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, H_2O_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_{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 H_2O_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.[1] It also protects the hive from microbial growth.[2] The history reveals that propolis has been used over centuries by Egyptians, Romans and Persians as medicine for various ailments.[3] The good quality propolis can be found from the trees of poplar, willow, elm, birch, alder, horse-chestnut, conifer and beech.[4] The chemical composition of propolis varies from one geographical region to the other and depends on its source of flora.[5] Till date more than 300 compounds have been isolated and identified which include sugars, polyols, hydroxy acids, fatty acids, cardanols and cardols, anacardic acids, flavan derivatives, triterpenes, prenylated flavanones and chalcones, aromatic acids and their esters.[6,7] Because of diversification in chemical composition, propolis exhibits various biological activities like hepatoprotective activity,[8] antidiabetic activity,[9] antiatherogenic and antiangiogenic

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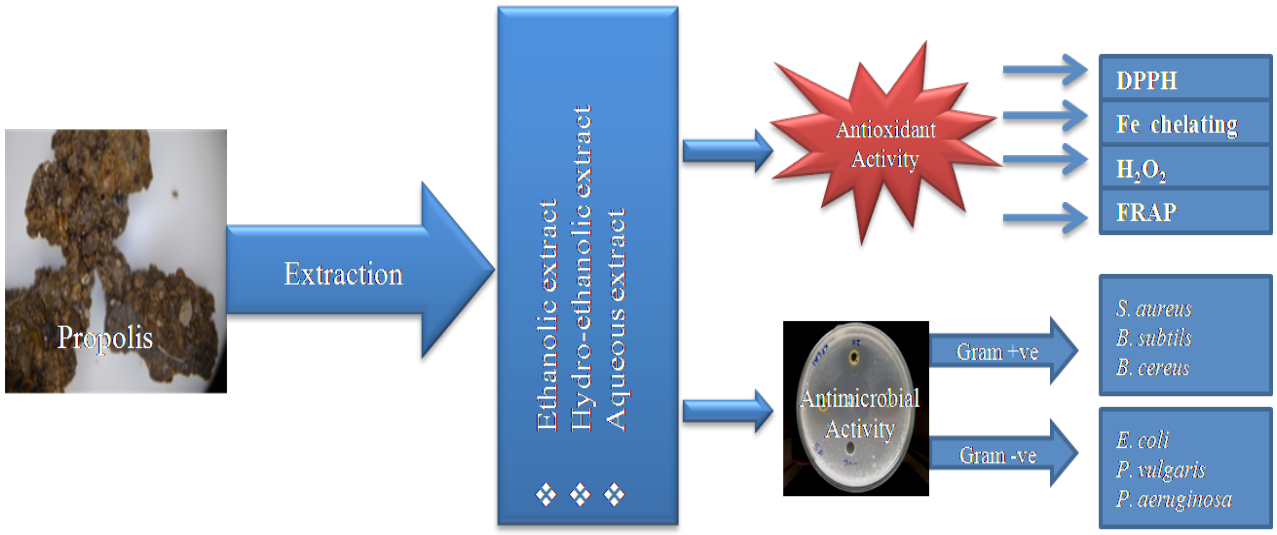

Graphical Abstract

activity,[10] anticancer activity,[11] anti inflammatory activity,[12] antimicrobial activity[13] and antioxidant activity.[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.[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.

$$Absorbance\ (\lambda 725) = 0.001 \times [GAE] + 0.016$$

Determination of total flavonoid content

The total flavonoid content in various extracts was determined by aluminium chloride colorimetric method.[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.

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

Extract	mg GAE/g[a]	mg QE /g[b]
KPEt	260 ± 20.00	105 ± 5.00
KPHa	200 ± 20.00	86 ± 6.63
KPAq	180 ± 10.00	45 ± 5.00

[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)

$$Absorbance\ (\lambda 415) = 0.002 \times [QE] + 0.059$$

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

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.

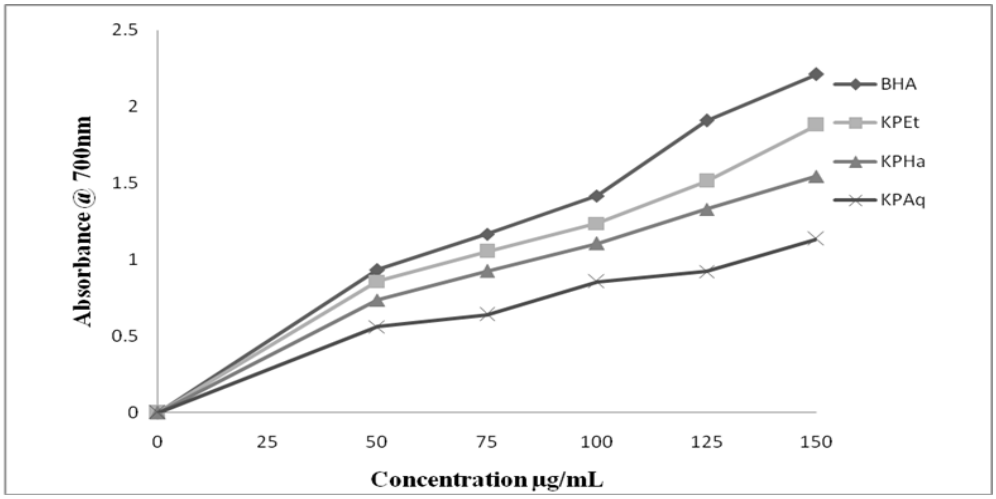

Figure 1: Total reduction capability assay of various extracts of Kashmiri Propolis

Table 2: Antioxidant potential (IC_{50}) of various extracts of Kashmir Propolis

Extract / Reference samples	DPPH* scavenging		Fe2+ Ion Chelating		H_2O_2 Scavenging	
	IC_{50} (µg/mL)	R^2	IC_{50} (µg/mL)	R^2	IC_{50} (µg/mL)	R^2
BHA	65.47 ± 4.76	0.976	-	-	88.47 ± 12.96	0.988
EDTA	-	-	34.08 ± 2.53	0.978	-	-
KPEt	76.15 ± 7.01	0.979	74.94 ± 5.51	0.997	109.93 ± 3.24	0.995
KPHa	93.51 ± 6.21	0.970	136.88 ± 7.41	0.973	126.93 ± 4.12	0.982
KPAq	102.17 ± 9.15	0.955	114.39 ± 9.34	0.948	145.42 ± 2.14	0.973

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

Quantity of extract (µg/disc)	Zone of inhibition diameter (mm)					
	Bacterial Strains					
	*S.aureus	*B. subtills	#P. aeruginosa	#Bacillus cereus	#P. vulgaris	*E.coli
KPEt						
50	18.06 ± 0.90	13.26 ± 0.46	11.69 ± 0.52	10.33 ± 0.60	-	-
100	21.33 ± 0.57	16.83 ± 0.76	17.36 ± 0.62	16.66 ± 0.57	10.28 ± 0.62	-
150	25.63 ± 0.63	23.33 ± 0.57	19.30 ± 0.60	20.40 ± 0.55	16.90 ± 0.17	9.46 ± 0.50
KPHa						
50	12.46 ± 0.50	8.66 ± 0.57	7.63 ± 0.63	-	-	-
100	16.66 ± 0.57	14.70 ± 0.60	14.05 ± 0.91	10.73 ± 0.64	-	-
150	18.63 ± 0.63	16.90 ± 0.85	16.70 ± 0.51	13.46 ± 0.50	10.76 ± 0.87	-
KPAq						
50	-	-	-	-	-	-
100	11.96 ± 0.95	10.63 ± 0.63	-	-	-	-
150	15.33 ± 0.57	14.36 ± 0.55	13.83 ± 0.76	7.92 ± 0.12	8.76 ± 0.68	-
Streptomycin						
10	29.66 ± 0.57	29.66 ± 0.57	25.30 ± 0.51	24.67 ± 0.59	19.65 ± 0.25	14.88 ± 0.29

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 *KPH*a 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, IC_{50} values obtained for *KPEt*, *KPHa* and *KPAq* were 76.15 ± 7.95 µg/ml (R^2: 0.987), 93.51 ± 6.21 µg/ml (R^2: 0.970) and 102.17 ± 9.15 µg/ml (R^2: 0.955) respectively where as for reference sample (BHA) the IC_{50} value was found to be 65.497.01 µg/ml (R^2: 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 IC_{50} values of propolis extracts were found to be 74.94 ± 5.51 µg/ml (R^2: 0.973), 136.88 ± 7.41 µg/ml (R^2: 0.948) and 114.39 ± 9.34 µg/ml (R^2: 0.997) for ethanolic extract, hydro ethanolic extract and aqueous extract respectively, where as the IC_{50} value for the reference sample EDTA was 34.08 ± 2.53 µg/ml (R^2: 0.978). Hydrogen peroxide scavenging potential of different extracts is shown in Table 2. The IC_{50} values calculated for different extracts were 109.92 µg/ml (R^2: 0.995) for *KPEt*, 126.69 ± 4.12 µg/ml (R^2: 0.982) for *KPHa* and 145.42 ± 2.14 µg/ml (R^2: 0.973) for *KPAq*, where as the IC_50 value of the reference sample BHA was 88.47 ± 12.96 µg/ml (R^2:0.988). 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.[22] It has been reported that flavonoids reveal their measures on membrane permeability and inhibition of membrane bound enzymes like ATPase and phospholipase A2.[23]

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.[24] 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 (Fe^{3+})/ferricyanide complex to ferrous (Fe^{2+}) 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.[25] 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 Fe^{2+} which gives pinkish red color. But in the presence of other chelating agents the reaction is limited that interrupts formation of ferrozine–Fe^{2+} 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.[26] 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.[27] In Hydrogen peroxide scavenging assay absorbance is deceased due to utilization of H_2O_2 by the antioxidant moieties present in the extracts. The results expressed that IC_{50} 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.[28] 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.[29] 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*.[30] 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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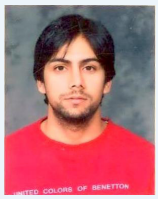

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