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Chemical Composition and Biological Activities of Honeybee Products From Algeria

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Abstract

Introduction: In the current study, the microscopic and chemical analysis of Algerian honey, pollen and propolis were investigated.

Materials and Methods: The chemical composition of the ethanolic extracts of honeybee products was determined via gas chromatography-mass spectrometry (GC-MS) analysis. Furthermore, their *in vitro* anticancer, antimicrobial, antioxidant activities, total phenolic content (TPC) and total flavonoid content (TFC) were evaluated. Anticancer activities were assessed using the MTT assay while the antimicrobial potential was studied using the microdilution method. The antioxidant activities were investigated using the 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH), hydrogen peroxide scavenging activity (H_2O_2) and ferric reducing antioxidant power (FRAP). The TPC and TFC were evaluated via Folin-Ciocalteu's and $AlCl_3$ assays, respectively.

Results: In the GC-MS analyses, 36 compounds were identified in the ethanol extract of pollen accounting for 92.73% of the total extract; linolenic acid was the most abundant compound (21.28%). Also, 23 compounds were identified in the ethanol extract of propolis representing 29.91% of the total extract; Z-nerolidol was the most abundant compound (8.96%). Moreover, 17 compounds were identified in the ethanol extract of honey representing 99.40% of the total extract while glyceraldehyde (27.07%) was the major abundant compound. The ethanol extract from pollen yielded the highest TPC with 1169.33 mg Gallic acid equivalent/g dry extract. In the DPPH assay, the SC_{50} values ranged from 50.74 to 53.05 µg/mL. Significant antimicrobial activities were associated with propolis with Gram positive bacteria as the most sensitive microorganisms. In addition, remarkable anticancer activities were observed for propolis against five human cancer cell lines with LD_{50} values in the range of 3-160 µg/mL.

Conclusions: Algerian Honeybee products, especially propolis, may be a potential source of naturally occurring bioactive compounds for the treatment of oxidative stress and cancer diseases.

Keywords: Honeybee Products, GC-MS Analyses, TPC, TFC, Antioxidant, Antimicrobial, Anticancer

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Introduction

All honeybee products, honey, propolis and pollen, are very rich in bioactive compounds; hence, they have antioxidant and other various beneficial biological activities.[1,2] In fact, honey has been used by human beings since around 8000 years ago. The ancient Egyptians, Greeks, Chines, Assyrians and Romans applied honeybee products for wounds and to cure some diseases.[3] Indeed, the medicinal importance of honey has been reported in the world's oldest medical literatures.[4,5]

Honey is a natural sweet viscous fluid produced by honeybees from the pollen and nectar of flowering plants or from the nectar of blossoms which honeybees collect and transform by combining with their salivary secretions and deposit, dehydrate and store in the honey comb to ripen.[6] Several reports have mentioned that honey contains more than 200 substances. Some honey substances are essential for human life such as sugars (the major sugar present in all the types of honey is fructose), proteins, vitamins, organic acids and minerals.[7-10] However, the quality of the honey largely depends on the climate and environmental conditions around the foraging area of bees. Furthermore, processing and improper storage conditions also indirectly affect the quality of honey.[11]

Propolis is a resinous material that is collected by honeybees from buds, leaves, bark and exudates of several trees and plants.[12,13] Currently, more than 300 compounds, such as phenolic acid, terpenes, cinnamic acid, caffeic acid, several esters and flavonoids have been identified as constituents

of propolis from different geographical origins.[14,15] Propolis exerts numerous pharmacological benefits such as antioxidant, antibacterial, antiviral, antitumor, anti-inflammatory, and immunomodulatory activities.[16-18]

Bee pollen, on the other hand, is a collection of pollen grains from various botanical sources, collected by the bees and mixed with nectar and secretions from the hypopharyngeal glands of honeybees such as β-glycosidase enzymes.[19] Health-boosting worth of bee pollen is evident due to the huge amount of secondary plant metabolites, such as tocopherol, niacin, thiamine, biotin and folic acid, in addition to the enzymes and coenzymes, found in the bee pollen.

Free radicals are highly energetic unstable molecules containing single electron able to attack cells and tissues in the human body.[20] Moreover, the accumulation of reactive species results in oxidative stress which is associated with several health related disorders such as cancer, inflammation, neurodegeneration and cardiovascular diseases.[21-24] Previous reports have demonstrated that some naturally occurring secondary metabolites, such as polyphenolics, are responsible for antioxidant action and have proven powerful the free radical scavenging activities.[25-28] Therefore, this study aimed to identify the botanical sources, chemical composition of honey, pollen and propolis from Algeria via gas chromatography-mass spectrometry (GC-MS) technique and to determine their antioxidant activities along with other biological activities.

Materials and Methods

Sample Collection

Three honeybee products (honey sample, propolis and pollen sample) were collected from the Laghouat region, of the Algerian Saharan Atlas, during the period of April to August in 2016 and were stored at 4°C until further processing.

Analysis for Propolis Samples

Propolis Extraction

The propolis extraction was carried out according to Popova et al.[29] Propolis was grated and a sample of 1 g was dissolved in 70% ethanol and mixed in an ultrasound bath. After extraction, the sample was filtered and the filtrate diluted to 100 mL with 70% ethanol.[29]

Analysis for Pollen Samples

Preparation of Pollen Slides for Microscopic Analysis

Dry pollen loads of 2 g of a sample were weighed into a 15 mL falcon centrifuge tube, mixed with 70% ethanol to a final volume of 13 mL and left for 30 minutes. The sediment was obtained after centrifugation at 3500 rpm for 20 minutes. A solution of distilled water/glycerin 1:1 was added to the sediment final volume of 13 mL. One drop of the well-mixed pollen grain suspension was applied on a microscope slide, covered with a 22 × 22 mm cover slide.[30]

Preparation of Pollen Ethanolic Extract

The pollen sample (2 g) was extracted using 15 mL of ethanol solution as an extraction solvent at the temperature of 70°C for 30 minutes with constant agitation. The supernatant separated and the solid residue was re-extracted. Then, the ethanol extract of pollen was combined and stored at 4°C.

Analysis for Honey Sample

Preparation of Honey Slides for Microscobic Analysis

Preparation of the honey sample for qualitative and quantitative melissopalynological analysis was performed according to Louveaux et al.[31] The total pollen number (TPN) of all samples was calculated according to the method described by Moar.[32] The honey samples (10 g) were classified according to TPN as group I: TPN<20 000; group II: 20 000<TPN<100 000; group III: 100 000<TPN<500 000; Group IV: 500 000<TPN<1 000 000 and group V: TPN>1 000 000. The used terms for the evaluation of the frequency classes in honeys were: dominant pollen (>45%), secondary pollen (16-45%), minor pollen (3-15%) and trace pollen (<3%).[33]

GC-MS Analysis of Honey Sample

A GC 6890N instrument from Agilent (Palo Alto, CA, USA) coupled with a mass detector (MS5973; Agilent) was used for the analysis of the propolis sample. Experimental conditions of the GC-MS system were as follows: a DB 5MS column (30 m × 0.25 mm, 0.25 µm film thickness) was used and the flow rate of the mobile phase (He) was set at 1 mL/min. In the GC part, temperature was kept at 35°C for 8 minutes and then increased to 60°C at 6°C/min intervals followed by 4°C/min to 160°C and 20°C/min to 200°C/min and was kept at 200°C for 1 minute. Organic compounds in propolis samples were identified in Wiley's NIST Mass Spectral Library, if the obtained comparison scores were higher than 95%. Otherwise, fragmentation peaks of the compounds were evaluated, and the compounds were identified using the memory background for the identification of the compounds that appeared in GC-MS chromatograms. Contents of individual compound in the ethanol extract were given in percent of the total compound in the sample. This was the standard way to quantify most organic compounds in the honey samples.[34] Variations were not higher than 5%.

Antioxidant Assays for Honey, Pollen and Propolis Samples

DPPH Free Radical Scavenging Activity Assay

The sample extract solutions (5-100 µg/mL) were prepared with ethyl alcohol. The DPPH free radical scavenging activity of the sample was determined according to Blois.[35] To 3.0 mL of various concentrations of sample, 1.0 mL solution of DPPH$^{\bullet}$ (0.1 mM) was added. After 30 minutes incubation in the dark, absorbance was recorded at 517 nm. Free radical scavenging activity was evaluated by drawing standard calibration graphics. Free radical scavenging activities of reaction mixtures were calculated by using absorbance estimations 30 minutes later and were compared with BHA (butylated hydroxy anisole), RUT (Rutin) and TRO (Trolox) as standard antioxidants. The decrease in absorbance is a demonstration of a high rate of free radical scavenging activity. The free radical scavenging activity of the sample is expressed as SC_{50} (µg/mL).[35]

Hydrogen Peroxide Scavenging Activity Assay

The hydrogen peroxide scavenging activity of the used sample in the study was done according to Ruch et al.[36] For this assay, 3.4 mL was taken from the sample (5-100 µg/mL) and 0.6 mL of 40 mM H_2O_2 (prepared with 0.04 M phosphate buffer (pH = 7.4)) was added. After 10 minutes, the absorbance of the mixture was measured at 230 nm compared to a blank sample. Phosphate buffer (0.04 M, pH = 7.4) which does not include hydrogen peroxide solution was used as a blank. The results were expressed as SC_{50} value (µg/mL) where it is inversely proportional to the hydrogen peroxide scavenging activity, and the obtained result compared with standard antioxidants.[36]

Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing capacity of the sample extract and standards were determined by Oyaizu.[37] At different concentrations (50–250 µg/mL), 2.5 mL of samples or standard were mixed with PBS (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1.0%). The obtained mixture was incubated at 50°C during 20 minutes and TCA (2.5 mL, 10%) were added to the mixture. Afterward, 2.5 mL of this solution was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). The ferric/ferrous transformation was investigated, and the absorbance values were measured at 700 nm in a spectrophotometer.[37] The reducing capacity of the different samples was determined using the following equation:

$$FRAP\ (\%) = (A_s/A_c) \times 100$$

Where, A_c was the absorbance of control, and A_s was the absorbance of samples.

Total Phenolic Content

The total amount of phenolic compound in the sample extract used in this study was determined according to Slinkard and Singleton using the Folin-Ciocalteu method.[38] A sample was taken in ethyl alcohol solution (1 mg/mL, 0.5 mL) and was then deionized water (7.0 mL). About 0.5 mL Folin C reagent was added, and the content of the tube was mixed thoroughly. After 3 minutes, Na_2CO_3 (2.0%, 2.0 mL) was added and the sample was stored at room temperature and was shaken occasionally for 2 hours. The absorbance of the mixtures was measured at 760 nm. Total phenolic content (TPC) of the sample was calculated with the aid of gallic acid calibration curve (R^2: 0.9987).[38]

Total Flavonoid Content

The total flavonoid content (TFC) of sample extract used in this study was measured according to the aluminum chloride colorimetric method.[39] Ethyl alcohol solution of the samples (1 mg/mL, 0.5 mL) was taken and deionized water (1.5 mL) was added. Then, $A1C1_3{\cdot}6H_2O$ (10.0%, 0.1 mL) and 1 M potassium acetate (0.1 mL) were added and were diluted using 2.8 mL deionized water. After it was incubated at room temperature for 30 minutes, its absorbance was immediately measured at 415 nm. The sample's TFCs were calculated with the aid of the catechin calibration curve ($R^2 = 0.99$).

Antimicrobial Activity

The species used in this study (6 bacterial and 2 fungal species), were obtained from the Microbial Culture Collection Center of Medicine School at The University of Jordan, Jordan, namely: *Staphylococcus epidermidis* ATCC 12228 (gram-positive bacterium), *Staphylococcus aureus* ATCC25923 (gram-positive bacterium), *Bacillus subtilis* ATCC11562 (gram-positive bacterium), *Escherichia coli* ATCC 29425(gram-negative bacterium), *Pseudomonas aeruginosa* ATCC 15442 (gram-negative bacterium), *Klebsiella pneumonia* ATCC43816 (gram-negative bacterium), *Candida glabrata* ATCC 22553 (fungus), and *Candida albicans* ATCC10231 (fungus).

To evaluate the antimicrobial potential of the honeybee products under investigation, the minimum inhibitory concentration (MIC) measurements, defined as the lowest concentration of an examined sample that inhibited bacterial or fungal growth after incubation at optimal temperature, were carried out in 96 flat bottom microtiter plates (TPP, Switzerland) as previously described.[20] An inoculum size of 1×10^5 CFU mL^{-1} for each microorganism was utilized in each microtiter plate well. Ampicillin and Amphotericin B were employed as positive controls while a negative control of untreated media was prepared under the same experimental conditions. Bacterial testing plates were incubated for 48 hours at 37°C, whereas *Candida* plates were incubated for 48 hours at 33°C, with shaking. Optical densities were determined at wavelength 600 nm (OD_{600}) using a Microplate Reader (Palo Alto, CA, USA).

Anticancer Activity

All cell lines (MCF7, MDA-MB-231, HeLa, PC3, and K562) were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM medium (Dulbecco's Modified Eagle's Medium), supplemented with 10% Fetal Bovine Serum, 100 U/mL of Penicillin, 100 µg/mL of Streptomycin, at 37°C with 5% of CO_2. The count of viable cells was determined using the Trypan blue method as previously described.[40]

The anticancer activities of the different honeybee products were determined in 96-well round bottomed microplates using the MTT assay (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) (Sigma-Aldrich, USA) as previously published.[40] In summary, cells were seeded in 96 well plates at cell density of $1{\times}10^4$ cells/mL and incubated for 24 hours to allow attachment. Different concentrations (0.1-1000 µg/mL) of the examined honeybee product were applied onto each well in triplicates and incubated for 48 hours. Afterwards, 10 µL of 0.5 mg/mL of MTT solution was added to each well and was further incubated for 4 hours before measuring the absorbance at 570 nm. Growth suppression was assessed according to the following equation:

inhibition (%)=100-(mean of Abs of test sample - mean of Abs of negative control)×100/(mean of Abs of positive control-mean of Abs of negative control).

The GraphPad Prism 8 software was used for data analysis to calculate inhibition percentage and results were expressed

as LD_{50} value, labelled as the concentration that yields 50% growth inhibition. Doxorubicin was used as a positive control that was employed under the same experimental conditions.

Results and Discussion

Microscopic Analysis of Pollen and Honey Samples

By the microscopic analysis, the pollen belonging to the Rhamnaceae family was found in dominant ratios in the honey samples (Table 1). Makhloufi et al. investigated the palynological and physicochemical analysis of 66 Algerian honey samples. They found 124 pollen species in total and reported the main pollen as *Eucalyptus* spp., *Olea europaea*, *Papaver rhoeas*, *Pimpinella anisum*, *Carduus* spp., *Hedysarum coronarium*.[41] Draiaia et al also investigated the Algerian honey and they also determined *Eucalyptus* pollen in secondary ratios by palynological analysis and stated that the examined honey samples represented multifloral honey.[42] In addition, Diafat et al analyzed 25 honey samples from Algeria and determined them as multifloral.[43] Similar to the results of the current study, they found *Daucus* and *Eucalyptus* pollen in the examined honey samples.[43]

By the microscopic analysis, the taxa belonging to the Asteraceae, Betulaceae, Boraginaceae, Brassicaceae, Cistaceae, Ericaceae, Fabaceae, Liliaceae, Myrtaceae, Salicaceae and Rosaceae were identified in the investigated pollen samples (Table 2). The dominant taxon belongs to the Brassicaceae family. So, the honeybees mostly prefer to collect Brassicaceae pollen for their feeding in this area. There is no data about characterization of Algerian pollen in literature. As shown in Tables 1 and 2, while honey bees prefer to collect nectar mostly from plant species belonging to the Rhamnaceae family, they choose to collect pollen mostly from the plants belonging to the Brassicaceae family.

Table 1. Microscopic Analyses of Honey: Plant Taxa of Identified Pollens and the Number of Counted Pollen Grains in Microscopic Honey Slide)

Plant Family	Plant Taxa	Honey
Apiaceae		11
	Daucus spp.	13
Asteraceae		28
	Carduus spp.	56
	Centaurea spp.	12
	Taraxacum spp.	12
Boraginaceae		
	Echium spp.	17
Brassicaceae		21
Cyperaceae	*Carex* spp.	7
Fabaceae		
	Astragalus spp.	126
	Lotus spp.	112
	Onobrychis spp.	54
Myrtaceae	*Eucalyptus* spp.	62
Oleaceae		7
Rhamnaceae		5138
Rosaceae		
Salicaceae	*Salix* spp.	29
TPN_{10} values		**162 388**

* TPN_{10}: Total pollen grain number in 10 g of honey.

Chemical Compositions of the Ethanolic Extracts of Pollen, Propolis and Honey Samples by GC-MS

Chemical composition of the ethanolic extracts of pollen, propolis and honey was investigated using the GC-MS analysis as indicated in Tables 3-5. The identification of chemical constituents was based on comparison of their mass spectral fragmentations pattern with those of the data reported in Wiley and NIST Libraries and those described by Adams.[44]

In the ethanol extract of pollen, 36 compounds were identified accounting for about 92.73% of the total peak area (Table 3, Figure 1). Linolenic acid was the most abundant compound (21.28%) followed by palmitic acid (13.47%), 4H-pyran-4-one (7.95%), 5-hydroxymethylfurfural (5.93%), 4H-pyran-4-one (5.35%), glyceraldehyde (3.77%), 1,2,3-propanetriol (Glycerin) (3.47%), 1H-imidazole (3.32%), cytidine (3.18%), 4(1H)-pyrimidinone (2.38%) and linoleic acid (1.56%), respectively. This is while, 23 compounds were identified in the ethanol extract of propolis representing 29.91% of the peak area (Table 4, Figure 2). Also, Z-nerolidol was the most abundant compound (8.96%) followed by E-nerolidol (5.29%), lauryl acetate (3.09%), styrene (2.61%), butyraldehyde (1.89%) and damascenone (1.47%).

In the ethanol extract of honey, 17 compounds were identified representing 99.40% of the peak area (Table 5, Figure 3). The major identified compounds were glyceraldehyde (27.07%), 5-hydroxymethylfurfural (27.0), 4H-pyran-4-one (6.21%), N-nitroso-N-methyl urea (6.18%), ethoxyethane (5.26%), 1,2,3-propanetriol (4.88%), cyclohexanamine (4.19%), 4-aminobutyric acid (3.93%), propylamine (3.93%) and butyraldehyde (3.12%).

In a study by Markiewicz-Żukowska et al, fatty acids and their derivatives were the main components of the tested pollen extracts from Poland including α-linolenic, linoleic, oleic and 11,14,17-eicosatrienoic acids.[45] The methanolic extract of Greek pollen was investigated for its chemical constituents. Results revealed the presence of fatty acids, fatty acid esters, and phenolic acids (*p*-coumaric acid, ferulic acid,

Table 2. Microscopic Analyses of Pollen : Plant Taxa of Identified Pollens and the Number of Counted Pollen Grains in Microscopic Pollen Slide

Plant Family	Plant Taxa	Pollen Sample
Asteraceae		68
	Taraxacum spp.	18
Betulaceae		17
Boraginaceae	*Cynoglossum* spp.	4
	Echium spp.	15
Brassicaceae		6802
Cistaceae		510
Ericaceae		51
Fabaceae		678
	Lotus spp.	102
	Trifolium repens	323
	Vicia spp.	18
Liliaceae		7
Myrtaceae	*Eucalyptus* spp.	8
Salicaceae		53
Rosaceae		19

Table 3. Chemical Composition of Ethanol Extract of Pollen

Rt	Area %	M.wt.	M.F.	Identified compound
3.265	0.82	98.14	$C_6H_{10}O$	4-Penten-2-one, 4-methyl
3.976	3.77	90.07	$C_3H_6O_3$	Glyceraldehyde
4.323	0.58	98.09	$C_5H_6O_2$	2-Furanmethanol
4.631	1.10	102.09	$C_3H_6N_2O_2$	Malonamide
4.912	2.73	103.11	$C_4H_9NO_2$	Propylcarbamate
5.397	1.75	98.09	$C_5H_6O_2$	2-Hydroxy-2-cyclopenten-1-one
6.230	0.31	133.15	$C_5H_{11}NO_3$	Ethyl methoxy(methyl)carbamate
6.272	2.01	144.12	$C_6H_8O_4$	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one
6.445	0.67	112.13	$C_5H_8N_2O$	3-Ethoxypyrazole $$ 1H-Pyrazole, 3 -ethoxy
7.281	0.48	87.07	$C_3H_5NO_2$	2-Oxazolidinone
7.748	2.84	59.06	C_2H_5NO	Acetamide
8.648	0.48	117.14	$C_5H_{11}NO_2$	Carbamic acid
8.848	5.35	96.08	$C_5H_4O_2$	4H-Pyran-4-one
8.967	1.33	104.10	$C_4H_8O_3$	Tetrahydro-3,4-furandiol
9.022	0.12	122.11	$C_4H_{10}O_4$	Erythritol
9.849	1.23	86.08	$C_4H_6O_2$	4-methyl-2-oxetanone
10.017	5.93	126.11	$C_6H_6O_3$	5-Hydroxymethylfurfural
10.278	3.47	92.09	$C_3H_8O_3$	1,2,3-Propanetriol (Glycerin)
10.713	3.32	68.07	$C_3H_4N_2$	1H- Imidazole
10.851	7.95	96.08	$C_5H_4O_2$	4H-Pyran-4-one
11.307	0.48	45.08	C_2H_7N	Ethanamine
11.431	1.28	285.25	$C_{10}H_{15}N_5O_5$	2R,3S-9-[1,3,4-Trihydroxy-2-butoxymethyl]guanine
11.728	1.47	103.11	$C_4H_9NO_2$	4-Aminobutyric Acid
12.263	2.38	96.08	$C_4H_4N_2O$	4(1H)-Pyrimidinone
12.910	0.40	142.17	$C_6H_6O_2S$	Thiopheneacetic acid- 2
13.032	0.05	164.20	$C_{10}H_{12}O_2$	4-Ethyl-1,3-dioxolane
13.556	3.18	243.21	$C_9H_{13}N_3O_5$	Cytidine
25.898	13.47	256.42	$C_{16}H_{32}O_2$	Palmitic acid
29.797	1.56	280.44	$C_{18}H_{32}O_2$	Linoleic acid
29.960	21.28	278.42	$C_{18}H_{30}O_2$	Linolenic acid
30.450	0.56	284.47	$C_{18}H_{36}O_2$	Stearic acid
30.571	0.76	306.48	$C_{20}H_{34}O_2$	Ethyl 9.alpha.-linolenate
33.794	0.63	399.65	$C_{27}H_{45}NO$	16,28-Secosolanid-5-en-3-ol
37.665	1.02	330.50	$C_{19}H_{38}O_4$	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
41.102	1.19	320.50	$C_{21}H_{36}O_2$	n-Propyl 9,12,15-octadecatrienoate
41.538	0.86	104.06	$C_3H_4O_4$	Malonic acid
	Total 92.73%			

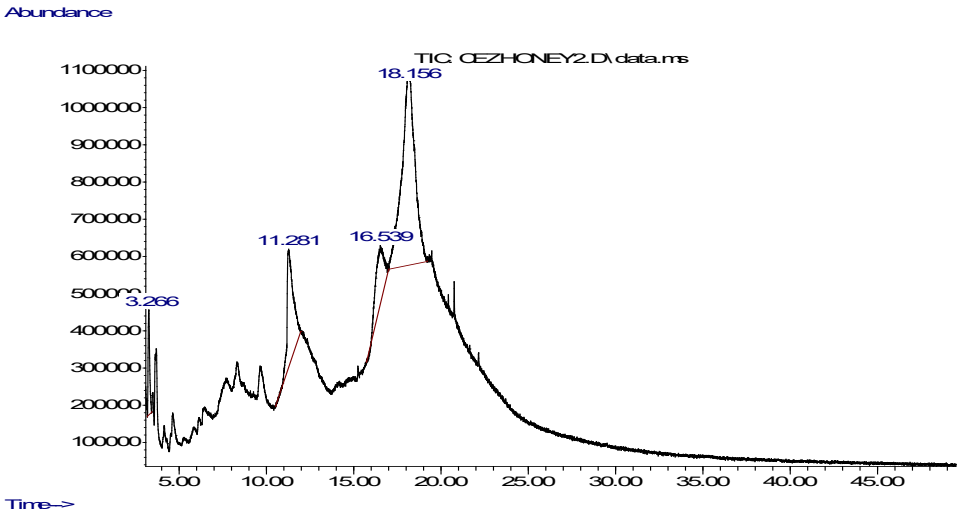

Figure 1. GC-MS Chromatogram of the Ethanol Extract of Pollen.

Table 4. Chemical Composition of Ethanol Extract of Propolis

Rt	Area %	M.wt.	M.F.	Identified Compound
4.522	0.02	200.31	$C_{12}H_{24}O_2$	Lauric acid
4.945	0.03	284.47	$C_{18}H_{36}O_2$	Stearic acid
5.105	0.03	116.15	$C_6H_{12}O_2$	4-Methylpentanoic acid
5.396	0.02	122.12	$C_7H_6O_2$	Benzoic acid
5.642	0.04	100.11	$C_5H_8O_2$	Acetyl propionyl
5.774	0.09	134.17	$C_9H_{10}O$	2,4-Dimethylbenzaldehyde
6.134	0.05	140.22	$C_9H_{16}O$	cis-6-Nonenal
7.791	0.04	136.23	$C_{10}H_{16}$	Limonene
12.758	0.06	154.24	$C_{10}H_{18}O$	Eucalyptol
18.856	0.44	284.47	$C_{18}H_{36}O_2$	Ethyl palmitate
19.370	0.76	152.23	$C_{10}H_{16}O$	Citral
19.622	0.85	140.22	$C_9H_{16}O$	cis-6-Nonenal
19.765	0.98	310.51	$C_{20}H_{38}O_2$	Ethyl oleate
19.879	0.94	130.14	$C_6H_{10}O_3$	Ethyl acetoacetate
20.496	0.74	154.24	$C_{10}H_{18}O$	Linalool
20.873	1.89	72.10	C_4H_8O	Butyraldehyde
21.005	1.22	222.36	$C_{15}H_{26}O$	Farnesol
21.862	2.61	104.14	C_8H_8	Styrene
23.091	1.47	190.28	$C_{13}H_{18}O$	Damascenone
23.691	3.09	228.37	$C_{14}H_{28}O_2$	Lauryl acetate
25.731	5.29	222.37	$C_{15}H_{26}O$	E-nerolidol
26.017	8.96	222.37	$C_{15}H_{26}O$	Z-nerolidol
28.772	0.29	180.20	$C_{10}H_{12}O_3$	Anisyl acetate
	Total 29.91%			

Table 5. Chemical Composition of Ethanol Extract of Honey

Rt	Area %	M.wt.	M.F.	Identified Compound
3.779	0.85	87.16	$C_5H_{13}N$	2-Amino-3-methylbutane
4.923	27.07	90.07	$C_3H_6O_3$	Glyceraldehyde
5.246	0.14	73.09	C_3H_7NO	Propanamide
6.173	3.93	59.11	C_3H_9N	Propylamine
6.653	3.12	72.10	C_4H_8O	Butyraldehyde
6.751	6.18	103.08	$C_2H_5N_3O_2$	N-Nitroso-N-methyl urea
6.773	5.26	72.10	C_4H_8O	Ethoxyethene
12.881	4.19	99.17	$C_6H_{13}N$	Cyclohexanamine
15.271	6.21	96.08	$C_5H_4O_2$	4H-Pyran-4-one
15.815	1.33	232.11	$C_4H_6O_3$	2-hydroxy-butanedial
17.911	2.96	85.10	C_4H_7NO	Methacrylamide
18.303	27.0	126.11	$C_6H_6O_3$	5-Hydroxymethylfurfural
18.843	4.88	92.09	$C_3H_8O_3$	1,2,3-Propanetriol
22.329	3.93	103.11	$C_4H_9NO_2$	4-Aminobutyric Acid
23.624	1.99	71.07	C_3H_5NO	2-Propenamide
25.452	0.26	101.14	$C_5H_{11}NO$	N-(n-Propyl)acetamide
30.372	0.10	148.07	$C_4H_4O_6$	Dihydroxymaleic acid
	Total 99.40%			

their glycerol esters and glycerol ester of caffeic acid). Also, the dichloromethane extract afforded fatty acids like linoleic acid, palmitic acid, methyl malonic acid and benzoic acid; fatty acid esters as palmitic acid methyl ester, linoleic acid methyl ester, linolenic acid methyl ester, linolenic acid ethyl ester, stearic acid methyl ester, arachidic acid methyl ester, behenic acid methyl ester and methyl palmitate.[19] In the same context, diterpenes, sesquiterpene esters of benzoic acids, aliphatic hydroxy acids, aromatic and fatty acids, triterpenes and anthraquinones were reported in ethanolic extracts of pollen from different locations like; Croatia, Sicilia, Greece, Malta, Algeria, Brazile and Cyprus.[46-48] Furthermore, the lipophilic composition of honeybee pollen from *Cistus ladanifer*, *Castanea sativa* and *Rubus* sp., was analyzed via GC-MS. The extracts are mainly composed by saturated and unsaturated fatty acids, sterols, long chain aliphatic alcohols, alkanes and alkenes.[49]

Different classes of chemical compounds were previously detected in bee pollen including polyphenols, flavonoids, proteins, amino acids, carbohydrates, vitamins and minerals. The variation in such chemical compositions depend on some factors like; geographic area, climate conditions and extraction methods.[50,51]

On the other hand, GC-MS analysis of propolis from Al-

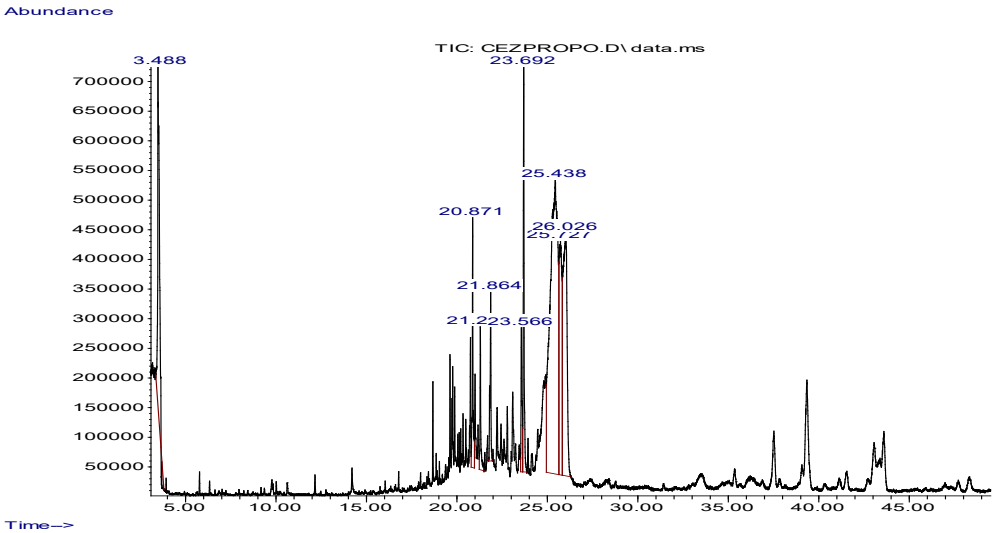

Figure 2. GC-MS Chromatogram of the Ethanol Extract of Propolis.

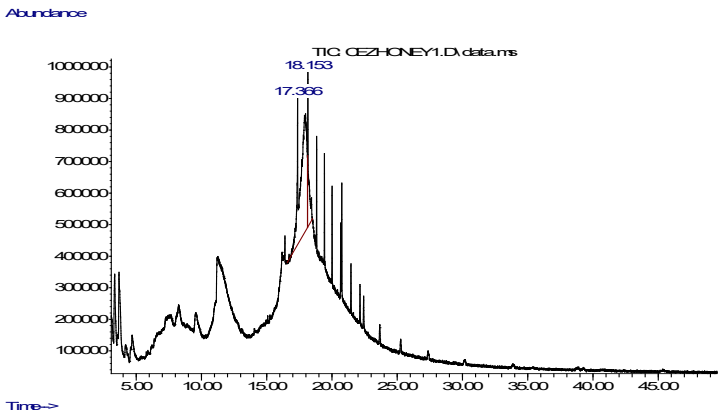

Figure 3. GC-MS Chromatogram of the Ethanol Extract of Honey.

Bahah, Saudi Arabia revealed that triterpenoids are the major components (74.0%) followed by steroids (9.8%) and diterpenoids (7.9%).[52] Globulol, δ-selinene, γ-gurjunene, ledene, aromadendrene, and α-cedrol were recorded as major terpenoidal compounds in the 70% ethanolic extract of Malaysian propolis, while gallic acid and eicosanoic acid methyl ester were recorded as major phenolic compounds.[53] By reviewing literature, flavonoids and terpenoids have been reported in manuka honey including; pinocembrin, chrysin, pinobanksin, 8-methoxykaempferol, luteolin, isorhamnetin, galangin, kaempferol, sakuranetin, quercetin, magniferolic acid and 3β-hydroxy-24-methylenecycloartan-26-oic acid.[54]

Algerian honey products are distinguished from other products by their unique bioactive ingredients among them phenolic derivatives (i.e., caffeate esters, isocupressic acid),[53] flavonoids (i.e., polymethoxyflavonol; pinostrombin chalcone, galangin, naringenin, tectochrysin, methoxychrysin),[53,54] diterpenes (i.e., labdane and clerodane),[53] prenylated coumarin (i.e., suberosin),[54] reducing sugars (i.e., glucose, and fructose),[55] and volatile constituents.[56]

Total Phenolic Content, Total Flavonoid Content and Antioxidant Activities of Honey, Pollen and Propolis

Algerian honey products belong to a group of honey products with relatively high amounts of flavonoids.[57] In the current study, the TPC and TFC of the ethanolic extracts of honeys, pollen and propolis were evaluated via the Folin-Ciocalteu and $AlCl_3$ assays, respectively. The ethanolic extract of pollen yielded the highest content of polyphenols with 1169.33 mg GAE/g dry extract, followed by 553.52 and 187.74 for propolis and honey extracts, respectively. Similarly, the ethanolic extract of pollen yielded the highest flavonoid content with 950.41 mg QE/g dry extract, followed by 721.87 and 42.60 for propolis and honey extracts, respectively (Table 6).

The antioxidant activities of the ethanolic extracts of honey, pollen and propolis were evaluated via three *in vitro* antioxidant models namely; free radical-scavenging activity (DPPH), hydrogen peroxide scavenging activity (H_2O_2) and FRAP.

In the DPPH assay, the SC_{50} values for the tested extracts ranged from 50.74 to 53.05 µg/ mL. The results of the tested extracts are in the following order: honey SC_{50} (50.74) > propolis SC_{50} (53.02) >pollen SC_{50} (53.05 µg/mL) (Table 6). Furthermore, the tested extracts showed variable antioxidant activity using H_2O_2 assay with SC_{50} values arranged in the following order: honey SC_{50} (56.82) > propolis SC_{50} (72.97) > pollen SC_{50} (89.35 µg/mL), data are recorded in Table 6.

Remarkably, in the FRAP assay the ethanolic extract of pollen showed high reducing power activity with 88.57 mM $FeSO_4$ equivalent/mg dry extract, followed by the ethanolic extract of honey (84.54 mM $FeSO_4$ equivalent/mg extract) and the ethanolic extract of propolis (82.29 mM$FeSO_4$ equivalent/mg extract), compared to quercetin (21.45 mM $FeSO_4$ equivalent/mg compound) (Table 6).

Antioxidant activity and TPC of pollen extracts from different geographical areas were previously evaluated and reported by many researchers.[19,45,55-57] The average value of TPC of pollen from Central Chile is 12.64 g GAE/kg.[58] On the other hand, Pérez-Pérez et al. (2012) evaluated the TPC of different solvent extracts of pollen from Venezuela. This study showed that the TPC values are 496.65, 755.0 and 1540.0 mg GAE/g pollen, respectively for water, methanol and ethanol

Table 6. Total Phenolic Content, Total Flavonoid Content, Free Radical-Scavenging Activity, Hydrogen Peroxide Scavenging Activity (and Ferric Reducing Antioxidant Power of Honey, Pollen and Propolis

Sample	TCP mg GAE/100 g Dry Extract	TFC mg QE /100g Dry Extract	DPPH SC_{50} (µg/mL)	H_2O_2 SC_{50} (µg/mL)	FRAP(mM $FeSO_4$ Equivalent/mg Extract
Honey	187.74 ± 0.92	42.60 ± 0.47	50.74 ± 0.79	56.82 ± 0.50	84.54 ± 0.27
Pollen	1169.33 ± 1.04	950.41 ± 0.59	53.05 ± 0.14	89.35 ± 0.17	88.57 ± 0.65
Propolis	553.52 ± 1.01	721.87 ± 0.64	53.02 ± 0.96	72.97 ± 0.79	82.29 ± 0.68

Results are expressed as mean values±SD (n=3). SC_{50}: Concentration of sample required to scavenge 50% of free radicals. SC_{50}: values are expressed as µg dry extract/ mL (µg/mL). SD: Standard deviation.

extracts.[59] In another study, the average TPC values of pollen samples are 32.15-18.55 mg/g, while the average TFC values are 10.14-3.92 mg/g.[60] Araújo et al reported that the TPC of the pollen extracts ranged from 33.73 to 75.60 mg GAE/g, while the TFC ranged from 1.42 to 9.05 mg QE/g of bee pollen extract.[61]

Eswaran and Bhargava reported that 90% ethanolic pollen extract showed FRAP value of 4.08 mg/mL indicting its ability to reduce ferric ions to ferrous ions in addition to its DPPH scavenging activity value of 45.69 g/mL.[56] It was reported that the antioxidant activity of pollen samples may be attributed to the presence of certain phenolic compounds.[58,62,63]

The ethanolic extract of green propolis from the state of Minas Gerais showed high free radical scavenging antioxidant potential against DPPH radical with SC_{50} value of 31.80.[64] Moreover, a previous study carried out by Mohdaly et al revealed that caffeic acid, ferulic acid, rutin and *p*-coumaric acid were detected as main phenolic compounds in methanolic extract of propolis which may be accounted for its antioxidant activities.[65]

Ita evaluated the TPC and total flavonoids content of honey samples from the Northern Savannah region and Southern rainforest ecosystems of Nigeria. The results revealed that TPC values ranged from 23.92 to 82.34 mgGAE/g for both ecosystems, while TFC varied between 2.52 27.21 mgQE/g for honey samples from the Northern Savannah zone and 9.17- 22.38 mgQE/g for honey samples from the Southern rainforest ecosystem.[66]

Evaluation of the Biological Activities of Algerian Honey Products

The Algerian honey products showed vital biological applications including antioxidant,[53] antimicrobial,[66] antitumor,[67] and preventive effects against the toxicity of cadmium sulfate.[68]

Antimicrobial Activity of Honey, Pollen and Propolis

All honey products examined in this study demonstrated antibacterial activity against gram-positive bacterial pathogens with MIC ranging from 32-128 µg/mL (Table 7). On the other hand, less bactericidal effect against gram-negative microorganisms was observed with MIC ranging from 128 to more than 512 µg/mL (Table 7). All examined samples exhibited moderate antifungal activities against the two tested fungal strains.

Several literature reports indicated that honey may have worthy activity against numerous microbial pathogens.[5] The proposed mechanism of action for the reported antibacterial activity involves degrading the bacterial cytoplasmic membrane leading to the loss of potassium ions and provoking cell autolysis.[67] Flavonoids, such as quercetin and rutin, were shown to increase membrane permeability, resulting in the loss of the bacterial capacity to synthesize ATP, crashing their membrane transport system, and reducing motility.[67]

In addition, propolis has been reported to be effective against many resistant strains of bacteria along with potential antiviral properties against herpes viruses.[68] Antimicrobial activity of bee propolis may be highly attributed to its content of phenolic compounds such as flavonoids. Propolis polyphenols were shown to interact with many bacterial proteins through forming hydrogen and ionic bonds, leading to disturbing the proteins three-dimensional (3D) structure and diminishing their functionality.[69]

Different patterns of pollen's antimicrobial activity were reported in many studies, which may be attributed to the variable chemical composition of pollen from different geographical locations. Evidenced in several reports in the literature, gram-negative bacteria were observed to be more resistant to the pollen's treatment than the gram-positive bacteria, which may be related to the impermeable outer layer membrane in the gram-negative bacteria.[70]

Anticancer Activity of Honey, Pollen and Propolis

The anticancer activities of the examined honeybee products are against several human cancer cell lines including the human breast adenocarcinoma MCF-7 cell line, the human mammary gland adenocarcinoma MDA-MB-231 cell line, the human epithelial adenocarcinoma HeLa cell line, the human prostate cancer PC3 cell line, and the human myelogenous leukemia K562 cell line. To the best of our knowledge, the anticancer activities of the Algerian honeybee products against these types of human cancers are being reported for the first time in the literature. Results demonstrated that the most efficient anticancer activities are associated with Propolis (Table 8). The LD_{50} values, defined as the concentration at which 50% of cell growth is inhibited, for propolis were in the range of 3-160 µg/mL with the prostate cancer as the most responsive to this treatment.

Propolis and its rich composition of polyphenolic compounds have gained large interest of many scientific reports for their potent antitumor properties.[71] Studies on the mechanism of the action of the propolis and its active

Table 7. Antimicrobial Activities of Honey, Pollen and Propolis Against 8 Pathogenic Microorganisms

Extract	*S. epidermidis*	*S. aureus*	*B. subtilis*	*E. coli*	*K. pneumonia*	*P. aeruginosa*	*C. albicans*	*C. glabrata*
Honey	32	64	64	256	256	>512	256	256
Propolis	32	32	32	128	128	256	32	64
Pollen	128	128	128	256	256	>512	64	128
Ampicillin	2	2	4	16	64	128	-	-
Amphotericin B	-	-	-	-	-	-	2	2

Results are expressed as MIC µg/mL.

Table 8. Anticancer Activities of Honey, Pollen and Propolis Against Five Human Cancer Cell Lines

Extract	MCF-7	MDA-MB-231	HeLa	PC3	K562
Honey	>1000	>1000	>1000	>1000	>1000
Propolis	160±4	55±2	82±3	3±0.8	10±1
Pollen	>1000	>1000	>1000	>1000	>1000

Results are expressed as LD_{50} (µg/mL)±SD.

constituents as anticancer agents were thoroughly reviewed in the literature.[72] It has been shown that propolis induces apoptosis pathways in many cancer cells. In addition, the suppression of cyclin complexes and initiation of cell cycle arrest are of the main proposed mechanisms. Our results come in great consistency with previous literature reports that support the anticancer potential of propolis suggesting that propolis may be useful as a potential naturally occurring chemotherapeutic or chemopreventive agent.

Conclusions

Ethanolic extracts of Algerian honeybee products e.g., pollen, propolis and honey showed good antioxidant activities using three different models and these activities may be attributed to the co-activity between their major and/or minor components. In addition, antimicrobial activities against clinically relevant pathogens along with remarkable anticancer potential against five different human cancer cell lines were associated with propolis. Therefore, honeybee products could be used as good sources of naturally occurring bioactive compounds with antioxidant, anticancer and antimicrobial activities.

Authors' Contributions

BB, İKT and AG prepared the samples, performed total phenolic and flavonoid contents as well as the antioxidant activities. ÖGÇ and SÇY: Performed GC-MS analysis. SKB: Performed the biological activities. BB, MAG and SKB. Wrote the manuscript and analyzed the results. All authors contributed to the manuscript revision, read and approved the submitted version.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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