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Antimicrobial and DPPH Free Radical- Scavenging Activities of the Ethanol Extract of Propolis Collected from India

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Abstract

Propolis is a natural product derived from plant resins collected by honey bees. In the present study, ethanolic extract of Propolis (EEP) collected from South India were tested for their antibacterial, antifungal and antioxidative activities. Propolis from *Apis mellifera* and *Trigona sp* were collected and compared with the commercial Propolis. EEP from *Apis mellifera* and commercial Propolis showed higher activity against *Staphylococcus aureus* and *Trigona sp* EEP showed higher activity against *Candida albicans* than commercial. In addition, the total flavanoid and total polyphenol content were analyzed. The chemical compositions of Propolis were identified from Gas Chromatography and Mass Spectrum (GC-MS). The compound 1,4 Di-O-Acetyl-2,3,5-tri-O-Methylribitol (C₁₂H₂₂O₇) was found to be first time in the propolis and the rest of the identified compounds were already reported. The results confirms the high DPPH free radical scavenging activity of Indian propolis. Thus Indian propolis, being a rich source of natural antioxidants, may be used in the prevention of various free radicals related diseases.

Key Words: Propolis, Antimicrobial activity, DPPH free radical-scavenging activity, GC-MS, 1,4 Di-O-Acetyl-2,3,5-tri-O-Methylribitol

Introduction

Propolis is a balm or resin like substance collected by honey bees from leaf buds and cracks in the bark. This resin is masticated and added with salivary enzymes. The partially digested material is mixed with bees wax and used in hive as a protective barrier against their enemies such as bacteria, fungi etc., [2]. Numerous studies have confirmed the pharmacological activity of propolis which includes bacteriostatic, immunostimulating, antifungal, antioxidant, local anaesthetic etc., [14]. They are also used as folk medicines for wound healing, tissue regeneration, treatment of burns, neurodermatitis, ulcers etc., So far polyphenolic compounds have been identified in the propolis collected by *Apis mellifera*. The main polyphenols are flavonoids, accompanied by phenolic acids and their esters, phenolic aldehydes, alcohols and ketones. The medical applications of propolis preparations have led to an increased interest in its chemical composition as well as to its origin.

The chemical composition of propolis is highly variable and the antibacterial compounds of bee glue are different with respect to their geographical region. For example propolis from Europe and China contains many kinds of flavonoids and phenolic acid esters where as contrarily Brazilian propolis has terpenoids and prenylated derivatives of P-coumaric acid [12]. Variations based on seasonal, latitudinal and geographical occurrence are also seen as well. These cause a significant problem in standardizing and commercialization of propolis.

Propolis was found to inhibit Gram positive organisms more effectively than Gram negative. Fungi, including yeast like fungi from the genus *Candida* and filamentous dermatophytes are also sensitive to ethanolic extracts of

propolis. The ethanol extract of propolis has an antibacterial effect due to the presence of very active ingredients. Propolis and some of its cinnamic acid and flavonoid components were found to uncouple the energy transducing cytoplasmic membrane and to inhibit bacterial mobility [1]. As an anti-inflammatory agent, propolis has been shown to inhibit the synthesis of prostaglandins, activate the thymus gland, aids the immune system by promoting phagocytactivity, stimulates cellular immunity and augments healing effects in epithelial tissues [10]. Propolis is an effective antioxidant since it contains a number of components including tocopherol, ascorbic acid, flavonoids and enzymes as glucose oxidase, catalase and peroxidase [15]. This property is one of the most important physiological functions of food, which is supposed to protect living organisms from oxidative damage, resulting in the prevention of various diseases such as cancer, cardio vascular diseases and diabetes. Flavonoids present in propolis are the major components which may reduce free radical formation and consequently might have a protective effect on serum lipids on oxidation.

The main objective of this work is to study the chemical composition and the radical scavenging ability of bee glue from regions of Coimbatore, Tamilnadu, India. To our knowledge this is the first report about the propolis from this region. In this study, we have estimated the total polyphenol content, flavonoid content and antimicrobial and antioxidant activity of South India propolis along with GC-MS analysis.

Experimental

Sample collection

The propolis samples were collected from *Apis mellifera* and *Trigona sp* from Bee Park in Tamilnadu Agriculture University, Coimbatore, India and commercial propolis was bought from Hitech Propolis India Ltd (New Delhi). The sample was collected by setting up a special mesh over the roof of the hive box with a whole size of about 3-5cm and left for about 3 months in the winter season. Then the mesh was kept in freezer where it becomes brittle so that propolis could be taken easily.

Extraction and sample preparation

Propolis samples were cut into small pieces and extracted with 80% ethanol (1:10, w/v) by shaking at 150 rpm for 3 days at room temperature. The Ethanol Extract of Propolis (EEP) was then filtered using Whatman #1 filter paper. The filtrate was centrifuged and the supernatant was restored to original volume with 80% ethanol [13].

Determination of Balsam Content

The EEP solution was evaporated under 105°C until dry. Weight was determined and expressed as weight percentage of balsam in the ethanolic extract solution [13].

Microorganisms and Preparation of Inoculum

The following microorganisms were used in this study to test antimicrobial activity of EEP. *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Candida albicans* and *Trichophyton rubrum*. A 48h old *T. rubrum* in Sabouraud Dextrose Agar, 8h old *C.albicans* in yeast glucose broth and 8h old bacterial cultures in Nutrient Broth were used for this study.

Measurement of Antimicrobial Activity

Antimicrobial activity of propolis ethanol extract was investigated by the well diffusion method except for *T.rubrum* where cross streak method was employed. The antimicrobial screening was performed using nutrient agar plate for bacteria and Sabouraud Dextrose Agar for the fungal pathogens. All tests were performed in duplicate, using an 80% ethanol as a control to test the inhibitory effect of the solvent. The pathogens were inoculated on the plate using sterile swab. The plates were incubated at 37°C and observed after 24 h for clear inhibition zones around the well.

Estimation of total polyphenol content

Total polyphenol contents in EEP were determined by Folin's Ciocalteu Colorimetric method [18] with some modifications. EEP solution (0.2 ml) was made up to 2ml with distilled water and was mixed with 0.1ml of Folin's ciocalteu reagent (Fischer Scientific, India) and incubated for 5 min at room temperature. To this mixture 0.3 ml of 2% Na₂CO₃ was added and the absorbance was measured at 765nm after 2 h incubation at 20°C. EEP samples were evaluated at the final concentration of 20µg/ml. Total polyphenol contents were expressed as mg/g (Gallic acid equivalents). The results were tabulated in percentage.

Estimation of total flavonoid content

Total flavonoid contents in EEP were determined by the method of Kumazawa *et al.* [12]. To 0.5 ml of EEP solution, 0.5 ml of 2% AlCl₃ ethanol solution was added. After 1 h incubation

at room temperature, the absorbance was measured at 420nm. EEP samples were evaluated at the final concentration of 20µg/ml. Total flavonoid contents were calculated as quercitin (mg/g) from a calibration curve. The results were expressed in percentage.

Measurement of α , α - Diphenyl-2-picorylhydrazyl (DPPH) Free Radical Scavenging activity

The reaction mixture containing 1mL of EEP solution and equal volume of 100µM DPPH was incubated for 30 min in dark at room temperature, the absorbance was recorded at 517nm. Results were expressed in percentage with respect to control value. EEP was evaluated at the final concentration of 100µg/mL and Butyl Hydroxy Toluene (BHT) at the ranging concentration was used as the reference.

GC- MS analysis

The GC-MS was performed with the Fisons Gas Chromatography GC8000 Series plus linked to Fisons MD800 Mass Spectrometer system equipped with a 30m X 0.25mm X 0.5mm dimension AB-35MS fused silica capillary column. The temperature was performed from 100°C to 250°C at a rate of 6°C/min. Helium gas was engaged as a carrier gas at the rate of 1mL/min. The injector temperature was 250°C and the spectrum was obtained in the EI mode with 70eV ionization energy. The identification was accomplished using computer searches on a NIST98 MS data library. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the basis of its mass spectral fragmentation. If available, reference components were co-chromatographed to confirm GC retention times. The components of ethanol extracts of propolis were determined by considering their areas as a percentage of the total ion current. Some components remained unidentified because of the lack of authentic samples and the library spectra of the corresponding compounds.

Results and Discussion

Color and Balsam content of Propolis

Two samples *Apis mellifera* and *Trigona sp* propolis were collected in the month of December 2009 from India. The color of the propolis was brown but it was odourless. In general the Brazilian propolis appeared green in color and Taiwanese propolis appeared yellowish brown in color, while the color of the Chinese propolis was dark brown [13]. Some propolis from Argentina, Australia, Brazil, Bulgaria, Chile, South Africa, China, United States, New Zealand and Thailand had a pleasant odour and were light yellow to dark brown in color [12]. In the present study, propolis was extracted with 80% ethanol. The fraction of the propolis soluble in alcohol was said to be 'Propolis Balsam' it leaves the alcohol insoluble or wax fraction separate (Ghisalberti, 1979). The balsam contents in Indian propolis were found to be 58.2% in *Apis mellifera*, *Trigona sp* has 37.6% and commercial shows 42.8% (Table 1). Difference was observed in the balsam content of propolis collected from two different hosts.

In general, a relatively higher content of balsam was noted in propolis collected in June, regardless of collecting area, than those collected in other months [12]. But samples collected in the month of December from India showed high content with only slight difference. Among the two samples from India (Tamilnadu), *Apis mellifera* showed highest balsam content.

Table 1. Balsam content and main biologically active compounds in ethanol extract of Propolis

Sample	% in the sample		IC ₅₀ values of DPPH free radical (µg/ml)	
	Balsam content(w/w)	Total ^a polyphenol		Total ^b flavanoid
<i>A. mellifera</i>	58.2 ± 0.00	15.5 ± 0.21	4.0 ± 0.06	75 ± 1.09
<i>Trigona sp</i>	37.6 ± 0.21	9.0 ± 0.19	2.4 ± 0.02	71 ± 0.44
Commercial Propolis	42.8 ± 0.11	13.3 ± 0.08	4.8 ± 0.10	65 ± 1.75

^a Levels calculated as gallic acid equivalents. ^b Levels calculated as quercetin equivalents. Results were presented as mean ± SD (n=3)

Antimicrobial activity of the EEP

It is reported that the antimicrobial activity of propolis reflected its constituent, which may differ from area to area, and season to season [17, 6, 7, 19]. Flavonoids and esters of phenolic acids are found to be responsible for the antimicrobial activity of propolis [19, 8]. Kujumgiev *et al.* [11] found that tropical propolis did not contain such substances but still showed similar antibacterial activity and indicated that different substance combination in the propolis are essential for its biological activity. On the other hand, Kedzia *et al.* [9] reported that the mechanism of anti-microbial activity is complicated and could be attributed to the synergy between flavonoid hydroxyacids and sesquiterpenes.

The antimicrobial activity of the EEP from *A.mellifera*, *Trigona sp* and commercial extract against test organisms are

summarized in (Table 2). *Staphylococcus aureus* was found to be highly susceptible to EEP of *A.mellifera* and commercial propolis with zone of inhibition at 27mm and 22mm respectively. The MIC values of both the samples were to be 1mg/ml for *S.aureus*. This value is consistent to Yaghoubi *et al.* [20]. The EEP of all the three samples showed low activity against *K. pneumoniae* and *S. typhi*. Yeast like fungal pathogen *C. albicans* was found to be highly susceptible to EEP of *A.mellifera* and *Trigona sp* than commercial product with zone of inhibition at 23mm and 20mm respectively (Table 2). The MIC values were found to be 10mg/ml which is similar to MIC obtained by Rafel *et al.* [16] against *C. albicans* and *C. tropicalis*.

Table 2. Antimicrobial activity of EEP Samples

Test Organism	Zone diameter in mm		
	<i>A. mellifera</i>	<i>Trigona sp</i>	Commercial
<i>Staphylococcus aureus</i>	27±0.8	14±1.3	25±1.6
<i>Escherichia coli</i>	22±1.2	10±2.0	24±1.2
<i>Klebsella pneumonia</i>	13±0.9	8±1.5	11±2.2
<i>Salmonella typhi</i>	10±1.4	9±1.0	13±1.1
<i>Candida albicans</i>	23±1.8	20±2.1	18±0.9
<i>Trichophyton rubrum</i>	18±0.3	-	19±0.2

Expressed as the x± S.D. mean diameter (mm)

An initial step was taken to check the inhibitory activity of EEP against the dermatophytic fungus *T.rubrum*. We have got a satisfactory result which initiates other researchers to use propolis for the dermatophytic infections. Antifungal activity was observed at various concentrations ranging from 100-500 mg/ml for both *A.mellifera* and commercial propolis and high activity was observed in 150 mg/ml of EEP, where the diameter of zone of clearance was 18mm for *A.mellifera* and 19 mm for commercial propolis (Table 2). But no activity was observed in stingless bee *Trigona sp* EEP. Among the two different species EEP, *A.mellifera* showed high activity. Antibacterial activity of all the propolis samples collected at the same time was similar which was in agreement with the report

of Sforcin *et al.* [19] who found there are no significant differences between the antibacterial activities of Brazilian propolis collected during different seasons.

Total polyphenol content

The Total Polyphenol content of the EEP samples from *A. mellifera*, *Trigona sp* and the commercial sample were estimated to be 15.5%, 9% and 13.3% respectively (Table 1). The percentage of polyphenol from temperate regions like Europe and China have been observed to be approximately in the range of 25% and propolis from regions like Brazil are also as high as 25% but the propolis from the regions of Thailand, South Africa, and Uzbekistan were 5%, 10% and 14% respectively, which was consistent with the results obtained in

the present study. The percentage of polyphenol content of propolis from various regions of Turkey is in the range of 8-26%. These observations confirm that the composition of polyphenols vary considerably not only from region to region [3, 12] but also species to species.

Total flavonoid content

The $AlCl_3$ calorimetric method primarily detects the flavones and flavonols in the given sample. The total flavonoid content of the EEP from *A. mellifera*, commercial propolis and the *Trigona sp* EEP samples were analysed using the $AlCl_3$ calorimetric method and compared with the calibration curve plotted with quercetin as standard. The EEP of *A. mellifera* and the commercial sample had 4% and 4.8% respectively and very low value was obtained in *Trigona sp* (Table 1). On comparing these results with the flavonoid content, the various propolis from Turkey was in the range of 1-8% and the total flavonoid content from regions like South Africa, Ukraine and Thailand were 5%, 6.3% and 0.2% respectively [12]. Our results were found to be consistent with the South Africa and Turkey propolis.

Effect of propolis samples on DPPH free radical

The model system of scavenging DPPH free radical is a simple method for evaluating the antioxidant activity of compounds. We evaluated two different species of propolis along with commercial propolis and the reference samples as BHT at the final concentration of 100 μ g/ml. The IC_{50} values of EEP from *A. mellifera*, commercial propolis and the *Trigona sp* EEP samples were estimated by plotting absorbance vs. percentage and was found to be 75 μ g/ml, 71 μ g/ml and 65 μ g/ml for respectively (Table 1). The IC_{50} values of Taiwanese propolis were estimated to be on the range 17-180 μ g/ml which is similar to our sample under study [13].

The propolis from various regions have been found to exhibit greater antioxidant potential than the known antioxidants like vitamin C and vitamin E. Dicafeicquinic acid derivatives were shown to be potent free radical scavengers which are found in various kinds of propolis samples. 3-[3,4-dihydroxy-5-prenylphenyl]-2-(E)-propenoic acid is another effective antioxidant compound which has highest potency (IC_{50} , 0.17 μ m), which was more effective than Butylated hydroxyl Toluene (IC_{50} , 0.36 μ m). Artipillin C (IC_{50} , 0.44 μ m) was also a potent antioxidant among the abundant of the compounds isolated from propolis [1]. South Indian propolis has also been found to possess higher antioxidant activity than Taipei and Australia [13, 12].

GC-MS analysis

The chemical composition of propolis, which was collected from the South Indian zone, was investigated by GC-MS. The peaks in the Chromatograms were subjected to mass spectral analysis and the mass spectrometry for each peak

have been displayed along with the compound which has maximum probable hit in the inbuilt library, the peak is also compared with the NIST 08 and WILEY library Databases.(Fig 1 and Fig 2)

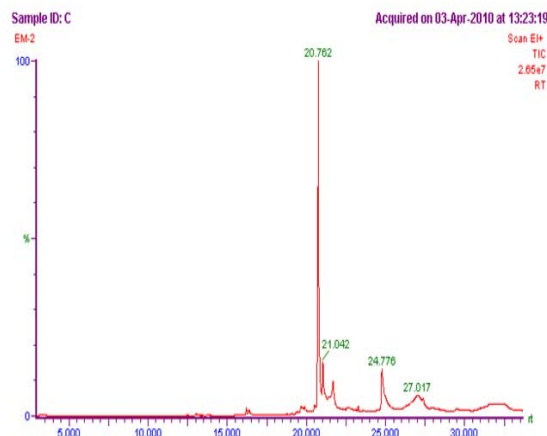


Fig. 1. GC-MS chromatogram of commercial EEP

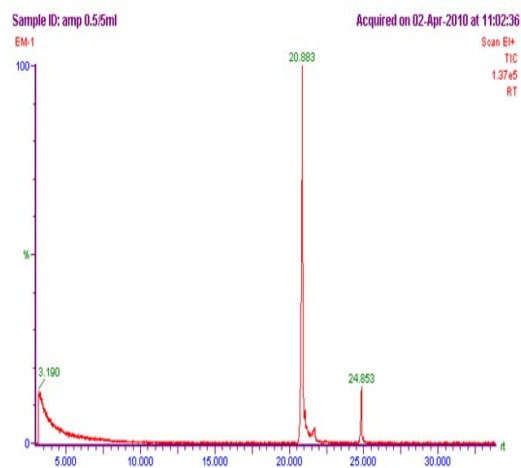


Fig. 2. GC-MS chromatogram of *Apis mellifera* EEP

From the above GC-MS result of *A. mellifera*, the most common compounds were found to be identified which listed in Table 3 and Table 4. Similar compounds were reported for their presence in the propolis samples [4]. The compound 1, 4 Di-O-Acetyl-2, 3, 5-tri-O-Methylribitol ($C_{12}H_{22}O_7$) was found to be first time in the *A. mellifera* propolis (Table 4). EEP of *Trigona sp* chromatogram barely legible and it was unable to identify the compounds (data not shown)

Table 3. Mass Spectrometry readings for Commercial EEP Sample

Retention time on chromatogram with Molecular Weight	Compound identified with Molecular formula
13.036-204	Azulene 1,2,3,4,5,6,7,8-Octahydro-4,4-Dimethyl-7-1-M(C ₁₅ H ₂₄)
16.205-202	4,4-Dimethyl-3-(3-Dimethyl-3-Buten-1-ylidene-2-Methyl(C ₁₅ H ₂₂))
16.367-204	Azulene 1,2,3,4,5,6,7,8-Octahydro- 1,4,Dimethyl-7-1-M(C ₂₄ H ₁₅)
20.762-158	4-Cyclooctatetranyl But-1-ene(C ₁₅ H ₁₄)
21.042-184	4,7-Methane_3,6,8-ethanocyclopentadiene 3,3 A (C ₁₄ H ₁₆)
21.693-159	1-(3-Butenyl) Cyclobutabenzene(C ₂₁ H ₁₄)
24.776-286	1,3,6,8-Nonatetraen-5-one,1,9-diphenyl-(C ₁₂ H ₁₈ O)
27.017-288	(1R,3S)-Cembra-4,7,11,15-Tetraen-3-ol(C ₂₀ H ₃₂ O)

Table 4. Mass Spectrometry readings for *A. mellifera* EEP Sample

Retention time on chromatogram with Molecular Weight	Compound identified with Molecular formula
3.190-181	4-Hydrazone-5-Hydroxyimino-4,5,7-tetrahydroben (C ₆ H ₇ O ₂ N ₅)
20.883-278	1,4 Di-O-Acetyl-2,3,5-tri-O-Methylribitol (C ₁₂ H ₂₂ O ₇)
24.853-153	Hydrazine(2-nitrophenyl)-hydrochloride (C ₆ H ₇ O ₂ N ₃)

Conclusion

India being a vast country has a number of varieties of propolis differing in chemical compositions and medicinal values. Even though country has development in the medical field it is to be still explored. To our knowledge, this is the first report describing the antimicrobial and antioxidant activity of South Indian propolis extracts. The results of this study demonstrate that ethanolic extracts of Indian propolis possess antibacterial, antifungal activities and DPPH radical-scavenging effects which varied from species to species. EEP of *A. mellifera* inhibits the growth of *T. rubrum* which gives a new pathway in the medicinal field for treating the fungal infection. Our sample collected from *A. mellifera* has the compound 1,4 Di-O-Acetyl-2,3,5-tri-O-Methylribitol (C₁₂H₂₂O₇), which is a new report for the propolis. To conclude, South Indian propolis is a promising potential source of biologically active substances and deserves further investigation.

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