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ORIGINAL RESEARCH ARTICLE

Antioxidant properties of Indian propolis

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Summary

Antioxidant properties of total ethanolic extract of Indian propolis were investigated in this study, and the antioxidant activity of hexane soluble portion, ethyl acetate soluble portion and ethanol soluble portion of the crude propolis were also determind. Although the alcoholic extract (TEEP) showed moderate antioxidant activity, it was found that ethyl acetate soluble fraction of propolis (EAFP) exhibited superior antioxidant activity to the activity of trolox at the same concentration. Also it showed the maximum DPPH scavenging activity (81%) and $NO^{\cdot}$ radical scavenging activity (60%) as compared to the other formulations which were higher. The ethyl acetate soluble fraction was found to exhibit strongest trolox equivalent antioxidant capacity and other free radical scavenging activities. From IC_{50} values it was deduced that antioxidant compounds were located in the ethyl acetate soluble fraction. Thus Indian propolis was shown to act as a natural antioxidant; these properties may make it useful for pharmaceutical industries and as a food supplement.

Keywords: Indian propolis, fractionation of propolis, antioxidant activity, trolox equivalent antioxidant activity, lipid peroxidation activity, free radical scavenging.

Introduction

Propolis (CAS No.9009-62-5), also referred as bee glue, is a sticky dark coloured complex mixture of compounds. These compounds are collected by honeybees from the surrounding flora (Garcia-Viguera *et al.*, 1992; Marcucci *et al.*, 1995). It is applied to internal walls of the hive to repair combs, to fill the crevices in brood frames and for making the entrance of the hive smaller. It is known to be used for 'embalming' dead bodies of intruders which can not be thrown out of the hive (Ghisalberti, 1978), suggesting that it possesses antimicrobial activity. During the last 50 years many reports have been published on the chemical composition, pharmacology and therapeutical uses of propolis. The summary of results generated from time to time is available in review articles. Origin of propolis, its types, methods of collection and uses were reviewed in the early seventies by Zile Singh (1972). Applications of propolis for healing of experimental burns and skin wounds, as an antifungal agent, for the stimulation of immunobiological activity, for inhibiting influenza virus, for the treatment of ear diseases, as a raw material for cosmetic and toiletries industries has been reviewed by Wells (1976). A detailed discussion of antibacterial, antifungal, anesthetic activities of propolis and its applications in the treatment of dermatological diseases like eczema, ulcer crucris and other diseases like ulcers, respiratory track infections, and pharyngitis was presented by Ghlisalberti (1978). Chemical constituents of propolis identified until 1987 were summarized by Walker and Crane (1987). Applications of propolis for removing corns, for treating cuts and wounds in animals, for giving local anesthesia were reviewed by Garg (1989). Information about chemical constituents of propolis published after 1995 was compiled by Bankova *et al* (2000). Recent findings concerning bioactive molecules in propolis were recently reported (Bankova, 2009).

Many other beneficial biological activities such as antibacterial, antifungal, antiviral properties were reported for propolis (Bankova *et al.*, 2000). Anti-inflammatory, antiulcer, local anesthetic (Ghisalberti, 1978), hepatoprotective anti-tumour

immunostimulating properties have also been explored (Marcucci *et al.*, 1995). Due to these attributes propolis is widely used as popular remedy in folk medicine, as a constituent of health food, as a bio-cosmetic and for numerous other purposes (Bankova *et al.*, 2000; Wollenweber *et al.*, 1997). A large range of aromatics, flavonoids, terpenoids, lignans and sugars are identified in various propolis samples collected from different regions (Bankova *et al.*, 2000).

The antioxidant activities of propolis of various geographic origins, such as Argentina, Australia, Brazil, Bulgaria, Chile, China, Hungary, New Zealand, South Africa, Thailand, Ukraine, Uruguay, United States, and Uzbekistan were compared in the past (Haydak, 1954). Besides this a large number of reports describe the antioxidant activity of propolis and the protocols involved. Biological activities of extracts of Korean propolis were evaluated and their quality compared to those from Brazil. Total polyphenol and flavonoid contents of propolis extracts were shown to be responsible for the activity (Garcia-Viguera *et al.*, 1992). In another comparative study propolis extracts were used for the treatment of oronasal infections and as antioxidant agents. Commercially available ethanolic extract of Brazilian propolis was assayed for their ability to scavenge DPPH radicals. These activities were correlated with their total phenolic content (Garg, 1989). Various types of propolis samples obtained from Europe, Asia, and Brazil were investigated to analyse their total polyphenol content and antioxidant activity by Folin-Ciocalteu method and DPPH radical scavenging ability respectively. European propolis samples mostly had a higher content of polyphenols and antioxidant activity than those of Brazilian origin (Helfenberg *et al.*, 1908).

Compared to other countries, much less effort has been made to investigate the biological properties and chemical constitution of Indian propolis. Propolis from Indian honeybees was shown to possess protective effect against alcohol-carbon tetrachloride induced hepatotoxicity in rats (Sharma *et al.*, 1997). Similarly its protective effect against paracetamol toxicity in the rat liver was demonstrated (Sharma *et al.*, 1998). It was, therefore, decided to undertake a detailed examination of the biological properties of extracts of Indian propolis. As the first step in this endeavour we report the antioxidant activity of extracts of Indian propolis and its various fractions.

Materials and methods

Collection of propolis

Crude propolis (100 g) was collected from apiaries housing *Apis mellifera* colonies, of the Khadi and Village Industry Board (KVIB) at Mahabaleshwar and Pune, Maharashtra state, India. Crude propolis was physically cleaned and powdered.

Chemicals

Linoleic acid, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), horse-radish peroxidase (HRPase), pyrocatechol, sodium nitroprusside, hydrogen peroxide were purchased from HiMedia laboratories Pvt. Ltd, Mumbai, India. 2-2′-Azinobis-3-ethyl benzothiazoline-6-sulphonate (ABTS), vitamin E analogue, 6-hydroxy-2,5,7,8-tetramethylchroman-2 -carboxylic acid (Trolox), 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich Inc., USA. All other routine chemicals used were of AR grade. Precoated TLC plates (60 F254) of size 20 cm x 20 cm and 0.2 mm thickness were purchased from Merck KGaA, Germany.

Extraction of propolis

Crude propolis was extracted separately with ethanol and with solvents of increasing polarity. Powdered propolis (18 g) was exhaustively extracted with 400 mL ethyl alcohol three times and the combined alcoholic solution was evaporated under reduced pressure. Another sample of 67 g crude propolis powder was successively extracted with 1 L each of hexane, ethyl acetate and ethyl alcohol exhaustively. The solvent in each case was evaporated under reduced pressure. Thin layer chromatogram and HPLC chromatogram of each extract were recorded.

From the plates purchased smaller plates of 10 cm x 5 cm were cut. The solution of propolis extract (1 mg/mL) was loaded on a small plate as 5 mm streak using Camag Linomat IV spotter. The plate was air dried and run in a suitable solvent system for each extract. The plate was dried and developed in an iodine chamber for visualisation.

High Performance Liquid Chromatograph

High Performance Liquid Chromatography (HPLC) was run on a ZORBAX, Eclipse, XDB-C8, 4.6 mm x 150 mm, 5 µm column using Agilent 1100 high performance pump and Agilent 1100 variable wavelength UV detector (254 nm). The solvent system at a flow rate of 1 mL/min, is specified where appropriate below.

Preparation of samples of propolis extract for analysis

Each propolis sample of 10 mg was dissolved in 10 mL of ethanol. From this stock solution (1 mg/mL), standard solutions of 0.5, 0.2 and 0.1 mg/mL were prepared by dilution with ethanol.

Evaluation of trolox equivalent antioxidant capacity (TEAC)

Total antioxidant activity of each of the propolis extracts was measured using standard TEAC assay method (Miller *et al.*, 1995) with minor modifications. In each experiment 2, 2′-azinobis- 3′-ethyl benzothiozoline-6-sulphonate (ABTS) radical cation was generated by interaction of 0.5 mL ABTS dianion with 0.5 mL of hydrogen peroxide under the influence of horse raddish peroxidase enzyme (2.2 units) at 40°C. For each of the test samples of 0.5 mL of propolis, was

mixed with ABTS cation radical generated and 2 mL of deionised water. The reaction mixture was allowed to stand for 10 min. Its quantification was done by recording the optical density at 734 nm using a UV-VIS spectrophotometer, Jasco V-530, Japan . Similar determination for trolox was carried out for reference. The TEAC value is based on the ability of the antioxidant to scavenge the blue-green 2, 2′-azinobis- 3-ethylbenzothiazoline-6- sulfonate ($ABTS^{\cdot+}$) radical cation relative to the ABTS· scavenging ability of the water soluble vitamin E analogue, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox). Decrease in absorption at 734 nm after addition of the reactants was used to calculate the TEAC value. The TEAC value is expressed as the millimolar concentration of Trolox solution having an antioxidant equivalent to a 1000 ppm solution of the sample under investigation. The lower the TEAC value of a sample, the stronger is its antioxidant ability.

Lipid peroxidation effect of propolis extract and fractions

Inhibition of lipid peroxidation was determined using the method of Liegeois *et al* (2000). A 30 µL aliquot of 16 mM linoleic acid dispersion was added to a UV cuvette containing 2.8 mL of 0.05 mM phosphate buffer, pH 7.4, at 40°C. The oxidation reaction was initiated at 37°C under air by adding 150 µL of 40 mM AAPH solution. Oxidation was carried out in the presence of 20 µL of 0.2 mg/mL propolis extract. Observations using 20 µL trolox solution in place of propolis extract served as a control. Rate of peroxidation at 37°C was monitored by recording increase in the absorbance at 234 nm caused by conjugated diene hydroperoxides. The percentage inhibition of lipid peroxidation was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{[A_0 - A_1]}{A_0} \times 100$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in presence of the extract.

Determination of antioxidant activity of propolis extract and fractions with the DPPH radical-scavenging

Hydrogen donating or radical scavenging ability of propolis extracts was measured according to Jung's method (Jung *et al.*, 2005) using the stable α, α-diphenyl-*β*-picrylhydrazyl (DPPH) radical. A 0.1 mL aliquot of ethanolic solution of the 0.2 mg/mL extract was placed in a cuvette and 2 mL 0.06 mM ethanolic solution of DPPH was added. Absorbance was immediately measured at 515 nm. It was allowed to stand at room temperature for 70 min and the absorbance was recorded again. Decrease in absorbance was determined. As a control the absorbance of DPPH radical without propolis extracts was measured daily. Percent inhibition of DPPH radical in samples was calculated according to the formula:

$$\% \text{ Inhibition} = \frac{(A_{C(0)} - A_{A(t)})}{A_{C(0)}} \times 100$$

where $A_{C(0)}$ is absorbance of the control at t = 0 min and $A_{A(t)}$ is absorbance of the antioxidant at t = 70 min. Trolox was used as a positive control.

Effect of propolis extract and fractions on scavenging of nitric oxide

Nitric oxide scavenging effect of propolis extracts was measured according to the method of Marcocci *et al* (1994). Aliquots of 4 mL of each of the 0.2 mg/mL propolis extracts were added to 1 mL of 25 mM sodium nitroprusside solution in a test tube and then incubated at 37°C for 1 h. Of this incubated solution 0.5 mL was removed and diluted with 0.3 mL of Griess' reagent. Absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with napthylethylenediamine dihydrochloride was immediately determind at 570 nm and compared with the absorbance of standard solutions of trolox treated in the same way with Griess' reagent.

Total polysaccharide content

Polysaccharide content of propolis extracts was determined using phenol-sulphuric acid method (Dubois *et al.*, 1956). To 2 mL of 0.2 mg/mL propolis extract containing polysaccharides was added 25 mL of 80% aqueous phenol solution followed by 1 mL 18.4 M sulphuric acid. The mixture was shaken and heated on a water bath for 45 min at 25°C. Absorbance of characteristic colour was then measured at 490 nm. Polysaccharide content was determined from a standard curve previously constructed using D-glucose.

Determination of total phenolic compounds

Total soluble phenolics in the propolis extracts were determined with Folin - Ciocalteu reagent according to the method of Slinkard and Singleton (1977) using pyrocatechol as the standard. The extract (0.1 mL) and 2 mL of 2% (w/v) aqueous sodium carbonate were mixed thoroughly. After 5 min, 0.1 mL 50% Folin - Ciocalteu reagent was added and allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. Concentration of total phenolic compounds in the extract was determined as microgram of pyrocatechol equivalent by using an equation:

Absorbance = 0.001 x pyrocatechol (µg) + 0.0033.

Determination of IC_{50} value

Half inhibitory concentration (IC_{50}) for total ethanolic extract and other fractions of propolis were calculated by extrapolation from the concentration/effect regression lines obtained from four different concentrations (0.1, 0.2, 0.5 and 1 mg/mL).

Stability of the extracts

Samples of each of ethyl acetate extract, ethanol extract and the ethanolic extract of crude sample of propolis were incubated at 4°C, 20°C and 40°C for 1 h and the trolox equivalent antioxidant activity, inhibition of lipid peroxidation, scavenging of nitric oxide, DPPH radical-scavenging activity were determined. Identical sets of extracts were kept in an incubator for 720 h at 4°C and 20°C and their activities were determined.

Statistical Analysis

Student's 't' test was applied using Sigmaplot version 8 to determine standard deviation and *p* values. Experimental results were mean ± SD of 10 parallel measurements. *p* values < 0.05 were regarded as significant and *p* values < 0.01 very significant.

Results

The extraction of 18 g of crude propolis with ethanol yielded total crude extract (TEEP; 9.8 g, 54.4%). TLC (using 2 : 8 of hexane : ethyl acetate) indicated seven distinct spots. Its HPLC analysis using acetonitrile : water (90 : 10) (Fig. 1) showed eight prominent peaks. Extraction of 67 g of propolis with hexane yielded a waxy solid (HEFP; 18.76 g, 28%). TLC (using 9.5 : 0.5 of hexane : ethyl acetate) indicated seven distinct spots. The HPLC results also showed seven prominent peaks in methanol (Fig. 2). On extraction with ethyl acetate a sticky mass (EAFP; 24.79 g, 37%) was obtained. It showed eight spots on TLC (using 7 : 3 of hexane : ethyl acetate) while its HPLC chromatogram (Fig. 3) showed nine prominent peaks in methanol : water (80 : 20). Ethanol extraction yielded a powdery extract (EFP; 3.35 g, 5%) showing five spots on TLC in 6.7 : 3.3 of hexane : ethyl acetate and six peaks in the HPLC chromatogram (Fig. 4) when recorded in methanol : water (80 : 20) system. The insoluble residue (approx. 21 g, 31.34%) was recovered.

The trolox equivalent antioxidant capacity (TEAC) along with lipid peroxidation inhibitory activity (LPO) of crude propolis extract (TEEP), ethyl acetate soluble fraction (EAFP) and ethanol soluble fraction (EFP) are presented in Table 1. The scavenging effect of propolis extract and its different fractions on DPPH and nitric oxide radicals are also given in the table. TEAC activity was expressed in mM concentration while the rest of results were expressed in % of the respective activity. Hexane soluble fraction (HEFP) did not show any antioxidant activity, hence is not included in the table. Yields of polysaccharide content and polyphenolic contents in the propolis extract and different fractions are shown in Table 2. The half inhibitory concentrations (IC_{50} values) determined for TEEP, EAFP and EFP are shown in Table 3.

Antioxidant activities of the propolis extracts and its fractions tested after incubation at three different temperatures, 4ºC, 20ºC and 40ºC for the stability studies are described in Table 4.

Correlation between polyphenolic content and TEAC activity and LPO activity of propolis extracts were determined (Fig. 5 and Fig. 6 respectively). The comparative levels of polyphenols and polysaccharides in TEEP, HEFP, EAFP and EFP are represented in Figure 7.

Table 1. Antioxidant activities of the extract and fractions of Indian propolis

Propolis extract	Concentration (mg/mL)	TEAC activity (mM ± SD)	LPO activity (% ± SD)	DPPH activity (% ± SD)	NOS activity (% ± SD)
TEEP	0.1	6.62 ± 0.047	6 ± 2.4	25 ± 1.04	16 ± 2.74
	0.2	5.14 ± 0.057	13 ± 2.66	47 ± 2.35	35± 3.81
	0.5	1.92 ± 0.084	26 ± 2.52	56 ± 1.44	59 ± 2.79
	1.0	0.39 ± 0.047	45 ± 1.86	78 ± 2.66	75 ± 3.56
EAFP	0.1	2.86 ± 0.012	19 ± 3.31	28 ± 2.11	55 ± 3.34
	0.2	1.81 ± 0.031	44 ± 2.14	76 ± 2.59	66 ± 3.03
	0.5	1.69 ± 0.041	54 ± 2.24	78 ± 2.37	60 ± 3.09
	1.0	1.62 ± 0.063	57 ± 2.33	80 ± 1.93	59 ± 3.09
EFP	0.1	5.20 ± 0.10	13 ± 1.94	23 ± 2.65	47 ± 3.04
	0.2	3.68 ± 0.12	32 ± 2.29	47 ± 3.71	53 ± 2.06
	0.5	2.91 ± 0.08	37 ± 2.39	53 ± 2.54	56 ± 3.16
	1.0	0.68 ± 0.11	44 ± 2.35	77 ± 2.59	57 ± 2.92
Trolox	0.2	3.98 ± 0.072	58 ± 1.35	75 ± 1.55	42 ± 1.28

TEAC- Trolox equivalent antioxidant capacity; LPO – Lipid peroxidation activity; DPPH- α,α-diphenyl-β-picrylhydrazyl scavenging activity; NOS- $NO^{\cdot}$ radical scavenging activity. Data are mean of 10 consecutive replicates.

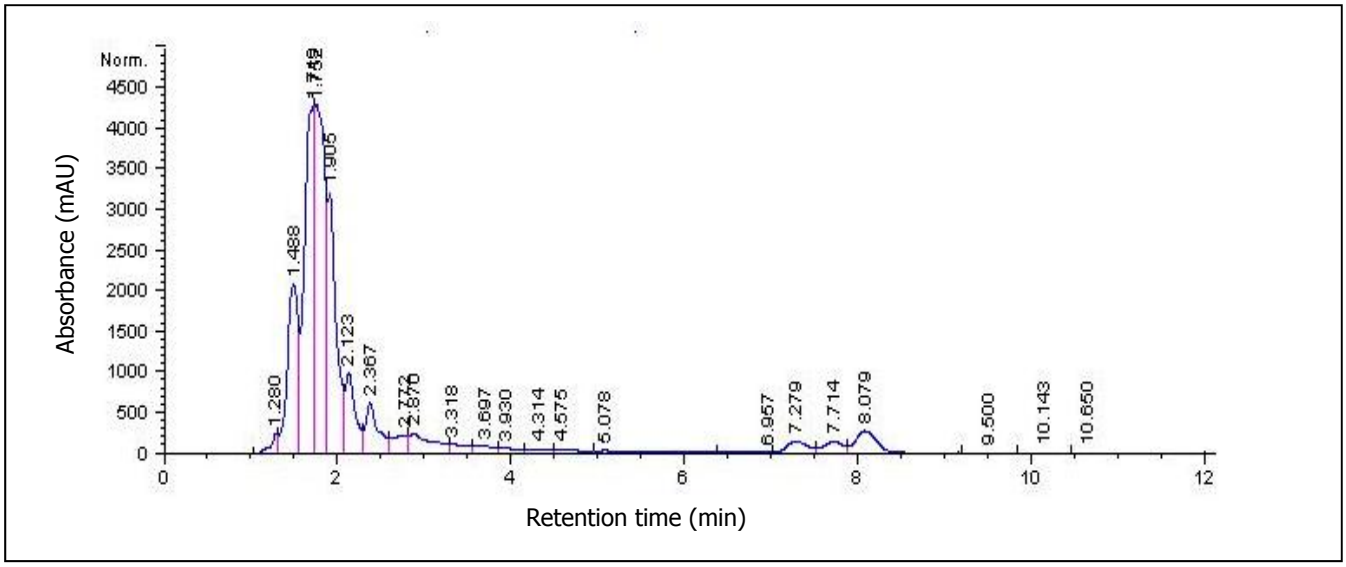

Fig. 1. HPLC chromatogram of total ethanolic extract of crude Indian propolis.

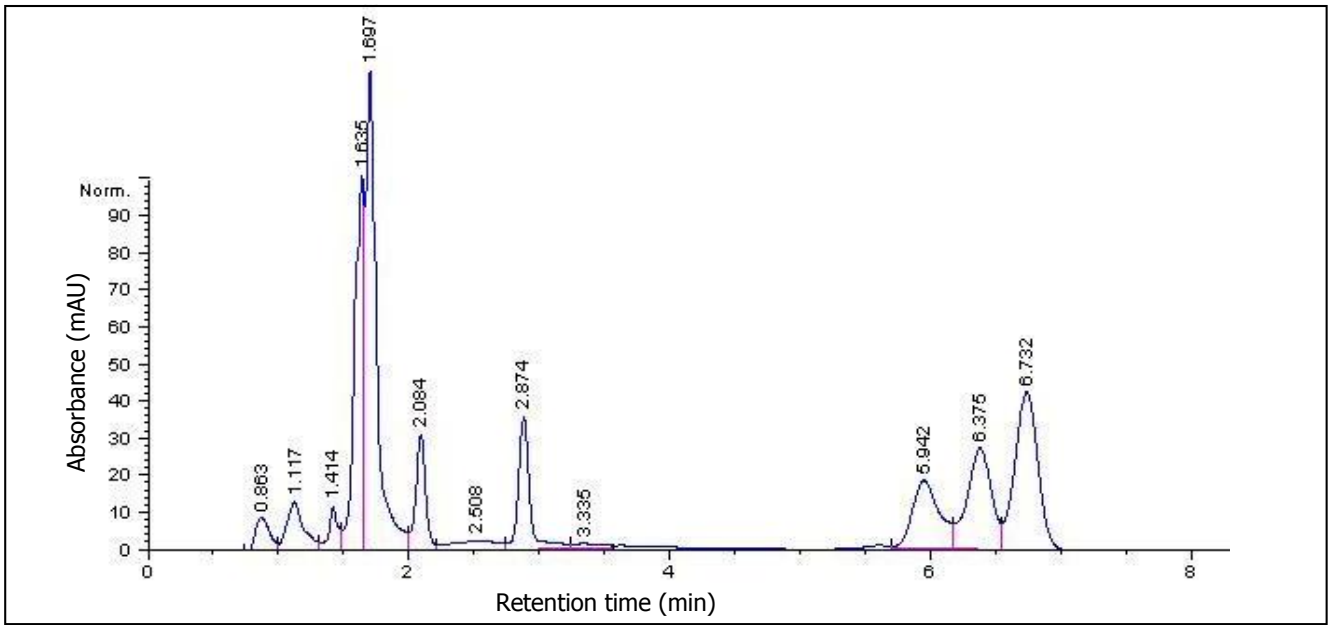

Fig. 2. HPLC chromatogram of hexane extract of Indian propolis.

Table 2. Yields of polysaccharide and polyphenolic content in extract and fractions of Indian propolis

Propolis extract	Polysaccharide content mg/mg ± SD	Polyphenolic content mg/mg ± SD
TEEP	0.236 ± 0.005	0.113 ± 0.006
EAFP	0.305 ± 0.009	0.161 ± 0.006
EFP	0.108 ± 0.016	0.160 ± 0.020

Data are means of the 10 consecutive replicates of extracts in assay performed.

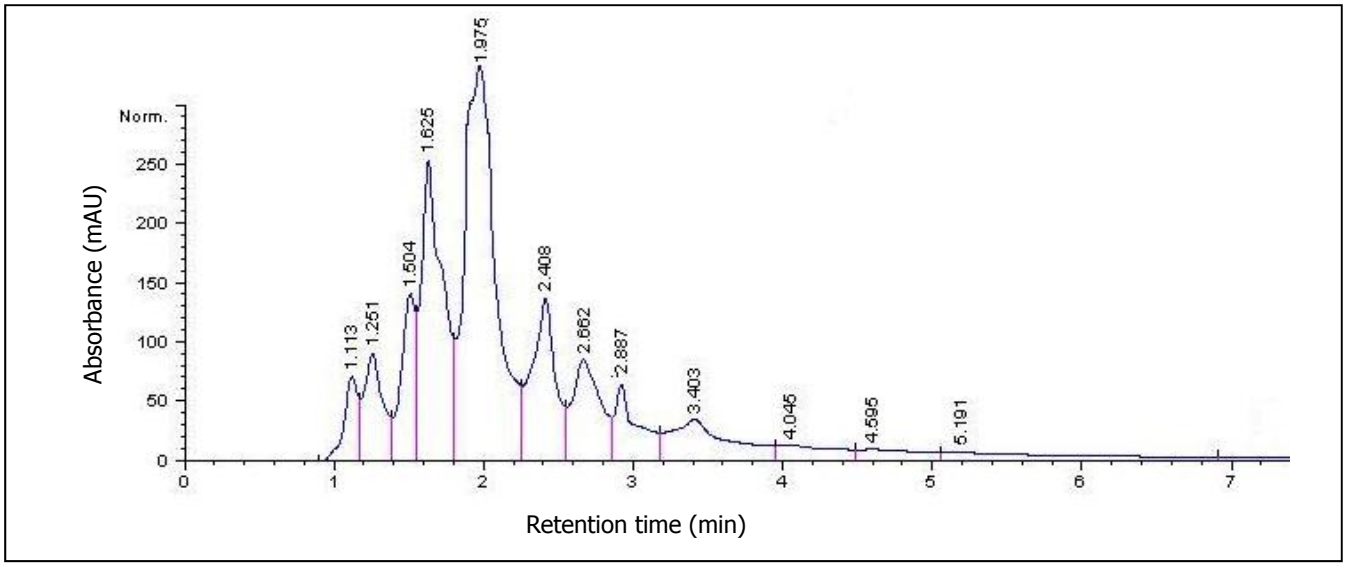

Fig. 3. HPLC chromatogram of ethyl acetate extract of Indian propolis.

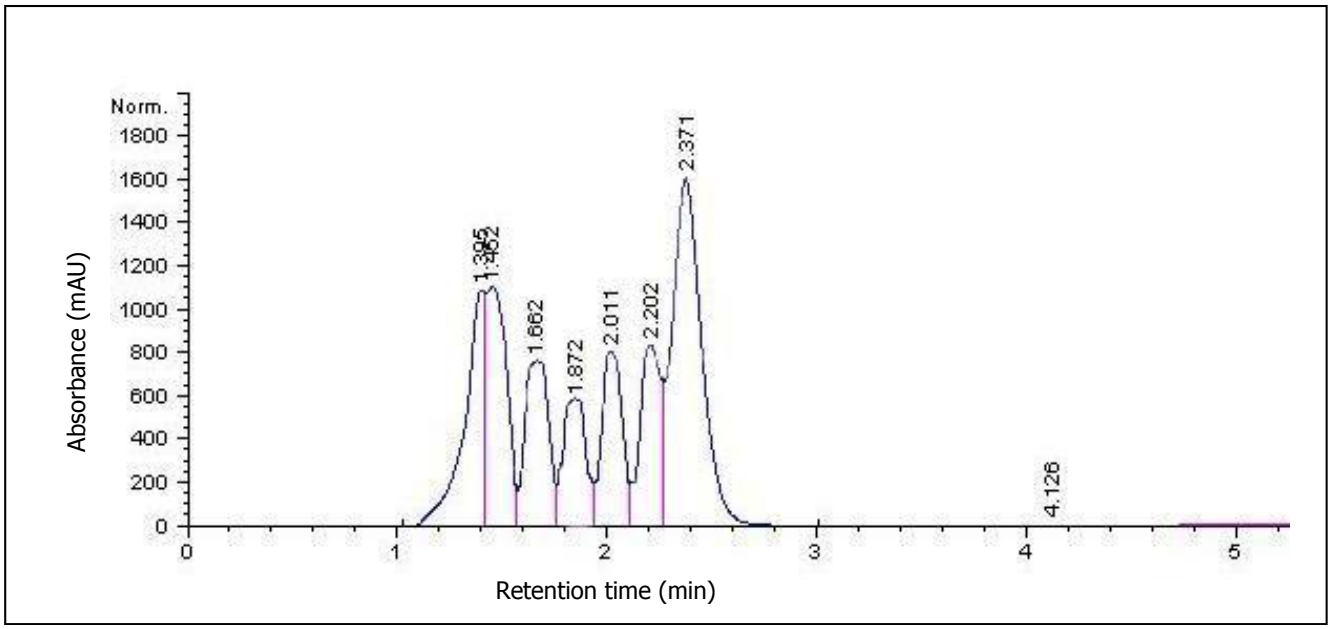

Fig. 4. HPLC chromatogram of ethanolic extract of Indian propolis.

Table 3. Half inhibitory concentration (IC_{50}) of the extract and fractions of Indian propolis for the antioxidant activity.

Propolis extract	LPO activity (mg/mL)	DPPH activity (mg/mL)	NOS activity (mg/mL)
TEEP	1.25	0.30	0.38
EAFP	0.48	0.14	0.06
EFP	1.39	0.31	0.18

Table 4. Thermal stability of propolis extracts and fractions

Propolis extract (0.2 mg/mL)	Temperature (°C)	Time (h)	TEAC activity (mM ± SD)	LPO activity (% ± SD)	DPPH activity (% ± SD)	NOS activity (% ± SD)
TEEP	4	1	5.15 ± 0.057	12 ± 1.94	45 ± 3.59	36 ± 2.84
	4	720	5.41 ± 0.041	13 ± 1.23	43 ± 3.29	32 ± 2.72
	20	1	7.10 ± 0.037	10 ± 1.58	50 ± 2.79	40 ± 1.89
	20	720	7.72 ± 0.039	9 ± 1.69	54 ± 3.37	38 ± 1.94
	40	1	7.18 ± 0.047	8 ± 1.89	71 ± 2.33	40 ± 2.93
EAFP	4	1	1.85 ± 0.035	45 ± 3.11	76 ± 2.98	66 ± 2.60
	4	720	1.91 ± 0.042	46 ± 2.79	70 ± 2.59	63 ± 3.15
	20	1	6.90 ± 0.059	12 ± 1.69	68 ± 3.34	45 ± 2.68
	20	720	7.32 ± 0.067	10 ± 1.54	66 ± 2.63	41 ± 1.83
	40	1	7.15 ± 0.057	12 ± 1.72	58 ± 2.62	38 ± 3.01
EFP	4	1	3.75 ± 0.087	30 ± 2.42	48 ± 3.09	52 ± 2.72
	4	720	3.88 ± 0.075	31± 2.58	45 ± 3.11	49 ± 2.44
	20	1	7.05 ± 0.034	13 ± 2.09	62 ± 2.55	45 ± 2.61
	20	720	7.22 ± 0.051	10 ± 1.94	57 ± 3.28	50 ± 2.83
	40	1	6.94 ± 0.036	11 ± 1.59	76 ± 2.71	35 ± 2.74

Data are mean of 10 consecutive replicates of extracts in assay performed.

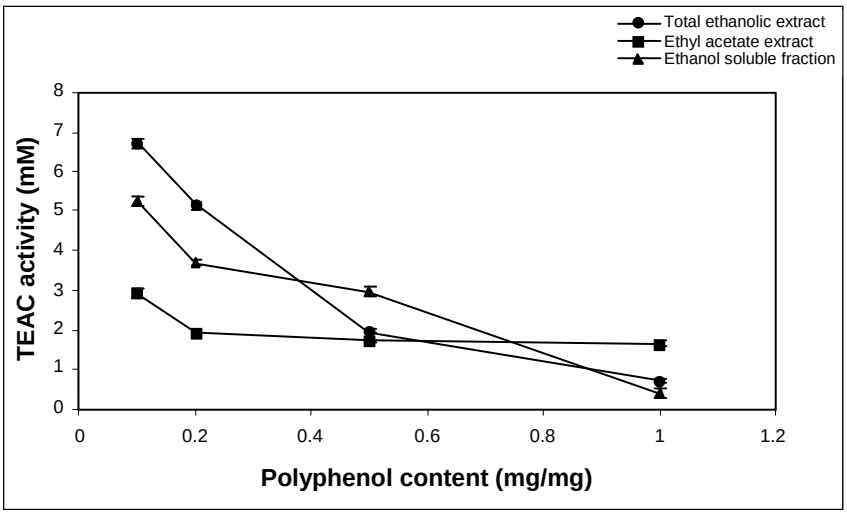

Fig. 5. Plot of TEAC activity against the polyphenol content of the Indian propolis extracts.

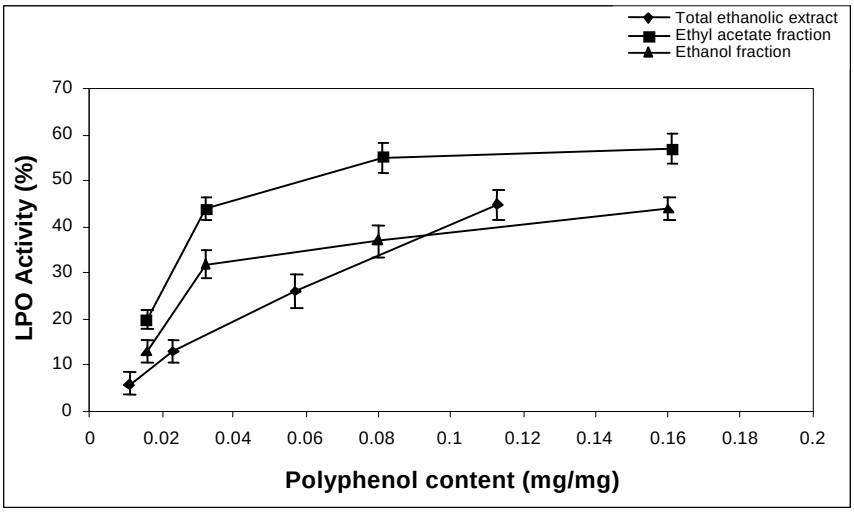

Fig. 6. Plot of LPO activity against the polyphenol content of the Indian propolis extracts.

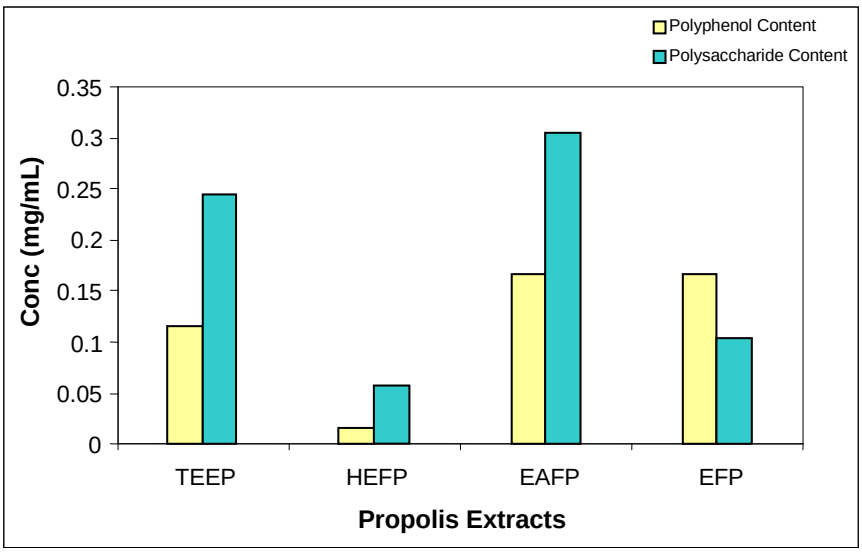

Fig. 7. Comparison of polyphenolic and polysaccharide content in the extracts of Indian propolis.

Discussion

TLC and HPLC profiles of total ethanolic extracts of propolis (TEEP) showed multiple spots and peaks indicating the complexity of the mixture of compounds present. Distribution of spots over TLC and retention times of peaks in HPLC chromatogram (Fig. 1) indicated the presence of five polar and three nonpolar compounds as major constituents of the extract. When extracted in the solvents of increasing polarity, the non polar and moderately polar compounds of propolis got extracted in hexane (HEFP) as seen from its HPLC chromatogram (Fig. 2). Ethyl acetate soluble fraction (EAFP) was the major fraction of propolis. This was also a complex mixture of compounds as indicated by appearance of eight spots in its TLC and nine peaks in its HPLC profile (Fig. 3). The HPLC chromatogram further indicated that the compounds present in this extract were polar and moderately polar. After removal of hexane and ethyl acetate soluble compounds, a very small portion of the residual propolis got extracted in ethanol (EFP) which also contained mostly the polar compounds as observed from its HPLC chromatogram (Fig. 4).

Trolox equivalent antioxidant capacity (TEAC)

TEAC assay gives an indication of the total antioxidant capacity of various samples. It was observed from the results (Table 1) that EAFP exhibited 1.92 mM (75.48%) trolox equivalent antioxidant activity at a concentration of 0.2 mg/mL. This was superior to the activity of the trolox standard used at the same concentration. EFP also showed activity equivalent to that of trolox at 0.2 mg/mL. A ranking order, EAFP > EFP > TEEP can also be generated from the results. TEAC values of all the samples were found to decrease with the increasing concentration of the formulation. The decrease was less for EAFP. The TEAC value was almost constant above 0.2 mg/mL for EAFP. From these observations it can be concluded that the ethyl acetate soluble fraction was active even at a lower concentration than that of standard. Conversely, EFP and TEEP formulations were active at the higher concentrations than that of the standard.

Polyphenols are one of the factors responsible for antioxidant capacity. Comparison of the total polyphenol content of TEEP, EAFP and EFP with TEAC activity of samples is shown Figure 5. It indicates that the anti-oxidant activity of TEEP and EFP was significantly correlated with the total polyphenol content ($r = 0.864$, total ethanolic extract, $p < 0.01$; $r = 0.949$, ethanol soluble fraction, $p < 0.01$). However, a similar correlation was not found for EAFP, the most active fraction. These findings again demonstrate that an increase in the concentration of EAFP formulation that is indirectly an increase in the polyphenolic content, does not affect the TEAC value.

Effect of propolis on lipid peroxidation

The lipid peroxidation activity (LPO) of Brazilian propolis has been determined (Nagai *et al.*, 2003). Here similar observations were seen in Indian propolis. The activity was found to increase with the increasing concentration of the extract. It suggests that lipid peroxidation is dose dependent. Correlation of the total polyphenolic content and LPO activity of propolis extracts was determind (Fig. 6). In the case of EAFP ($r = 0.610$; $p < 0.05$) and EFP ($r = 0.666$; $p < 0.05$), the increase in LPO activity was marginal while a significant correlation was observed for TEEP ($r = 0.989$; $p < 0.01$).

Free radical scavenging activity

Both European and Brazilian propolis are known to possess DPPH scavenging activity (Kumazawa *et al.*, 2001). Cuban propolis also shows a similar type of scavenging action against different species of oxygen radicals generated by specific chemical reactions. The results indicate that the antioxidant properties of propolis extracts could be attributed to their free radical scavenging activity against alkoxy

radicals and against superoxide (Pascual, 1994). DPPH is a stable free radical in aqueous or methanolic solution and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It is usually used as a substrate to evaluate the antioxidant activity. Antioxidant potency can be evaluated through free radical scavenging with the propolis extracts. The antioxidant activity of propolis extracts and trolox have been presented in Table 1. The DPPH scavenging effects of dilute solution of propolis extract (0.1 mg/mL) are comparatively less (approximately 25%). However, the effect was higher for the higher concentrations. More importantly the effect was much more pronounced for the EAFP (> 75%) at the concentrations 0.2 mg/mL and above, and was approximately equivalent to that of trolox. It was also observed that the activity for all the formulations was similar at a concentration 1 mg/mL.

It is interesting to note that the nitric oxide scavenging activity was largely consistent around 60%, for all the concentrations of EAFP. For EFP it varied over a small range of 10%. The activity was much more pronounced for EAFP (66%) than the standard trolox (42%) at 0.2 mg/mL while it was also higher in case of EFP (53%) than that of trolox at the same concentration.

These results suggest that all these formulations of Indian propolis have good antioxidant capacity. At certain concentrations they proved to be superior to a standard antioxidant, and have potential to be used as antioxidative agents.

50% Inhibition concentration (IC_{50} value)

IC_{50} value of EAFP formulation of propolis was found to be 0.48 mg/mL for 50% lipid peroxidation inhibition, 0.14 mg/mL and 0.06 mg/mL for 50% inhibition of scavenging of DPPH and nitric oxide free radicals respectively, which are lower values than other formulations of propolis. The active compounds, thus, are likely to be present in the EAFP formulation of propolis as the 50% inhibition was achieved at lower concentrations.

On the basis of the results it is possible that Indian propolis could be of value as an easily accessible source of natural antioxidant as a food supplement or in the pharmaceutical industry. However, at present the active components in these extracts that are responsible for the antioxidant activity are unknown. Further work on the isolation and purification of the active components responsible for the antioxidant activity in the crude extracts of propolis is in progress.

Polysaccharide and polyphenolic content

The estimation of polysaccharide and polyphenolic content is important since it is reflected in antioxidant activity. Such a correlation was shown for propolis samples from Argentina, Australia, Brazil, Bulgaria, Chile, China, Hungary, New Zealand, South Africa, Thailand, Ukraine, Uruguay, United States and Uzbekistan (Kumazawa *et al.*, 2004).

It can be seen from Table 2 and Figure 7 that polysaccharide content was higher than polyphenolic content in TEEP and EAFP, while it was lower in EFP. The polysaccharide content was highest in EAFP and polyphenolic content was the same in EAFP and EFP, but lower in TEEP.

The polysaccharide and polyphenolic content of EAFP was high. It was proved that the ethyl acetate soluble fraction of propolis was most active fraction in all respects. The higher percentage of polyphenolic content may be responsible for its activity. Examination of Figure 7 shows that the EFP also has the polyphenolic content equivalent to EAFP. Therefore there must be additional compounds present in the EAFP formulation which contribute to antioxidant activity. Further study of bioactive compounds and their isolation is in progress.

Stability of extracts

Activities of the extracts incubated at 4°C for 1 h were unaltered, indicating stability of the extract. To confirm this further activity of the extracts incubated at 4°C for 720 h was checked and also found to be unchanged. The DPPH scavenging activity of EAFP was reduced, while it was found to increase for TEEP and EFP when incubated at 40°C at 1 h and at 20°C for 1 h as well as 720 h. Nitric oxide scavenging activity was reduced in EAFP and EFP, while there was a slight increase in the activity in TEEP when incubated at 40°C for 1 h and at 20°C for 720 h. The extracts and fractions incubated at 40°C for 1 h and at 20°C for 720 h showed marginal TEAC and LPO activity. The investigation of the stability of extracts indicates that the appropriate temperature to store propolis extracts and fractions is 4°C to retain their antioxidant activity.

The antioxidant activity of propolis has long been a topic of interest. Although reports of screening of propolis are available since the middle of last century (Hydak, 1954), a large number of reports have appeared in the recent past. Some representative recent references may be cited to illustrate this. The antioxidant activity of propolis from three species of stingless bees was reported very recently (Sawaya, 2009). The antioxidant activity of propolis from various regions of China was determined by DPPH and ABTS assays (Mok-Ryeon *et al.*, 2007). The recent publications of antioxidant activity of Iranian (Shiva *et al.*, 2007), Croatian (Ivona *et al.*, 2007, Sandra *et al*, 2006), Brazilian (Sheng *et al.*, 2007, Mendes da-Silva *et al.*, 2006), Venezuela (Trusheva *et al.*, 2005), Korean (Choi *et al.*, 2006), Chile (Russo *et al.*, 2004), Japanese (Tomoko *et al.*, 2004) propolis reveal the interest of researchers in this area. Our present results on the antioxidant activity of Indian propolis supplement the existing knowledge.

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