



Original Research

Chemical profiling, HPLC characterization and *in-vitro* antioxidant potential of Pakistani propolis collected from peripheral region of Faisalabad

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Abstract: Propolis is a highly adhesive and resinous product of honey bee (*Apis mellifera* L.) which is produced from the exudations of plants. Bee propolis being a source of bioactive compounds like polyphenols and flavonoids imparts numerous biological properties including, antioxidant, anti-inflammatory, anti-microbial and anticancer activities. Present study was designed to elucidate the composition and antioxidant status of locally available propolis using *in-vitro* conditions. Propolis collected from locally found apiaries and its hydroalcoholic extract of propolis was prepared using different concentrations of ethanol and methanol. The results regarding proximate composition of propolis showed a higher proportion of ether extract (85.59±0.87%) and lowest contents of crude fiber (0.31±0.08%). Among the mineral's sodium, potassium and calcium was found in a concentration of 11.33±0.91, 52.10±2.9 and 10.53±0.83.59±0.23mg/Kg respectively whilst zinc was noticed as 3.59±0.23mg/Kg. HPLC characterization indicates a highest concentration of Chlorogenic acid 31.80±2.56mg/Kg whereas gallic acid (0.21±0.01mg/Kg) was found in lowest concentration among the polyphenols. Ethanol extract represents more phenolic contents, DPPH activity and antioxidant status as 327.30±14.89mg/gGAE, 73.18±4.43% and 60.59±4.38% accordingly in comparison to methanol and water extract. Bee propolis found an effective source of natural antioxidants which retards the production of free radicals and reactive oxygen species thus help to cope oxidative stress.

Key words: Antioxidant; Composition; Phenolic compounds; Propolis.

Introduction

Honeybee propolis is a natural produce of bee that is formulated from plant seeps, bud exudates and barks of trees. Bee use propolis to fill small holes and fissures in the hives to protect from foreign intruders and microbial infection (1). Propolis composition and associated biological properties highly influenced due to phytogeographical diversity, climate change, seasonal variations and specie of the queen bee in the hive (2). Chemically, it is made up of about 40-45% resins, 25-30% fatty

acids, 10% essential oils, 5% pollens and 5% minerals with other compounds of organic and inorganic nature (3). In resinous component, large spectra of compounds including polyphenols (phenolic acid, esters of phenolic acid, flavonoids), steroids, amino acids and terpenoids identified as part of propolis (4). Among these compounds, phenolic compounds possessed numerous biological properties like scavenging of free radicals, those are associated with majority disorders such as diabetes, cancer and cardiovascular problems (5). Propolis composition and its biological activities determined in its

extracts prepared with different solvents like ethanol, methanol, chloroform, acetone in different proportions which showed different properties (6). The concentration of solvent used for preparation of propolis extracts greatly influenced phenolic composition and properties including antioxidant, antimicrobial, anticancer and other related activities (7). Besides this, propolis contains inorganic substances like iron, zinc, copper, magnesium and manganese and vitamins B₁, B₂, E and vitamin C (8). Presently, bee propolis is being explored in natural medicines as an antioxidant, anti-inflammatory and antibacterial agent in different parts of the world around the globe (9). The phenolic contents found in propolis has been utilized in different food formulations and considered functional food ingredients and food additives and marked as Generally Recognized as Safe (GRAS) for human consumption (10). During past few years, among researcher and physicians bee propolis has become a potent research component agent due to its natural composition and health benefits including its safe production and consumption. Almost twelve different forms of propolis identified from Brazil and one of the most popular form is green propolis which is heavily explored as functional ingredient in food formulations (11). Among propolis producing countries, China, Brazil, New Zealand and Japan produced and export propolis on a large scale. China is the largest producer of propolis which produced 350 tons of propolis annually and accounts for 80% of total world propolis production (12). Propolis possessed health promoting activities including biological properties including antioxidant, anti-inflammatory, immunomodulatory, anti-cancer, antibacterial, antiviral, antifungal and antiparasitic activities and most of the biological properties associated with its flavonoids, phenolic acids and their derivatives (13).

The production of reactive oxygen species (ROS) involved in onset of wide range of diseases due to oxidative stress. An unequal distribution of antioxidants and ROSs causes oxidative destruction of cellular components such as lipids, proteins and nucleic acid which ultimately leads for development of atherogenic disease (14). Therefore, incorporation of antioxidants from natural sources like plants secondary metabolites for functional food formulations has gained popularity for health promotion and medical aspects. Among the antioxidants, polyphenols supposed to be effective for the management of diseases and health perspectives (15). Phenolic compounds showed their effect as singlet oxygen quencher, free radical scavengers, hydrogen donor and metal ion chelators hence they retard oxidative stress by managing the balance of ROSs and antioxidant status (16). Propolis contains a wide variety of compounds including phenolic aldehydes, phenolic acids, polyphenols and flavonoids those are responsible for its biological properties. Furthermore, propolis also retards the production of superoxide anion, reverts glutathione metabolism to suppress the free radical's activity (17). Previously, published data showed that propolis and its extracts prepared using different solvents in different parts of the world possess numerous properties such as pharmacological activity, hepatoprotective, anticancer properties, antifungal and antibacterial activities. Due to these beneficial aspects propolis extracts has

been used in folk medicines since last few decades to manage different ailments and health problems around the globe (18).

In continuation of this fact, the present study designed to explore the chemical composition of locally available propolis and its extracts prepared with different concentrations of ethanol, methanol and aqueous extraction by using *in vitro* medium to explain associated properties of propolis which is the first effort of such kind in the region in this regard.

Materials and Methods

Sample collection and preparation of extract

Raw bee propolis, collected from local apiaries, was evaluated for its proximate composition and mineral contents. The weighed amount of sample was continuously agitated with 95, 80, and 65% of ethanol and methanol at room temperature for 24 hours under dark conditions. The extracts were filtered twice with Whatman paper # 2 and centrifuged at 3000 g for 15 minutes. Polyphenols rich supernatant was collected and concentrated through rotary evaporator under reduced pressure at 40°C. (19,20).

HPLC analysis of extracts

Polyphenols characterization was performed using Shimadzu, series 10 A, Japan, HPLC system equipped with C18 column (250 mm x 4.6 mm, 5.0 µm particle size). A 10 µL of sample was injected using and column temperature adjusted at 40°C throughout the analysis. Water and acetic acid in 94:6% (A) and 100% acetonitrile (B) used as mobile phase by maintaining gradient elution at a flow rate of 1 ml/min and peaks were recorded through UV/Vis detector (21).

Total phenolic contents (TPC)

Total polyphenol contents (TPC) in hydroalcoholic extract of propolis (HAEP) were estimated by Folin-Ciocalteu procedure using gallic acid as standard. 50 µL sample mixed with 250 µL of Folin-Ciocalteu reagent with 750 µL of 20% Na₂CO₃, volume was made up to 5 mL with distilled water and absorbance was noted at 765 nm with UV/visible Spectrophotometer (CECIL CE7200) (22).

Free radical scavenging activity of HAEP using DPPH assay

Free radical scavenging activity of HAEP was measured by using 1,1-diphenyl-2-picrylhydrazyl (DPPH). Four mL sample was mixed with 1 mL of DPPH solution, mixture was incubated for 30 minutes at room temperature and absorbance was measured at 520 nm (23).

Antioxidant activity of HAEP using β-carotene assay

3 mg of β-Carotene was mixed with 30 mL of chloroform. 3 mL of mixture was added in 40 mg of linoleic acid and 400 mg of Tween 40. (3 mL) of aliquots, the β-carotene/linoleic acid, was mixed with 50 µL of HAEP and incubated in a water bath at 50°C. Antioxidant activity of HAEP was monitored by spectrophotometrically at 470 nm after 60 minutes of incubation (24).

Results and Discussion

The results regarding proximate composition of pro-

Table 1. Proximate profile of locally collected honeybee propolis.

Proximate composition (%)						
Crude fat	Crude protein	Crud fibre	Ash content	Water content	Nitrogen free extract	
85.59±0.87	1.84±0.09	0.31±0.08	1.03±0.04	2.22±0.14	9.01±0.05	
Mineral profile of locally collected propolis (mg/Kg)						
Sodium	Potassium	Calcium	Iron	Copper	Magnesium	Zinc
11.33±0.91	52.10±2.9	10.53±0.8	29.3±1.7	1.50±0.1	32.13± 2.3	3.59±0.23

Values are expressed as means of triplicates ± standard deviation.

propolis revealed moisture content, crude protein, crude fat, crude fiber, ash content and nitrogen free extract (NFE) as 2.22±0.14, 1.84±0.09, 85.59±0.87, 0.31±0.08, 1.03±0.04 and 9.01±0.05%, respectively (Table.1). Similarly, the results pertaining to mineral contents depicted that propolis contains calcium (10.53±0.80 mg/Kg), potassium (52.10±2.90 mg/Kg), sodium (11.33±0.91 mg/Kg), magnesium (32.13±2.30 mg/Kg), iron (29.3±1.70 mg/Kg), zinc (3.59±0.23 mg/Kg), copper (1.50±0.10 mg/Kg) and manganese (0.67±0.03 mg/Kg) (Table.1). The results related to proximate composition of propolis during the study were in accordance with the previous findings of Kim *et al.* (25), investigated the composition of propolis for moisture contents, crude protein, crude fat, ash contents and carbohydrate contents as 3.00, 0.70, 90.90, 0.20, and 5.30%, respectively. Similarly, outcomes of Jeong *et al.* (26), are in harmony with our results, who described the composition of propolis as crude fat (86.40%), crude protein (2.71%), crude fiber (0.20%), ash (1.05%) and NFE (7.32%). Likewise, Song and Gil examined the composition of bee propolis from falsacacia and chestnut tree and results revealed that moisture content (3.60-3.90%), crude lipids/fats (81.1-86.9%), crude protein (2.0-2.50%), crude fiber (3.50-4.00%) and ash content (1.10-1.50%) (27). The non-momentous difference was observed in proximate composition of propolis as a part of environmental and phytogeographical variations. The results regarding mineral contents propolis are supported by early investigations of Nicolae and Tatiana, who concluded a maximum value of magnesium as 179.0-255.80mg/Kg and minimum value of potassium 0.75-1.00 mg/Kg [16]. Bonvehi and Bermejo observed

inorganic elements in different propolis samples as 93-225 mg/kg, 735-4790, 301-1405, 301-1405, 163-1,364, 312-1270 mg/kg for sodium, potassium, magnesium, zinc and iron respectively (27, 28).

The results regarding total phenolic contents (TPC) indicate significant variations among different extracts. The highest values of 327.30±14.89 mg/gGAE in ethanol extract followed by 292.10±15.27 mg/gGAE in methanol extract whereas minimum value 134.02±12.67 mg/gGAE was obtained in aqueous extract (Table.3). The results regarding the polyphenols quantification marked variations as part of solvent used for extract preparation. Maximum concentration 43.17±2.89mg/Kg was recorded for 4-Hydroxy benzoic acid followed by chlorogenic acid (31.80±2.56mg/Kg) whilst the minimum value (0.21±0.01mg/Kg) was observed for gallic acid (Table.2). The results pertaining to polyphenol quantification of propolis extracts were comparable with previous findings of Tosi *et al.*, who observed that propolis from Argentina contains coumaric acids and syringic acid in concentration of 0.05 to 2.1% and 0.59 to 12.1% respectively (10). Similarly, Christov *et al.*, determined the ferulic acid, caffeic acid and *p*-coumaric acid as 1.00 to 3 to 10%, 0.8% and 3.4 to 18.8% in ethanol extract of propolis collected from different regions of Canada (20). Kumazawa *et al.*, narrated the amount of caffeic acid and *p*-coumaric acid by 0.2 to 7.2mg/g and 0.9 to 27.4mg/g in different propolis extracts (29). Mello *et al.*, indicated the contents of ferulic acid and caffeic acid by 0.92 to 1.56µg/mL and 0.25 to 1.04µg/mL in various propolis samples of Brazil and inferred that variations among the results as a function of climate and geographic distribution (30).

Table 2. Polyphenols identification and quantification of propolis extracts using HPLC (mg/Kg).

Polyphenols	WE	EEP ₉₅	EEP ₈₀	EEP ₆₅	MEP ₉₅	MEP ₈₀	MEP ₆₅
Caffeic acid	ND	8.21±0.06	ND	21.66±2.10	5.33±0.04	4.13±0.80	6.39±0.02
Gallic acid	0.21±0.01	ND	ND	13.77±1.29	0.75±0.06	1.06±0.80	ND
<i>p</i> -Coumaric acid	6.65±0.04	12.31±0.09	2.86±0.01	6.27±0.01	14.98±1.21	ND	11.76±0.03
<i>m</i> -Coumaric acid	8.57±0.30	ND	1.33±0.08	ND	ND	ND	13.57±0.30
Vanillic acid	ND	ND	19.53±1.06	26.81±2.18	12.67±1.21	21.69±1.73	ND
Syringic acid	3.82±0.02	ND	2.36±0.16	20.43±1.89	ND	ND	23.36±0.02
Chlorogenic acid	7.45±0.48	19.15±0.48	ND	ND	ND	6.33±0.42	31.80±2.56
Ferulic acid	ND	4.74±0.19	5.86±0.47	ND	5.40±0.38	ND	24.32±1.89
4-Hydroxy benzoic acid	ND	ND	ND	43.17±2.89	ND	ND	ND

Results expressed as mean of triplicates ± SD. ND= Not detected, WE: water extract EEP₉₅: 95% ethanol extract, EEP₈₀: 80% ethanol extract, EEP₆₅: 65% ethanol extract, MEP₉₅: 95% methanol extract, MEP₈₀: 80% methanol extract, MEP₆₅: 65% methanol extract.

Table 3. Assessment of in-vitro antioxidant properties of HAEP of locally available propolis.

	W.E	EEP ₉₅	EEP ₈₀	EEP ₆₅	MEP ₉₅	MEP ₈₀	MEP ₆₅
TPC (mg/g)	134.02	239.10	251.71	327.30	225.88	246.87	292.10
GAE	±12.67 ^d	± 13.23 ^c	±22.43 ^c	±14.89 ^a	±17.63 ^c	± 19.37 ^c	± 15.27 ^b
(%)DPPH	44.73	61.26	66.93	73.18±4.43 ^a	59.01±2.81 ^c	60.51±3.37 ^{bc}	70.06±5.28 ^a
Inhibition	±3.32 ^d	±4.58 ^{bc}	±3.62 ^{ab}				
β-carotene	39.21	50.14	49.94	60.59	48.70	50.43	57.01
assay(%)	±2.83 ^d	±3.37 ^{bc}	±2.86 ^{ab}	±4.38 ^a	±2.52 ^c	±3.31 ^{bc}	±4.38 ^a

The results described as means of triplicates ± SD. letters indicate significant difference among treatments ($p \leq 0.05$). W.E: water extract, EEP₉₅: 95% ethanol extract, EEP₈₀: 80% ethanol extract, EEP₆₅: 65% ethanol extract, MEP₉₅: 95% methanol extract, MEP₈₀: 80% methanol extract, MEP₆₅: 65% methanol

The free radical scavenging activity using DPPH assay is widely accepted and supportive feature for the estimation of antioxidant potential of natural extracts. The principle of DPPH radical ability is the conversion of deep violet color to pale yellow color due to formation of non-radical (31).

Maximum DPPH inhibition was observed in the ethanol extract of propolis as 73.18±4.43% followed by methanol extract (70.06±5.28%) whereas minimum value was noticed in aqueous extract as 44.73±3.32%. Nonetheless, antioxidant activity perspective, β-carotene assay represents a good index for the ability of natural extracts. It indicates the efficacy of antioxidants to cope oxidative damage imposed by ROSs. The antioxidant potential for HAEP depicted a higher antioxidant value for ethanol extract (60.59±4.38%) and methanol extract (57.01±4.38%), however minimum values (39.21±2.83%) were recorded for aqueous extract (Table.3).

Phytochemicals are associated with numerous biological properties including anti-inflammatory, anti-radical, anti-oxidant and antimicrobial activities. Polyphenols are leading group in this context and considered as active component of bee propolis (32, 33). The outcomes of total phenolic contents of HAEP are supported by the previously observations of Ahn *et al.*, who examined the TPC of propolis from china and values ranged 42.90±0.80 to 302±4.30mg/g GAE (21). One of their peers, Kumazawa *et al.*, investigated the polyphenol contents as 200 to 300mg/g GAE those are in line with the results of current study (29). Choi *et al.*, observed TPC values as 120.07±3.5 to 212.7±7.4mg/g GAE in propolis samples collected from different regions of Korea. The radical quenching ability of polyphenols is mainly due to hydrogen transferring potential to free radicals produced during oxidative reactions (34). Sulaiman *et al.*, inferred about free radical scavenging activity of propolis as a function of its phenolic compounds as observed in the current study (31).

The outcomes of DPPH activity of bee propolis from different regions of Canada are in accordance with the findings of present study (19). Our results for the percent inhibition of DPPH were supported by earlier findings of Choi *et al.*, who inferred DPPH inhibition is the direct function of phenolic contents (34). Afterwards, Ahn *et al.*, reported the DPPH free radical scavenging activity of propolis upto 70% which is in harmony to the findings of current study (21). Furthermore, Kumazawa *et al.*, concluded that antioxidant activity of propolis extract using linoleic acid oxidation system possess correlation with flavonoids and phenolic contents as obser-

ved during the current study (29).

Likewise, Choi *et al.*, examined propolis extract from Korea exhibit higher antioxidant activity in contrast to Brazilian propolis as a function of its composition (34). Comparably, Gregoris and Stevanato reported antioxidant activity of bioactive compounds derived from propolis showed different values for lipid peroxidation using linoleic acid system and noticed higher values for caffeic acid phenyl ester as compared to other constituents (32). In a similar way, Orsolice *et al.*, examined the antioxidant status of propolis using β-carotene degradation system and depicted potential of different parameter varies greatly according to nature and concentration of solvent used for extraction as mentioned for the findings of current study (32,35,36).

In our findings, bee propolis found in Pakistan contains appreciable amount of minerals. Hydroalcoholic extracts prepared using 65% ethanol imparted highest antioxidant status as compared to other extracts due to higher phenolic contents. Therefore, there is a need of further research to analyze the constituents of HAEP and their related benefits.

Contribution of authors

Muhammad Shahbaz designed and implement the research throughout project and Tahir Zahoor supervised the whole project during studies. Ayesha Sameem, Saima Rafique, M. Javaid and Adnan Amjad involved in concept building and writing of the manuscript. Atif Liaqat, Shamas Murtaza and Umar Farooq involved in data analysis and results description of studies.

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