	–	11.25
3-Methyl-3-butenyl-*trans*-4-coumarate	0.04	–
trans-3-Penteny-*trans*-4-coumarate	0.09	–
Tetradecanyl-*trans*-4-coumarate[b]	0.04	–
Hexadecanyl-*trans*-4-coumarate[b]	0.09	–
Heindecenyl hydroferulate[b,c]	–	0.79
Tridecatrienyl hydroferulate[b,c]	–	0.31
Tridecatrienyl hydroferulate (isomer)	–	6.00
Tridecyl hydroferulate[b,c]	–	0.35
Tridecyl hydroferulate (isomer)	–	0.81
Tridecyl hydroferulate (isomer)	–	11.24
Tridecenyl hydroferulate[b,c]	–	0.30
Pentadecyl hydroferulate[b,c]	–	3.14
Pentadecyl hydroferulate (isomer)	–	10.39
Pentadecenyl hydroferulate[b,c]	–	0.50
Octenyl ferulate[b]	0.40	–
Octadecatrienyl ferulate[b]	1.11	–
Isopentenyl-caffeate	0.22	–
2-Methyl-2-butenyl-caffeate	0.33	–
Di and Triterpenes	–	–
Dehydroabietic acid	0.08	0.24
Diterpene ,$[M]^+ = 274$	0.08	–
Diterpene ,$[M]^+ = 274$	0.15	–
Cycloartinol	–	2.05
Cycloartinol isomer	–	0.17
Cycloartinol isomer	–	2.12
Cycloartinol with another double bond	–	0.83
Cycloartinol with another double bond	–	1.73
Lanosterol acetate[b]	0.06	–
α-Amyrin	0.14	2.53

Table III.

Compounds	El-Saff Propolis	Ismailia Propolis
	% TIC [a]	
β-Amyrin	0.44	2.24
Triterpene (lupane type)[b]	0.44	
Triterpene of β-amyrin type	0.15	3.61
Triterpene of β-amyrin type	0.05	2.00
Triterpenic acid methyl ester (oleanane type)	0.08	–
Triterpenic acid methyl ester(ursane type)	0.50	–
Triterpenic acid methyl ester (ursane type)	0.05	–
Flavonoids		
2′,6′-Dihydroxy-4′-methoxychalcone (Pinostrobin chalcone)	0.01	–
Hexamethoxy flavone	0.04	0.26
5,7,4′-Trihydroxyflavanone	0.04	–
Others	–	–
2,3- Butanediol	0.02	–
Glycerol	1.66	0.71
Phosphoric acid	0.06	–
Xylitole	0.01	–
2-Pentanyloxy-4-pentenylphenethanol [bc]	0.20	–
2,6-bis-(Pentanyloxy)-4-pentanylphenethanol[b,c]	27.00	–
2,6-bis(t-Butyl)-4-(dimethyl benzyl) phenol[b]	–	0.04
2-Ethyl-1,3-dihydroxypropane	–	0.01
2,4,6-Tri(dimethylbenzyl) phenol[b]	–	0.17
Gluconic acid	0.06	–
2-O-Glycerylgalactose	0.04	–
Tropine (alkaloide) [b]	0.03	–
1,2,3-Trihydroxy butanal	0.09	0.11
2,4-bis(Dimethylbenzyl)-6-t-butyl phenol	0.05	0.14

[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.

methyl esters of oleanane and ursane types while the triterpenoids in this study were: cycloartinol, cycloartinol isomers, cycloartinol with another double bond and its isomers, α-amyrin, β-amyrin and triterpenes of β-amyrin type. Egyptian propolis from Banisweif provence revealed the presence of 17.8% of the triterpnoids: lanosterol, cycloartinol, β-amyrin and triterpenes of β-amyrin type (Christov *et al*., 1998).

Phenethyl alcohol was identified in European propolis by Greenaway *et al.* (1987) but that is the first time to identify derivatives from this compound from El-Saff propolis, namely 2-pentanyloxy-4-pentenylphenethanol (0.20%) and 2,6-bis-(pentanyloxy)-4-pentanylphenethanol (27.0%).

There are more plants that bees could accept as sources for propolis in Egypt where its chemical composition can differ (Hegazi and Abd El Hady, 2001; Abd El Hady and Hegazi, 2001). So further investigation is needed on resin-producing plants from the vicinity of the hives.

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