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, antimicrobial 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 concentration of 11.33±0.91, 52.10±2.9 and 10.53±0.8 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 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, anticance and other related activities (7). Besides this, propolis contains inorganic substances like iron, zinc, copper, magnesium and manganese and vitamins B₃, B₆, 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 effectual for the management 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 retards 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, revert 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 possesses numerous properties such as pharmacological activity, hepatoprotective, anticance 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 continuous this fact, the present study designed to explore chemical composition of locally available propolis and its extracts prepared with different concentrations of ethanol, methanol and aqueous extraction by using invitro medium to explain associated properties of propolis which is first effort of such kind in in the region in this regard.

Materials and Methods

Sample collection and preparation of extract

Raw bee propolis, collected from local apiaries, evaluated for its proximate composition and mineral contents. The weighed amount of sample continuously agitated with 95, 80, and 65% of ethanol and methanol at room temperature for 24 hours under dark conditions. The extracts filtered twice with Whatman paper # 2 and centrifuged at 3000 g for 15 mins. 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 Schimadzu, series 10 A, Japan, HPLC system equipped with C18 column (250 mm x 4.6 mm, 5.0 μm particle size). A 10μL 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 elusion at a flow rate of 1mL/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 250μL of Folin-Ciocalteu reagent with 750μL of 20% Na₂CO₃, volume was made upto 5 mL with distilled water and absorbance was noted at 765nm with UV/visible Spectrophotometer (CECIL CE7200) (22).

Free radical scavenging activity of HAEP using DPPH assay

Free radical scavenging activity of HAEP 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 520nm (23).

Antioxidant activity of HAEP using β-carotene assay

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

Results and Discussion

The results regarding proximate composition of pro-
polis 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 (2.0-2.50%), crude fiber (3.50-4.00%) and ash content (1.00-3.00%) (28). 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 11.33±0.91 mg/Kg and minimum value of potassium 3.59±0.23 mg/Kg, whilst the minimum value (0.21±0.01 mg/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 feraulic 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 7.2 mg/g and 0.9 to 27.4 mg/g in different propolis extracts (29). Mello et al., indicated the contents of feraulic 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 1. Proximate profile of locally collected honeybee propolis.

<table>
<thead>
<tr>
<th>Proximate composition (%)</th>
<th>Crude fat</th>
<th>Crude protein</th>
<th>Crude fibre</th>
<th>Ash content</th>
<th>Water content</th>
<th>Nitrogen free extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>85.59±0.87</td>
<td>1.84±0.09</td>
<td>0.31±0.08</td>
<td>1.03±0.04</td>
<td>2.22±0.14</td>
<td>9.01±0.05</td>
</tr>
</tbody>
</table>

### Table 2. Polyphenols identification and quantification of propolis extracts using HPLC.

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

Results expressed as mean of triplicates ± SD. ND= Not detected, WE: water extract EEP<sub>65</sub>: 95% ethanol extract, EEP<sub>95</sub>: 80% ethanol extract, EEP<sub>65</sub> 65% ethanol extract, MEP<sub>65</sub>: 95% methanol extract, MEP<sub>95</sub>: 80% methanol extract, MEP<sub>65</sub>: 65% methanol extract.
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 with the results of current study (29).

The presented work is a part of PhD studies of the author. The author thanks the beekeepers of Faisalabad region for propolis sample and also Higher Education Commission of Pakistan for providing financial support to conduct the current research project.

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

<table>
<thead>
<tr>
<th></th>
<th>W.E</th>
<th>EEP&lt;sub&gt;95&lt;/sub&gt;</th>
<th>EEP&lt;sub&gt;80&lt;/sub&gt;</th>
<th>EEP&lt;sub&gt;65&lt;/sub&gt;</th>
<th>MEP&lt;sub&gt;95&lt;/sub&gt;</th>
<th>MEP&lt;sub&gt;80&lt;/sub&gt;</th>
<th>MEP&lt;sub&gt;65&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC (mg/g)</td>
<td>134.02</td>
<td>239.10</td>
<td>251.71</td>
<td>327.30</td>
<td>225.88</td>
<td>246.87</td>
<td>292.10</td>
</tr>
<tr>
<td>GAE</td>
<td>±12.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>±13.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±22.43&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±14.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>±17.63&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±19.37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±15.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(% DPPH)</td>
<td>44.73</td>
<td>61.26</td>
<td>66.93</td>
<td>73.18±4.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.01±2.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60.51±3.37&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>70.06±5.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inhibition</td>
<td>±3.32&lt;sup&gt;d&lt;/sup&gt;</td>
<td>±4.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>±3.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>±4.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>±2.52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±3.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±4.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-carotene assay (%)</td>
<td>±2.83&lt;sup&gt;d&lt;/sup&gt;</td>
<td>±3.37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±2.86&lt;sup&gt;b&lt;/sup&gt;</td>
<td>±4.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>±2.52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±3.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±4.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The results described as means of triplicates ± SD. Letters indicate significant difference among treatments (p<0.05). W.E: water extract, EEP<sub>95</sub>: 95% ethanol extract, EEP<sub>80</sub>: 80% ethanol extract, EEP<sub>65</sub>: 65% ethanol extract, MEP<sub>95</sub>: 95% methanol extract, MEP<sub>80</sub>: 80% methanol extract, MEP<sub>65</sub>: 65% methanol.

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, Orsolic 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 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.

**Acknowledgements**
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