Antioxidant Activity of Indian Propolis - An *In Vitro* Evaluation

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Abstract. Antioxidants from the natural products are essential to prevent the progression of free radical mediated diseases. In the present study, ethanol extract of propolis collected from different geographical origin were evaluated for their free radical scavenging potential by employing different in-vitro assays such as DPPH, ABTS, Nitric oxide and Hydrogen peroxide. All the tested samples contained considerable amount of total phenols and vitamin C content. In the entire assay, the percentage of inhibition increased with the increase in concentration. Among the propolis samples collected, the highest activity was found in Tamil Nadu, Kerala, Karnataka and Haryana. The difference in the antioxidant activity level was obtained from the assay may reflect a relative difference in the ability of antioxidant compounds to scavenge different free radicals in the extract. Phenols and vitamin C are the major contributors to antioxidant activity in propolis. The propolis from these locations may be of considerable interest in preventing the ill effects of excessive free radical generation in the human body.

Abbreviations: IC₅₀ - half maximal inhibitory concentration; DPPH - 2,2-diphenyl-1-picrylhydrazyl, ABTS - 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); H₂O₂- Hydrogen peroxide; NO – Nitric Oxide

Introduction

Propolis is a resinous substance collected by honeybees from various plant sources such as leaf, buds, twigs, tree barks and exudates and combine with salivary secretions and wax. The main role is to construct, repair and protect their hive against infection [1]. Since propolis is a complex mixture of plant derived compounds, it generally composed of 50% resin and balsams, 30% wax, 10% essential and aromatic oil, 5% pollen and 5% various organic compounds such as aliphatic acids, aromatic acids, fatty acids, carbohydrates, amino acids, ketones, vitamins etc [2]. Worldwide propolis has a tremendous popularity and has become a subject of interest concerning the chemistry and biological activities. On the other hand, the variation in the chemistry of propolis from different ecosystems has made it a source of active molecules mainly as antimicrobial [3], anti-inflammatory [4], antioxidant [5], hepatoprotective [6], immunostimulating [7] and cytotoxic [8, 9]. Propolis contains more than 300 chemical constituents in which polyphenol and flavonoids play a role in the pharmacological activities. Several scientists revealed the potentiality of propolis in the development of new drug and used as a constituent in pharmacognosy, cosmetics, dietary supplement and veterinary medicines [10].

The developing countries use propolis as raw materials in many industries especially in food and health care products. The Indian propolis has different chemical composition due to varied climate and rich plant biodiversity and known for its high medicinal value [11]. The chemical composition of propolis and its importance in health care in India has not been understood except few preliminary investigations. Hence, in the present study, DPPH, ABTS, Nitric oxide and Hydrogen peroxide scavenging assays were employed to assess the radical scavenging potential of ethanol extract of propolis collected from 10 different locations of India. The antioxidant activity of the propolis extracts were compared with phenols (gallic acid) and vitamin C (ascorbic acid) which were used as reference samples.

Materials and Methods

The propolis samples were collected from 10 different Indian states like Haryana, Himachal Pradesh, Uttaranchal, Punjab, Delhi, Maharashtra, Tamil Nadu, Karnataka, Kerala and Andhra Pradesh by scrapping the frames of *Apis mellifera* bee hives. Raw propolis samples were stored in cool place for further investigations.

Preparation of ethanol extract of propolis

The ethanol extract of propolis was prepared by crushing 10 gm of propolis into small pieces and extracted with 100 ml of 70% ethanol and left overnight followed by intermittent shaking at room temperature. The suspension obtained was filtered and the extraction was repeated twice. The extracts were combined and evaporated to dryness under pressure by using rotor evaporator and the dried extract was weighed to determine the yield of soluble components. The resulting extract was kept in refrigerator at 4°C to assess the antioxidant potential through various assays [12].

Determination of vitamin C

The estimation of vitamin C was carried out by a colorimetric method [13]. To 1 ml of propolis extract, 2.5 ml of oxalic acid (4%), 0.5 ml of sulphuric acid (5%), 2 ml of ammonium molybdate and 3 ml of distilled water was added, the extract was incubated in water bath with the temperature maintained at 60°C. The resulting solution was cooled and measured at 515nm. The calibration curve was plotted using a freshly prepared solution of ascorbic acid.

Determination of total phenols

The procedure used was based on the Folin-Ciocalteu method [14]. A calibration curve was plotted using standard aqueous solutions of gallic acid. One millilitre of sample extract was added to 500 µl of sodium carbonate and 250 µl of the Folin–Ciocalteu reagent in a test tube, homogenized and allowed to react for 30 minutes at a room temperature and absorbance was measured at 710nm on a spectrophotometer.

Determination of Antioxidant Activities

DPPH free radical-scavenging activity

Antioxidant activity of ethanol extract of propolis samples were estimated by using 1-1-diphenyl-2-picrylhydrazyl [15]. One millilitre of various concentrations of propolis extracts were added to 3 ml of 60 mM methanolic solution of DPPH. Absorbance measurements were read at 517 nm after 30 min of incubation time at room temperature. Absorbance of a blank sample containing the same amount of methanol and DPPH solution acted as the negative control. The percentage inhibition was calculated against the gallic acid (phenol) and ascorbic acid (vitamin C) content and IC₅₀ values were determined.

ABTS free radical-scavenging activity

The determination of ABTS radical scavenging activity was carried out by the reaction of (7 mM) ABTS aqueous solution with potassium persulphate (2.45 mM) in the dark for 16 hrs adjusting the absorbance at 734 nm to 0.700 at room temperature. The ABTS (2 ml) was added to 1 ml different aliquots of ethanol extract of propolis samples (100,200,300,400,500µg/ml), the absorbance at 734 nm was read after 30 min of incubation. Several concentrations were measured, and the percentage inhibition was plotted against gallic acid and ascorbic acid content and IC₅₀ values were determined [16]

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging potential was determined by adding 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture incubated at 25 °C for 150 min. From the incubated mixture 0.5 ml was taken out and 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) was added and incubated at room temperature for 5 min. Finally, 1.0 ml Naphthylethylenediamine dihydrochloride

(0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance at 540 nm was measured with a spectrophotometer. The percentage and IC₅₀ values of nitric oxide radicals scavenging activity was calculated [17].

Hydrogen peroxide scavenging activity

The ability of the propolis extracts to scavenge hydrogen peroxide was determined by preparing solution of hydrogen peroxide (40 mM) in phosphate buffer (pH 7.4) and 2 ml of various concentrations of propolis extract was mixed with 1 ml of hydrogen peroxide solution (10 mM) and incubated at 37°C for 10 minutes [18]. The absorbance of reaction mixture was measured at 260 nm and percentage of hydrogen peroxide scavenged from propolis extract was calculated.

The percentage of inhibition of these radicals at the concentration of 500µg/ml were calculated according to the formula- % inhibition = (Ac-At)/Ac) x100

At and Ac is the respective absorbance of tested samples and control. The IC₅₀ value (The half maximal inhibitory concentration) was calculated by linear regression analysis.

Statistical Analysis

All the analysis was performed in triplicate for each sample and at each concentration and the data has been statistically analyzed by using SPSS and Mega state software.

Result and Discussion

The percent yield of extractable compounds in ethanol extract of selected Indian propolis is presented in Table 1. The ethanol extract of propolis from Himachal Pradesh, Kerala and Tamil Nadu recorded the maximum yield of 13.75%, 12.80% and 12.34% respectively followed by Maharashtra (11.50%), Karnataka (10.90%), Punjab (10.60%), Haryana (10.20%), Uttaranchal (9.84%), Delhi (5.90%) and Andhra Pradesh (3.95%).

The estimation of vitamin C and total phenols is important since it is reflected in antioxidant activity. It is implicated in the prevention of some oxidative stress related diseases and is an essential principal component responsible for antioxidant activity [19]. Almost all the samples indicated considerable amount of vitamin C that ranged between minimum 16.80±4.15µg/ml (Delhi) to maximum 28.53 ± 3.21µg/ml (Uttaranchal) in terms of ascorbic acid equivalent (AAE).

The analyzed samples also recorded significantly higher amount of total phenol that varied from 8.25±3.85µg/ml from Delhi sample to 15.56 ± 2.34µg/ml from Haryana sample, in terms of gallic acid equivalent (GAE). The phenolic compounds present in the natural products have higher antioxidant activities than those of synthetic antioxidant [20]. The quantitative assessment of vitamin and total phenol content of Indian propolis has been summarized Table 1. The variation in chemical contents of propolis is mainly attributed to the difference in plants collected by honey bees [21].

Table 1: Yield percent, vitamin C and total phenol content in ethanol extract of propolis

Sl. No.	Place of collection	Yield (%)	Vitamin C (µg/ml)	Phenols (µg/ml)
1	Haryana	10.20	21.20 ± 4.06	15.56 ± 2.34
2	Himachal Pradesh	13.75	27.20 ± 6.04	11.83 ± 2.48
3	Uttaranchal	9.84	28.53 ± 3.21	11.08 ± 2.27
4	Punjab	10.60	22.10 ± 7.23	14.83 ± 1.26
5	Delhi	5.90	16.80 ± 4.15	8.25 ± 3.85
6	Maharashtra	11.50	25.85 ± 5.80	12.44 ± 1.56
7	Tamil Nadu	12.34	27.22 ± 3.70	13.50 ± 2.03
8	Karnataka	10.90	21.22 ± 3.99	9.55 ± 2.03
9	Kerala	12.80	18.80 ± 5.75	9.75 ± 0.90
10	Andhra Pradesh	3.95	21.00 ± 2.18	10.17 ± 1.89

A comparison of percentage of inhibition in antioxidant activity of ethanol extract of propolis samples from various locations of India was evaluated with gallic acid (phenol) and ascorbic acid (vitamin C) as standards for scavenging DPPH, ABTS, NO and H₂O₂ free radicals are presented in Table 2 and Figure 1.

Table 2: IC₅₀ value of ethanol extract of propolis samples with ascorbic acid (vitamin C) and gallic acid (phenol)

Propolis samples and standards	DPPH IC ₅₀ ($\mu\text{g/ml}$)	ABTS IC ₅₀ ($\mu\text{g/ml}$)	NO IC ₅₀ ($\mu\text{g/ml}$)	H ₂ O ₂ IC ₅₀ ($\mu\text{g/ml}$)
Haryana	339.40	313.22	615.43	691.78
Himachal Pradesh	478.02	498.98	536.19	765.75
Uttaranchal	370.60	839.22	620.57	740.15
Punjab	462.51	860.32	708.71	565.80
Delhi	516.51	808.66	757.75	721.20
Maharashtra	451.21	525.23	455.17	706.75
Tamil Nadu	333.48	298.86	415.32	325.30
Karnataka	368.38	478.98	450.60	487.07
Kerala	350.48	463.68	582.71	453.87
Andhra Pradesh	600.88	694.44	657.99	585.64
Gallic acid	192.20	298.03	276.67	307.87
Ascorbic acid	284.92	345.25	252.60	348.95

Figure 1: Comparison of antioxidant activity of propolis with DPPH, ABTS, NO and H₂O₂ assay with gallic acid (phenol) and ascorbic acid (vitamin C)

Free radical scavenging properties of propolis extracts was measured using DPPH, which is frequently used for the evaluation of the ant oxidation potential of various sample extracts [22]. The DPPH radical is a stable organic free radical in methanolic solution with absorption maxima at 517 nm. It loses this optimal absorption when accepting an electron, resulting in colour variation from purple to yellow. The degree of discoloration indicates the scavenging potential of antioxidant compounds [23]. According to the result (Table 2), all the samples showed free radical scavenging activity less than the standard. The sample collected from Tamil Nadu (333.48 $\mu\text{g/ml}$), Haryana (339.40 $\mu\text{g/ml}$), Kerala (350.48 $\mu\text{g/ml}$), Karnataka (368.38 $\mu\text{g/ml}$) and Uttaranchal (370.60 $\mu\text{g/ml}$) revealed the highest radical scavenging potential (IC₅₀). The samples from Maharashtra

(451.21 $\mu\text{g/ml}$), Punjab (462.51 $\mu\text{g/ml}$) and Himachal Pradesh (478.02 $\mu\text{g/ml}$) had moderate and the samples from Delhi (516.51 $\mu\text{g/ml}$) and Andhra Pradesh (600.88 $\mu\text{g/ml}$) showed lower antioxidant activity.

ABTS assay is based on the inhibition of the absorbance of the radical cation. ABTS is converted to its radical cation by addition of potassium persulfate and absorbs light at 734nm. During the reaction, blue coloured ABTS radical cation change to colourless when the free radicals were scavenged by antioxidant [24]. The ethanol extracts of propolis samples collected from Tamil Nadu, Haryana, Kerala showed higher ABTS radical scavenging activity with the IC value 298.86 $\mu\text{g/ml}$, 313.22 $\mu\text{g/ml}$ and 463.68 $\mu\text{g/ml}$ respectively followed by Karnataka (478.98 $\mu\text{g/ml}$), Himachal Pradesh (498.98 $\mu\text{g/ml}$), Maharashtra (525.23 $\mu\text{g/ml}$), Andhra Pradesh (694.44 $\mu\text{g/ml}$), Delhi (808.66 $\mu\text{g/ml}$), Uttaranchal (839.22 $\mu\text{g/ml}$) and Punjab (860.32 $\mu\text{g/ml}$) samples exhibited good antioxidant activity.

The nitric oxide or reactive nitrogen species generated during their reaction with oxygen to produce nitrite ions are responsible for altering the structural and function behaviour of many cellular components. The antioxidant component in the extract competes with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. According to the result (Table 2) the sample collected from Tamil Nadu and Karnataka region recorded greater antioxidant activity (415.32 $\mu\text{g/ml}$ and 450.60 $\mu\text{g/ml}$) whereas, Andhra Pradesh, Punjab and Delhi samples (657.99 $\mu\text{g/ml}$, 708.71 $\mu\text{g/ml}$ and 757.75 $\mu\text{g/ml}$) had lower antioxidant activity.

Hydrogen peroxide is a weak oxidizing and reducing agent and can inactivate few enzymes directly by oxidation of essential thiol (-SH) groups. H_2O_2 probably reacts with ferric and copper ions to form hydroxyl radical which may be the origin of many of its toxic effects [25]. It is therefore hydroxyl radical scavenging activity of natural extracts was measured. The scavenging ability of ethanol extract of propolis samples (IC₅₀) were evaluated and compared with standards ascorbic acid and gallic acid (Table 2). The samples collected from Tamil Nadu and Kerala region had greater antioxidant activity with 325.30 $\mu\text{g/ml}$ and 453.87 $\mu\text{g/ml}$ IC₅₀ value respectively. On the other hand, the moderate Nitric oxide scavenging activities were recorded from the propolis extract collected from Karnataka, Punjab, Andhra Pradesh, Maharashtra, Haryana and minimum scavenging activity was recorded in Uttaranchal (740.15 $\mu\text{g/ml}$) and Himachal Pradesh (765.75 $\mu\text{g/ml}$).

The percentage of inhibition at 500 $\mu\text{g/ml}$ concentration of ethanol extract of propolis samples collected from ten different geographical regions of India and compared with the standards ascorbic acid (vitamin C) and gallic acid (phenol) were calculated and plotted in Fig. 1. The concentration of the sample necessary to decrease initial concentration (IC₅₀) of DPPH, ABTS, NO and H_2O_2 radicals under the experimental condition was calculated. Lower the value of IC₅₀ indicates higher antioxidant activity, whereas, the extracts were found to have less antioxidant activity than the standards. The content of active compounds present in the ethanolic extract of propolis might have contributed towards the scavenging of free radicals. In the entire assay, the percentage of inhibition increased with the increase in concentration. The DPPH, ABTS, NO and H_2O_2 assays have indicated a comparable result for the antioxidant activity of collected propolis samples. The highest activity was found in Tamil Nadu, Kerala, Karnataka and Haryana propolis samples. The difference in the antioxidant activity level was obtained from the assay may reflect a relative difference in the ability of antioxidant compounds to scavenge different free radicals in the extract. Phenolics and vitamin C are the major contributors to antioxidant activity in propolis. The propolis from these locations may be of considerable interest can be used in preventing the ill effects of excessive free radical generation in the human body.

Conclusion

The present investigation indicates that the ethanol extracts of propolis were evaluated by in-vitro assays like DPPH, ABTS, Nitric oxide and Hydrogen peroxide. The tested propolis samples contained considerable amount of total phenols and vitamin C content. Phenols and vitamin C are the major contributors to antioxidant activity in propolis. The propolis from different geographic regions would be of considerable interest for drug research and development.

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