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Comparison of Gymnosperms and Angiosperms Plants on Quality and Antibacterial activity of propolis

Houshang Afrouzan[a], Vassya Bankova[b], Gholamhosein Tahmasebi[a]
Millena Popova[b]

[a]Honeybee Department, Animal Sciences Research Institute Karaj, Iran.
[b]Phytochemistry of Natural products, Institute organism chemistry Bulgariya Academic Science, Bulgaria
Corresponding author:afrouzan1@hotmail.com

ABSTRACT- In this study, the effect of two kinds of plants sources (gymnosperms and angiosperms) on quality of propolis were compared. For this purpose, two areas with poplar (angiosperms) and Cypress (gymnosperms) plantations were selected in Khojir and Telo located near Tehran, Iran respectively. The propolis samples were analyzed by GC/MS the Folin- Ciocalteu, $AlCl_3$ and DNP methods. Further, the antimicrobial activity of the samples was also tested against *Staphylococcus aureus*. Thirty eight compounds of Cypress propolis and thirty-four compounds of Poplar propolis were identified. The results indicated that poplar plants are very important for propolis production.

KEY WORDS- Antibacterial, Cypress, Poplar, Propolis

INTRODUCTION

Propolis (bee glue) is a sticky, gummy, resinous substance gathered by the honey bees (*Apis mellifera L.*) from gum of some plant species (pine, cypress willow, birch, several species of poplar, ash, rivet and similar), substances of which the phenolic flavonoids and compounds are most important. Propolis is available for purchase as an ingredient in capsule form, lip balm, skin cream, tincture and toothpaste. Bees collected propolis to seal hole in the hives, smoothen the interior of the nest, strengthen comb attachments, to cover and embalm intruders in the hive that are too large to carry out. Row propolis is composed of approximately 50% resin 30% wax, 10% essential oils, 5% pollen and 5% various organic and inorganic compounds (3,4). Now a days, it is well documented that in temperate zone all over the world, the main sources of bee glue is the resinous exudates of the buds of poplar trees, mainly the black poplar *poplus nigra* (2, 3), but in some cases other poplar species can be used as a source of propolis (7, 8, 9).Therefore, chemical composition of propolis and connected with it, biological activity will be changed. In the present study, the composition of 70 % ethanolic extracts of two propolis samples collected from different plants (Poplar and Cypress) of Iran were investigated.

MATERIALS AND METHODS

Propolis origin - Propolis samples were collected from two different plants, cypress and poplar trees, in Telo and Khojir near the capital of Iran, respectively, in autumn 2003 and summer 2004 respectively. These two areas were far away about 20 kilometers from each other. Ten honey bee colonies were applied in each area and the fiber method was used to produce propolis (1). Collected propolis samples were desiccated in the dark untill processing (16).

Chemicals

Sample preparation - The extract was prepared using 70% ethanol and was evaporated to dryness. About 5 mg of the residue was mixed with 75 ml of dry pyridine and 50 µl of bis(trimethylsilyl) trifluorocacetamide (BSTFA), heated at 80^0 C for 20 min and analyzed by GC-MS.

GC-MS procedure

The GC-MS analysis was performed with a Hewlett Packard Gas Chromatograph 5890 Series II Plus Linked to Hewlett Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm ID, 0.5µm film thickness HP5-MS capillary column. The temperature was programmed from 80°C to 315°C at a rate of 5°C/min, with a 10 min hold at 315°C. Helium was used as a carrier gas, flow rate 0.8 ml. min^{-1}. Split ratio 1:50, injector temperature-324 for galangin µg/ ml; 0.22 -1.80 mg/ml for pinocembrin; was used, 37 – 326 µg/ ml for the 2:1 pinocembrin- galangin mixture. Three independent determinations were performed at each concentration and absorbance was plotted against concentration.

Calibration - Calibration curves were constructed according to the International Conference of Harmonization (1996) by using series of five working standard solutions with concentrations in the respective concentration ranges: (4-324 for galangin μg/ml;0.22-1.80mg/ml for pinocembrin; was used, 37-326μg/ml for the 2:1 pinocembrin- galangin mixture). Three independent determinations were performed at each concentration and absorbance was plotted against concentration.

Propolis samples were collected from two different areas with poplar and Cypress trees, in Khojir and Telo, respectively.

Balsam content

Preparation of propolis samples for quantification of phenolics and Flavonoids

Propolis (froze, kept overnight in a freezer) was powdered (coffee mill) and exactly measured sample of 1g was dissolved in 30 ml 70% ethanol in a 50 ml flask and left for 24 h at room temperature. It was then filtered, and the procedure was repeated. Preliminary experiments showed that a third extraction under the same conditions was not necessary since the last extract gave negative reaction to 5% ferric chloride thus confirming that two successive extractions ensure complete recovery of phenolics (incl. Flavonoids). The extracts were filtered (paper filter, Filtrak 388), combined and diluted to 100 ml with 70% ethanol in volumetric flask. They were then analyzed to determine the total flavanone and dihydroflavonol content (14).

An aliquot (3ml) of the same solution was transferred into a volumetric flash, and diluted to 50 ml with methanol. It was further, analyzed to determine the total phenolics, and the flavones and flavonols. For each sample, three parallel extractions were performed

Determination of balsam content (16)

From each of the three parallel extracts, 2 ml were evaporated in vacuum to dryness to constants weight (g). The percentage of balsam (P) in propolis sample was calculated by Formula:

$p = \frac{g}{2M}$ 100%, M = the weight of the propolis sample

The mean of the three values was determined.

Spectrophotometric measurement of Flavone and flavonol content(15)

2 ml of the test solution, 20 ml methanol and 1 ml 5% $AlCl_3$ in methanol (w/v) were mixed in a volumetric flask and the volume was made up to 50 ml with methanol. The mixture was left to stay for 30 min and the absorbance at 425 nm was measured (6). Each assay was carried out in triplicate.

Flavanone and dihydroflavonol content(14)

Initially, 1 ml of the test solution and 2 ml of DNP solution (1 g DNP in 2 ml 96% sulfuric acid, diluted to 100 ml with methanol (volumetric flask) were heated at 50^0C for 50 min). After cooling to room temperature, the mixture was diluted to 10 ml with 10% KOH in methanol (w/v). 1 ml of the resulting solution was add to 10 ml methanol and was diluted to 50 ml with methanol (volumetric flask). Absorbance was measured at 486 nm (14). Each assay was carried out in triplicate.

Total phenolic substances

The procedure of Woisky and Salation (15) was employed using a reference mixture of pincembrin and galangin, in 2:1(w/v) for calibration (14). In Brief, 1 ml of the test solution was transferred to a 50 ml volumetric flask, containing 15 ml distilled water, and 4 ml of the Folin- Ciocateu reagent and 6 ml of a 20 % sodium carbonate solution (w/v) were added. The volume was made up with distilled water to 50 ml. The sample was left for 2 h and the absorbance at 760 nm was measured. Each assay was carried out in triplicate.

Minimum Inhibitory Concentration against *S. aureus*:

The test strain used was *Staphylococcus aureus 209*. Minimum Inhibitory Concentration (MIC) was determined by the macro dilution tube methods (11). The MIC of balsam was determined by diluting solution of dry balsam to various concentrations (0.0-2000 μg/ml) using meat-peptone broth in test tubes. Each test tube was inoculated with a bacterial suspension containing 1×10^6 cells per ml and incubated at 37°C for 24 hours. The MIC was regarded as lowest concentration of the extract that did not permit any visible growth when compared with drug free broth inoculated with the bacterial suspension. For more precise detection, tubes that showed no visible growth were streaked on fresh meat peptone agar plates, incubated at 37°C for 24 h and examined for growth.

RESULTS AND DISCUSSION

The results of analysis of propolis samples by GC- MS Methods in Telo (Cypress trees predominated in the vicinity of the hives) and Khojir (poplar trees predominated in the vicinity of the hives) were presented in Table-1. Some flavonoides such as Apigenin, Kaempferol methyl ether and Isosakuranetin were identified in Telo samples that probably they have been originated from the bud exudates of gymnosperms trees, while in Khojir samples have no such compounds. Furthermore, the other flavonoides

such as Sakuranetin and Dihydroxymethoxy-flavone were probably originated from the angiosperms trees (propolis samples of Khojir) were not found in propolis of Telo (Table - 2). Meanwhile, α-Eudesmol, α-Bisabolol, Doecanoic acid, Pentose, Hexose, Pentenyl *p*-coumarate, Beutenyl caffeate, Diterpenic acid, Hexyl Z-*p*-coumarate, Hexyl caffeate, Disaccharide, Trisaccharide were identified in Telo samples,

Table 1. Chemical compositions of ethanol extracts of Telo and khojir propolis samples-2003 (% of total ion* current, GC-MS)

(Telo) Compounds	%	RT	(Khojir) Compounds	%	RT
α-Eudesmol	0.1	18.3	Alanine	0.2	5.16
α-Bisabolol	0.1	19.0	Sesquiterpene	0.9	20.2
Doecanoic acid	0.3	19.0	Fructose	1.0	22.5
Glucose	0.5	22.3	Glucose	0.3	24.0
Pentose	1.9	22.5	*p*-Coumaric acid	2.2	24.2
Hexose	0.2	23.1	Hexose	0.2	25.9
p-Coumaric acid	0.7	24.3	Dimethoxycinnamic acid	0.6	26.0
Hexose	0.7	25.9	Hexadecanoic acid	0.4	26.3
Dimethoxycinnamic acid	0.1	26.0	Ferulic acid	0.3	27.0
Hexadecanoic acid	0.1	26.3	Isoferulic acid	0.2	27.2
Ferulic acid	0.1	27.0	Caffeic acid	1.1	28.1
Isoferulic acid	0.1	27.2	Pentenyl caffeate	0.2	28.9
Caffeic cid	1.6	28.1	Oleic acid	0.3	29.2
Pentenyl *p*-coumarate	0.2	28.2	Isopentenyl ferulate	0.2	31.6
Oleic acid	0.2	29.2	Pentenyl caffeate	0.6	31.7
Pentenyl caffeate	0.2	29.7	Dimethylallyl ferulate	0.4	31.8
Beutenyl caffeate	0.1	30.0	Isopentenyl caffeate	1.0	32.4
Diterpenic acid	0.2	30.6	Dimethylallyl caffeate	1.3	32.6
Isopentenyl caffeate	7.0	31.0	Hydroxyoctadecanoic acid	0.2	32.7
Hexyl Z-*p*-coumarate	1.2	31.8	Pinostrobin chalcone	2.1	33.9
Pentenyl caffeate	2.3	32.1	Pinocembrin chalcone	4.0	34.4
Dimethylallyl caffeate	11.4	32.4	Pinocembrin	7.0	34.5
Hexyl .E-*p*-coumarate	2.2	32.7	Pinobanskin	4.0	35.4
Hexyl caffeate	2.1	32.9	Sakuranetin	3.2	36.3
Pinostrobin chalcone	7.4	33.3	Pinobanksin acetate	9.2	36.6
Pinocembrin chalcone	6.0	33.9	Benzyl caffeate	0.8	37.0
Pinocembrin	3.3	34.4	Chrysin	9.7	37.4
Isosakuranetin	0.6	35.2	Dihydroxymethoxiflavone	1.5	37.5
Pinobanksin acetate	4.3	36.6	Galangin	8.3	37.7
Disaccharide	5.3	36.6	Pinobanskin butanoate	1.0	38.0
Benzyl caffeate	0.9	36.9	Phenethyl caffeate	1.5	38.2
Chrysin	5.7	37.1	Pinobanskin pentanoate	1.6	39.2
Galangin	2.4	37.4	Cinnamyl caffeate	0.5	41.3
Pinobanksin butanoate	0.3	3737	Triterpene	1.0	46.7
Phenylethyl caffeate	3.2	38.0	-	-	-
Apigenin	0.3	38.2	-	-	-
Pinobanksin pentanoate	0.1	39.1	-	-	-
Kaempferol methyl ether	1.2	39.2	-	-	-
Triterpene	0.1	42.5	-	-	-
Trisaccharide	0.3	43.0	-	-	-

**The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation*

Table 2. The flavonoids of Telo and Khojir propolis samples determined/ Analyses by GC_MS

Row	Telo	Khojir
1	Pinostrobin chalcone	Pinostrobin chalcone
2	Pinocembrin chalcone	Pinocembrin chalcone
3	Pinocembrin	Pinocembrin
4	Chrysin	Sakuranetin
5	Galangin	Chrysin
6	Pinobanskin butanoate	Dihydroxymethoxy flavone
7	Pinobanskin pentanoate	Galangin
8	Pinobanksin acetate	Pinobanskin butanoate
9	Apigenin	Pinobanskin pentanoate
10	Kaempferol methyl ether	Pinobanksin acetate
11	Isosakuranetin	Pinobanskin

Table 3. Compounds of propolis samples of Telo and Khojir

Telo	Khojir	Compounds
62%	47%	Balsam
28%	21.1%	phenolics
7.3%	8.4%	Total flavones and flavonols
3.7%	4.6%	Total flavanones and dihydroflavonols
11%	13 %	Flavonoids

But, were not in propolis of Khojir samples. Also, Alanine, Sesquiterpene, Fructose, Isopentenyl ferulate, Pentenyl caffeate, Dimethylallyl ferulate, Hydroxyoctadecanoic acid and Cinnamyl caffeate were identified in propolis of Khojir samples, but were not in propolis of Telo samples.

The chemical composition of propolis is quite complicated and over 150 components have been identified (13, 7). Among these compounds flavonoids were suggested to be responsible for biological activity (12, 5). Flavonoids (Total flavones and flavonols, Total flavanones and dihydroflavonols) are considered as an important index for evaluating propolis quality. The compound of propolis samples of Telo and Khojir were identified by the Folin – Ciocalteu, $AlCl_3$ and DNP methods are listed in Table 3.

Both the samples of propolis obtained from Khojir and Telo have 11 kinds of Flavonoids, however the quality of Flavonoids producing in Khojir was better than Telo one (Table 3). Total flavones and flavonols, and also total flavanones and dihydroflavonols (Flavonoids) in khojir (13%) samples were higher than the Telo (11%). The Flavonoids are main compounds in propolis which have treatment characters (10). The obtained propolis of poplar and cypress plants can be used at medicine. Despite of higher balsam and phenolics production in Telo, It seems that phenolics as balsam had a little role at quality of propolis.

The antibacterial activity of propolis samples of Telo and Khojir against *Staphylococus aureus* showed that the quality of Khojir samples were better than Telo samples with 31.25 and 62.5 μg/ ml MIC, respectively.

The GC-MS, Folin – Ciocalteu , $AlCl_3$, DNP and MIC tests indicated that, quality of propolis samples of Khojir (poplar trees) were better than that the propolis samples of Telo (Cypress trees).

In Iran, beekeepers usually move their colonies to different areas two or four times every year. In order to gain the propolis of each region separately, fiber traps method was applied (1). Therefore, they gathered honey, pollen and propolis by honeybees are from different regions.

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