In vitro antioxidant and antimicrobial activities of propolis from Kashmir Himalaya region

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ABSTRACT

Introduction: Propolis from Kashmir has not been explored as far as its phytochemistry and pharmacological activities are concerned. This piece of work is the first approach to explore it for various activities. Methods: Total phenolics, flavonoids contents and in vitro antioxidant assay such DPPH, \( \text{H}_2\text{O}_2 \) scavenging assay, ferrous metal ion chelating activity and total reduction capability assay were performed. Investigation for antimicrobial activity of different extracts (Ethanolic, hydro-ethanolic and aqueous) was also carried out. Results: The results showed highest total phenolics and flavonoids content in ethanolic extract (260 ± 10.00 mg GAE/g and 105 ± 5.00 mg QE/g respectively). All the extracts showed free radical scavenging potential but ethanolic extract exhibited significant scavenging potential having an IC\text{50} value of 65.49 ± 7.01 µg/mL for DPPH, 74.94 ± 5.51 µg/mL for ferrous metal ion chelating activity and 109.93 ± 3.24 µg/mL for \( \text{H}_2\text{O}_2 \) scavenging assay. In total reduction capability assay ethanolic extract showed highest reducing power. In addition, ethanolic extract showed greater antibacterial activity against Staphylococcus aureus having zone of inhibition diameter 25.63 ± 0.63 mm. Conclusion: The presence of flavonoids in extracts maximum in ethanolic extract which could be responsible for antioxidant, antibacterial activities and can be used in the management of the human pathosis originated due to oxidative stress and in the treatment of microbial infections.

Keywords: In vitro antioxidant potential, Kashmiri Propolis, Staphylococcus aureus, Total phenolics content, Total Flavonoids content.

INTRODUCTION

Propolis “bee glue” is a resinous material collected from the beehive which bees obtain from their surrounding flora; it has tendency to maintain internal stability, reduce vibration, check in flow of air and prevent putrefaction in the hive. It also protects the hive from microbial growth. The history reveals that propolis has been used over centuries by Egyptians, Romans and Persians as medicine for various ailments. The good quality propolis can be found from the trees of poplar, willow, elm, birch, alder, horse-chestnut, conifer and beech. The chemical composition of propolis varies from one geographical region to the other and depends on its source of flora. Till date more than 300 compounds have been isolated and identified which include sugars, polyols, hydroxy acids, fatty acids, cardanol and cardols, anacardic acids, flavan derivatives, triterpenes, prenylated flavanones and chalcones, aromatic acids and their esters. Because of diversification in chemical composition, propolis exhibits various biological activities like hepatoprotective activity, antidiabetic activity, antiatherogenic and antiangiogenic

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activity,\textsuperscript{10} anticancer activity,\textsuperscript{11} anti inflammatory activity,\textsuperscript{12} antimicrobial activity\textsuperscript{13} and antioxidant activity.\textsuperscript{14}

The literature survey reveals that no previous studies have been reported on propolis from Kashmiri region. In the present work, it is for the first time that the propolis of Kashmir region has been evaluated for its total phenolic content, total flavonoid content, In vitro antioxidant and antimicrobial activities.

MATERIALS AND METHODS

Propolis Material

Propolis used for study was directly collected from the honeybee colonies (Apis mellifera) placed at Rangil, Ganderbal (Jammu and Kashmir). The same was identified under voucher specimen number AU/DR/NAE-II/137 from research and training center for pollinator, pollinizer and pollination management, Sher-i-Kashmir University of Agricultural Sciences & Technology of Kashmir, India.

Preparation of different extracts

Ethanolic extract (KPEt)

Finely chopped Propolis (10.0 g) was extracted with 50 mL of ethanol at room temperature with constant agitation for 24 hrs. The residue was further extracted with ethanol (50 mL x 3 times) after every 3 hrs, and then filtered and recovered under reduced pressure. The extract obtained was kept in refrigerator.

Hydro ethanolic extract (KPHa)

Finely chopped Propolis (10.0 g) was extracted with 50 mL of 50% ethanol (1:1 in water) at room temperature with constant agitation for 48 hrs. The residue was further extracted with 50% ethanol (50 mL x 3 times) after every 3 hrs, and then filtered and recovered under reduced pressure. The extract obtained was kept in refrigerator.

Aqueous extract (KPAq)

Finely chopped Propolis (10.0 g) was extracted with 50 mL of milli Q water at room temperature with constant agitation for 48 hrs, then filtered and recovered under reduced pressure and the extract obtained was kept in refrigerator.

Determination of total phenolic content

The total phenolic content in various extracts (KPEt, KPHa and KPAq) was determined by Folin-Ciocalteu method.\textsuperscript{15} Gallic acid was used as standard for constructing calibration curve (50 to 150 mg/mL).

The total phenolic content in various extracts was expressed as milligrams of gallic acid equivalent (GAE) per gram using gallic acid calibration curve equation.

\[
\text{Absorbance (\lambda 725)} = 0.001 \times [\text{GAE}] + 0.016
\]

Determination of total flavonoid content

The total flavonoid content in various extracts was determined by aluminium chloride colorimetric method.\textsuperscript{16} Quercetin was used as standard for constructing calibration curve (50 to 150 mg/mL).

The total flavonoid content in various extracts was expressed as milligrams of quercetin equivalent (QE) per gram using quercetin calibration curve equation.
Antioxidant Activities
The antioxidant assays of the various extracts of propolis were investigated by DPPH assay, Total Reduction Capability assay, Ferrous Metal Ion Chelating Activity and Hydrogen peroxide scavenging assay. All these assays were performed in triplicate and mean values were taken.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity
The free radical scavenging activity was investigated by the method.\(^{17}\)

Total Reduction Capability assay

Table 1: Total phenolics and flavonoids content of various extracts of Kashmir Propolis

<table>
<thead>
<tr>
<th>Extract</th>
<th>mg GAE/g(^a)</th>
<th>mg QE/g(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPEt</td>
<td>260 ± 20.00</td>
<td>105 ± 5.00</td>
</tr>
<tr>
<td>KPHa</td>
<td>200 ± 20.00</td>
<td>86 ± 6.63</td>
</tr>
<tr>
<td>KPAq</td>
<td>180 ± 10.00</td>
<td>45 ± 5.00</td>
</tr>
</tbody>
</table>

\(^a\)Total phenolics content is expressed in terms of gallic acid equivalent (µg of GA/g).  
\(^b\)Total flavonoids content is expressed in terms of quercetin equivalent (µg of QE/g).

\[Absorbance (\lambda_{415}) = 0.002 \times [QE] + 0.059\]

The total reduction capability of the various extracts was investigated by the method.\(^{18}\)

Ferrous Metal Ion Chelating Activity
The ferrous ion chelating potential of various extracts was investigated by the method.\(^{19}\)

Hydrogen peroxide scavenging assay
The capacities of the various extracts to scavenge hydrogen peroxide were investigated according to the method\(^{20}\) with some modifications.

Microbiological Assay
Investigation for antibacterial activity of different solvent extracts was carried out on *Staphylococcus aureus* (MTCC 11949), *Escherichia coli* (MTCC 10312), *Proteus vulgaris* (MTCC 7299), *Bacillus cereus* (MTCC 10650), *Bacillus subtilis* (MTCC 11554) and *Pseudomonas aeruginosa* (MTCC 10636) according to National Committee for Clinical Laboratory Standards (NCCLS) using Muller Hinton agar as medium.\(^{21}\) All the assays were performed in triplicate and mean values were taken.

Table 2: Antioxidant potential (IC\(_{50}\)) of various extracts of Kashmir Propolis

<table>
<thead>
<tr>
<th>Extract / Reference samples</th>
<th>DPPH* scavenging (µg/mL)</th>
<th>Fe2+ Ion Chelating (µg/mL)</th>
<th>H(_2)O(_2) Scavenging (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC(_{50})</td>
<td>R(^2)</td>
<td>IC(_{50})</td>
</tr>
<tr>
<td>BHA</td>
<td>65.47 ± 4.76</td>
<td>0.976</td>
<td>-</td>
</tr>
<tr>
<td>EDTA</td>
<td>-</td>
<td>-</td>
<td>34.08 ± 2.53</td>
</tr>
<tr>
<td>KPEt</td>
<td>76.15 ± 7.01</td>
<td>0.979</td>
<td>74.94 ± 5.51</td>
</tr>
<tr>
<td>KPHa</td>
<td>93.51 ± 6.21</td>
<td>0.970</td>
<td>136.88 ± 7.41</td>
</tr>
<tr>
<td>KPAq</td>
<td>102.17 ± 9.15</td>
<td>0.975</td>
<td>114.39 ± 9.34</td>
</tr>
</tbody>
</table>

Each value were presented as the mean ±S.E.M. (n=3).  
\(^{a}\)The values given in the above table were expressed as µg/ml.  
\(^{b}\)EDTA was only used as reference sample in Ferrous metal ion chelating activity.
Table 3: Antibacterial capacity of various extracts of Kashmir Propolis using agar disc diffusion variant method

<table>
<thead>
<tr>
<th>Quantity of extract (µg/disc)</th>
<th>Zone of inhibition diameter (mm)</th>
<th>Bacterial Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S.aureus</td>
<td>B. subtilis</td>
</tr>
<tr>
<td>KPEt 50</td>
<td>18.06 ± 0.90</td>
<td>13.26 ± 0.46</td>
</tr>
<tr>
<td>100</td>
<td>21.33 ± 0.57</td>
<td>16.83 ± 0.76</td>
</tr>
<tr>
<td>150</td>
<td>25.63 ± 0.63</td>
<td>23.33 ± 0.57</td>
</tr>
<tr>
<td>KPHa 50</td>
<td>12.46 ± 0.50</td>
<td>8.66 ± 0.57</td>
</tr>
<tr>
<td>100</td>
<td>16.66 ± 0.57</td>
<td>14.70 ± 0.60</td>
</tr>
<tr>
<td>150</td>
<td>18.63 ± 0.63</td>
<td>16.90 ± 0.85</td>
</tr>
<tr>
<td>KPAq 50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>11.96 ± 0.95</td>
<td>10.63 ± 0.63</td>
</tr>
<tr>
<td>150</td>
<td>13.33 ± 0.57</td>
<td>14.36 ± 0.55</td>
</tr>
<tr>
<td>Streptomycin 10</td>
<td>29.66 ± 0.57</td>
<td>29.66 ± 0.57</td>
</tr>
</tbody>
</table>

Results in the table are expressed in millimeter (mm) and each value is represented as mean ± S.E.M.; – represents no zone of inhibition; *Aerobic bacteria; # Anaerobic bacteria

RESULTS

Table 1 depicts the total phenolic content present in KPEt, KPHa and KPAq extracts of propolis. The concentration of phenolic moieties in various extracts was expressed in mg GAE/g; KPEt was found to contain 260 mg GAE/g followed by KPHa with 200 mg GAE/g and KPAq with 180 mg GAE/g. The flavonoids content was expressed in mg QE/g; the result revealed that KPEt, KPHa and KPAq contained 105 ± 5.00 mg QE/g, 86 ± 6.63 QE/g and 45 ± 5.00 mg QE/g respectively Table 1. The antioxidant potential of the various extracts of propolis was investigated by their ability to scavenge the stable free radicals and defend against damage caused by oxidizing agents. The results were compared with the scavenging capability of reference samples of butylated hydroxyanisole (BHA) and ethylene-diamine-tetra-acetic acid (EDTA). In this assay the free radical scavenging activities are shown in Table 2. The values obtained for KPEt, KPHa and KPAq were 76.15 ± 7.95 µg/ml (R²: 0.987), 93.51 ± 6.21 µg/ml (R²: 0.970) and 102.17 ± 9.15 µg/ml (R²: 0.955) respectively where as for reference sample (BHA) the IC₅₀ value was found to be 65.497.01 µg/ml (R²: 0.979). In the Figure 1 shows the total reduction capability of KPEt, KPHa and KPAq. With increase in the concentration of the extracts there was increase in the absorbance which indicated greater reducing potential. Therefore, the activity of the extracts and reference sample followed the order BHA > KPEt > KPHa > KPAq. The IC₅₀ values of propolis extracts were found to be 74.94 ± 5.51 µg/ml (R²: 0.973), 136.88 ± 7.41 µg/ml (R²: 0.948) and 114.39 ± 9.34 µg/ml (R²: 0.997) for ethanolic extract, hydro ethanolic extract and aqueous extract respectively, where as the IC₅₀ value for the reference sample EDTA was 34.08 ± 2.53 µg/ml (R²: 0.978). Hydrogen peroxide scavenging potential of different extracts is shown in Table 2. The IC₅₀ values calculated for different extracts were 109.92 µg/ml (R²: 0.995) for KPEt, 126.69 ± 4.12 µg/ml (R²: 0.982) for KPHa and 145.42 ± 2.14 µg/ml (R²: 0.973) for KPAq, where as the IC₀ value of the reference sample BHA was 88.47 ± 12.96 µg/ml (R²: 0.988). As depicted in Table 3, all the three extracts inhibited the growth of the microbes against both Gram-positive bacteria (Staphylococcus aureus, Bacillus subtilis and Bacillus cereus) and Gram-negative bacteria (Escherichia coli, Proteus vulgaris and Pseudomonas aeruginosa) to some extent.

DISCUSSION

It has been found that all extracts contains mixture of phytocompounds, predominantly flavonoids, thus establishing that the in vitro antioxidant potential and antimicrobial activity as exhibited by KPEt due to maximum flavonoids are due to the cumulative effect of the different compounds in the extract.

The phenolic compounds are found extensively in kingdom flora; the recent research revealed phenolic compounds have prospective protective role against oxidative diseases. The phenolics, in nature, have ability to donate hydrogen and form a stable radical intermediate. The preponderance moieties present in the propolis reported till date is Polyphenols.

In the total phenolics content assay there is transport of
electrons in basic medium from the phenolic moieties and the reducing group to molybdenum which forms blue colored coordination complexes between metal and the phenolic moieties present in the extract. It has been reported that flavonoids reveal their measures on membrane permeability and inhibition of membrane bound enzymes like ATPase and phospholipase A2.

In summary the results revealed that the phenolics and flavonoids content in KPAq were least. The phenolics content and flavonoids content was highest in the KPEt of propolis from Kashmir region.

In DPPH analysis, the purple color of DPPH solution is reduced to pale yellow color because of donation of hydrogen atom to reducing moieties. DPPH radical is reduced by interaction with reducing moieties present in the extract, that causing decreasing absorbance at 517 nm. The result suggests that scavenging potential of the KPEt is highest followed by KPHa and least for KPAq. The utmost scavenging potential of the KPEt was perhaps due to the presence of rich phenolic and flavonoids content as revealed by the tests performed earlier. The total reduction capability assay may provide an important sign of antioxidant capacity. In total reduction capability assay reductive capacity of ferric (Fe³⁺)/ferricyanide complex to ferrous (Fe²⁺) in the extracts was due to transfer of an electron which is an important mark for its antioxidant activity. With increase in the concentration of the extracts there was increase in the absorbance which indicated greater reducing potential. The results established that all the extracts had reducing capacity and significant electron transferring properties to convert reactive free radicals into stable products. In ferrous metal ion chelating activity ferrozine forms a complex by forming chelates with Fe²⁺ which gives pinkish red color. But in the presence of other chelating agents the reaction is limited that interrupts formation of ferrozine–Fe²⁺ complexes which in turn results in decrease in the color intensity via Fenton type reactions. Chelating agents act as secondary antioxidants as they reduce the reduction potential and result in maintaining the oxidized form of the metal ion. These results exhibited that the KPEt possesses the stronger chelating power then all the extracts. Hydrogen peroxide is the byproduct of oxygen metabolism produced in vivo by numerous oxidizing enzymes known as peroxidases. When the concentration of hydrogen peroxide rises in excess, it could cause tissue destruction and cell demise. Therefore elimination of the-OH is very essential for shielding the in vivo system. In Hydrogen peroxide scavenging assay absorbance is deceased due to utilization of H₂O₂ by the antioxidant moieties present in the extracts. The results expressed that IC₅₀ value was least in KPEt followed by KPHa and KPAq; therefore KPEt had highest hydrogen peroxide scavenging ability and KPAq had the least. It has been reported that about 25% of the recent medicines used in the management of diseases are derived from nature. The KPEt was found to possess significant antibacterial activity than the other two extracts. The reason for highest microbial activity in the KPEt may be due to the maximum presence of flavonoids and phenolics; this could exert antagonistic effects on microbes. Other reasons might be that the maximum secondary metabolites from the nature show good solubility in the organic solvents; therefore ethanolic extract shows good microbial activity followed by KPHa and KPAq. In present study, the antibacterial screening revealed that the KPEt of propolis is more potent against gram positive bacteria than gram negative bacteria because it has been reported that gram negative bacteria are more resistant than gram positive bacteria.

**CONCLUSION**

This piece of work is the first approach to research propolis from Kashmir Himalaya region. The Propolis from Kashmir contains a good quantity of phenolics and flavonoids. Besides, it was also observed that KPEt had good antioxidant capacity as revealed by various in vitro tests. Moreover, the KPEt exhibited itself as a good antimicrobial representative against gram positive bacteria than gram negative bacteria. In conclusion, the presence of rich quantity of phenols and flavonoids in the KPEt, which is responsible for antioxidant and antibacterial activities, Kashmir propolis is having encouraging pharmacological activities.

Our group is further investigating isolation and identification of bioactive compounds from propolis that are responsible for the antioxidant potential, antibacterial potential and other pharmacological properties of propolis.

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**CONFLICT OF INTERST**

We declare that we have no conflict of interest.
Highlights of the paper

- Kashmiri propolis is rich source of phenolic acid and flavonoids content.
- Prominent free radical scavenging potential was observed in KPEt.
- KPEt showed greater antibacterial activity against Staphylococcus aureus.

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