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ORIGINAL ARTICLE **OPEN ACCESS**

Determination of Physicochemical, Antimicrobial and Antioxidant Properties of Indian Propolis

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ABSTRACT

The aim of this work was to study four propolis samples from Western Ghats of Maharashtra, India. The data from physicochemical analysis (moisture, soluble and insoluble solids content, pH, conductivity, ash content, wax, total phenolics and flavonoids content, antioxidant activity) was treated using multivariate statistical tools as cluster heat map. Microbiologically, the commercial quality was satisfactory, since the samples didn't contain deterioration or pathogenic microorganisms. All the samples studied presented antimicrobial activity. The antimicrobial activity was strictly related to the physicochemical composition. This work will allow connecting a particular chemical propolis type to a specific biological activity which is essential for the use in therapeutic applications.

Keywords: *Propolis, physicochemical analysis, antioxidant activity, antimicrobial activity, therapeutic application*

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INTRODUCTION

Propolis or bee glue is a resinious mixture that honey bees produce by mixing saliva and beeswax with exudate gathered from tree buds, saps, or other botanical sources. It has been used for thousands of years in folk medicine for several purposes. Propolis contains a variety of compounds, some of which are terpenes, flavonoids and other phenolics. [26].The chemical composition and nature of propolis depend on environmental conditions and harvested resources. The composition of propolis varies from hive to hive, from district to district, and from season to season. Normally, it is dark brown in color, but it can be found in green, red, black, and white, depending on the sources of resin found in the particular region. It is rich in vitamin A, which is essential for human cell development. It also acts as an antioxidant and has some free radical scavenging ability. The phenolic compounds are formed by an aromatic ring linked to a hydroxyl group. The flavonoids are formed by an aromatic ring linked to a cinnamoil group.[13]. Propolis has a wide range of biological activities, antibacterial, anti-inflammatory [14] antioxidant,antiulser and tumoricidal activities.[19]. The antimicrobial activity of propolis against a wide range of bacteria, fungi and viruses has been investigated lately. These studies have reported that *Staphylococcus aureus* is one of the bacterial species that appears to be susceptible to propolis[25]. Propolis is under preliminary research for the potential development of new drugs.

The aim of our study was to elucidate the significant properties of propolis collected from different regions and investigation about important bioactive compounds by chemical analysis like FTIR , HPLC. As well as the antimicrobial, anti-inflammatory,and anti-oxidant activity of propolis.

MATERIAL AND METHODS

Sample collections: Propolis samples were collected by beekeepers from different region of Western Maharashtra. Each sample was scrutinized in order to find out rests of bees, pupa of moth, plant etc.The major visible impurities were removed from then samples.The raw propolis samples were stored in cool place until analysis.[1,3,5,7,9].

Preparation of extract of Propolis: 10gm of propolis were powdered and then mixed with 70% ethanol and then kept on magnetic stirrer for 24hour. After that, solutions were filtered and concentrated under vacuum using the rotatory evaporator (40°C).Then stored in dark amber colored bottles.[3,5]

Physicochemical analysis

All the tests were performed in triplicate.

Physical Analysis

Moisture

Moisture content was determined by the standard method (AOAC Official Method 934.01). Ten grams of propolis were dried in a hot air oven at 105^0 for 1 h. After that, it was removed and let cool to room temperature and weight back. The procedure was repeated just to stabilize the weigh.

The water content was determined using the equation, in which A1 = weigh of sample;

A2 = weigh of sample dried:

$$\text{Moisture \%} = \frac{100 \times (A1-A2)}{A1}$$

Wax content

It was weighed 10 g of each sample and added 75 ml of methanol. The mixture

Was placed in a freezer at - 20^0C. Then the solution was filtered.

The wax was expressed in percentage (W %) using the sample weigh (SW) and the wax weigh (WW). The equation used was:

$$W\% = \frac{WW}{SW} \times 100$$

Conductivity

Electrical conductivity of a propolis solution at 20% (w/v) (dry matter basis) in methanol was measured at 20^0C. Results were expressed as milliSiemens per centimeter (mS/cm).

pH

pH of propolis was measured by pH meter with three standard buffer solutions.

Soluble and Insoluble soilds

0.5gm of propolis was added to 125ml of ethanol and kept on shaker for 30 min and then the solution was filtered and insoluble solids weighed. The difference between sample weigh (SW) and insoluble weigh (IW) gave the soluble soilds(SS). The result was expressed in percentage, by equation for soluble soilds:

$$SS\% = \frac{SW-IW}{SW} \times 100$$

$$\text{For insoluble solids: } IS\% = \frac{IW}{SW} \times 100$$

Chemical Analysis

Determination of Flavonoids

Flavonoids were estimated by modified aluminum chloride method [2]. 0.5ml of EEP was mixed with 1.5 ml 95% ethanol, 0.1 ml 10% aluminum chloride, 0.1ml of 1mol potassium acetate and 2.8 ml water and kept for incubation at room temperature for 30 min. Absorbance of the reaction mixture was measured at 415nm**.** Total flavonoids content were expressed as mg of catechin equivalents per g of propolis (CAEs).

Determination of Total Phenol

Total Phenol content was determined by Folin Ciocaltue method [2]. 4ml Folin Ciocaltue mixed with 15ml DW and 1ml Ethanol extract of propolis then add 6ml 20% disodium carbonate solution. Make final volume of 50ml with distilled water. Then Keep it for 2 hrs incubation at room temperature and observe colour change and take absorbance at 760nm. Total phenolic content (TPC) was quantified and reported as Caffeic acid equivalents (CAF) in mg of Caffeic acid.

Anti-oxidant activity (DPPH assay) [16]

The antioxidant activity of extracts of propolis was determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. Extracts are prepared in methanol at different conc. (100 to 400µg/ml). Addition of 1 ml 0.1mM DPPH solution .Mixture were shaken and stand at room temperature for 30min.Absorbance was taken at 517 nm .Ascorbic acid (vitamin c) is used as standard.

The scavenging activity was calculated by the following equation:

Scavenging activity (%) = [(Absorbance of control − Absorbance of test sample) / Absorbance of control] ×100 From the calibration curves, obtained from blotting different concentrations of extracts against corresponding scavenging activity, the IC50 was determined. IC50 value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals

Anti-inflammatory activity [17,18]

Membrane stabilization

Blood sample collected from human were washed with 0.4% saline and centrifuge for 10 min at 3000rpm. Suspension of RBC made were with 10% saline phosphate buffer. [20]. The reaction mixture consisted of 1ml propolis extract of different concentrations (100 - 500 µg/ml) and 1ml of 10% RBCs suspension. In control instead of test sample only saline was added and aspirin was used as a standard drug and all were incubated in water bath at 50ºC for 30min. The tubes were cooled, centrifuged at 2500rpm for 5min and the absorbance of the supernatants were measured at 560nm.[21].

The experiments were performed in triplicates for all the test samples.

The percentage inhibition of protein denaturation and inhibition of haemolysis was calculated as: Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control.

Inhibition of albumin denaturation assay

Albumin denaturation technique was used to determine the anti-inflammatory activity of ethanol extract of propolis was studied by using inhibition of albumin denaturation technique [20]. The reaction mixture consists of 1ml of propolis extract and 1% aqueous solution of bovine albumin fraction, the sample extracts were incubated at 37ºC for 20min and then heated to 70º C for 20min, after cooling the samples the turbidity was measured at 660nm using UV Visible Spectrophotometer.

Screening for antimicrobial activity

24 hrs fresh culture was used as inoculum and was adjusted to 10^8 CFU/ml by comparing with 0.5 McFarland standard turbidity .Freshly prepared with DMSO was used as stock solution. Micro organisms used were *Staphylococcus aureus-ATTC 6538, Bacillus subtilis, E.coliATTC8739, Pseudomonas aeroginosa ATCC 9027.* The addition of 1ml inoculum to each tube and incubated at 35+2◦C for 16 to 20 hrs.

High Performance Liquid Chromatography (HPLC)

Propolis extracts were dissolved in 2ml of methanol. Then it was given for HPLC analysis. [3]

Fourier Transform Infrared Spectroscopy(FTIR)

Dried propolis sample were given to FTIR analysis. The melting point of each sample was checked before analysis. [12].

RESULT

Physical Properties of Propolis

Physical properties of propolis showed wide differences in colour between different samples of propolis in that area. (Table 1)

Table (1) summarizes the important physical properties of propolis from different localities:

Sample	Place	Colour	Texture	Odour
1	Shivajinagar honey institute,Pune	Dark brown	Rigid waxy	Aromatic
2	Kurduwadi, Solapur	Brown	Waxy	Aromatic
3	Bhor ,Pune	Yellowish brown	Waxy	Very aromatic
4	Mahisgaon,Solapur	Brown	Waxy	Mildly aromatic

Table 2 Physical analysis of Propolis

Sr. No	Parameter (%)	Sample 1	Sample 2	Sample 3	Sample 4
1	Moisture content	1.7	6.2	8.5	13.5
2	Wax content	86.97	95	76.25	98.75
3	Soluble and	0.1	0.1	0.22	0.1
4	Insoluble solids	0.4	0.458	0.28	0.461
5	Conductivity (mS/cm)	1.2	2.4	2.2	1.6
6	pH	5.5	5.6	5.3	4.7

Table 3. Chemical analysis of Propolis

Sr. No	Parameter	Sample 1	Sample 2	Sample 3	Sample 4
1	Total phenol concentrations (mg GAE g^{-1})	0.36	0.30	0.49	0.36
2	Total Flavonoids concentrations (mg Quercetin g^{-1})	0.038	0.036	0.037	0.038
3	Antioxidant activity (DPPH CE50in µg L-1)	50	86	67	56

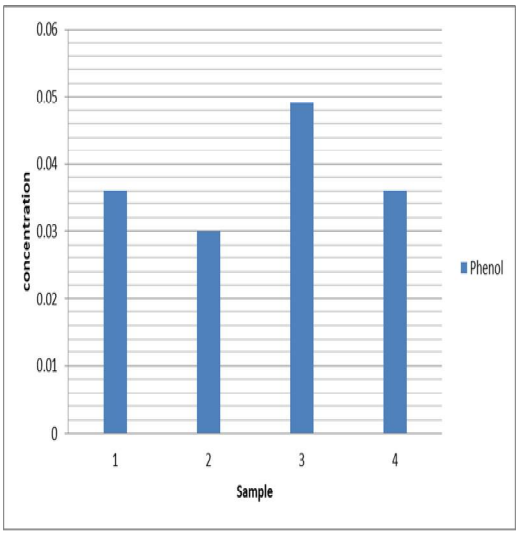


Fig 1. Total Phenol

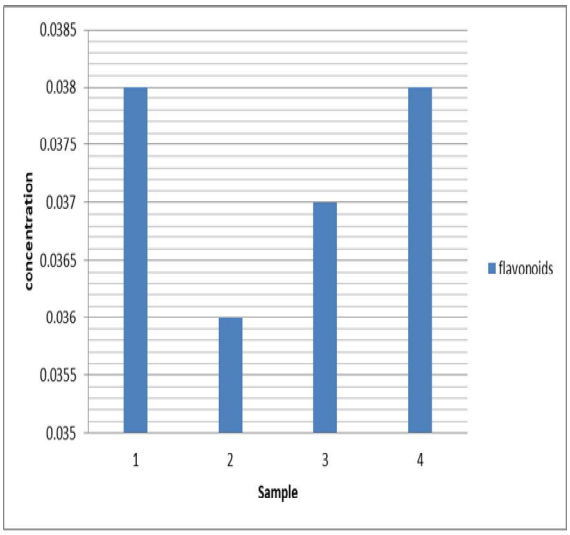


Fig 2. Total Flavonoids

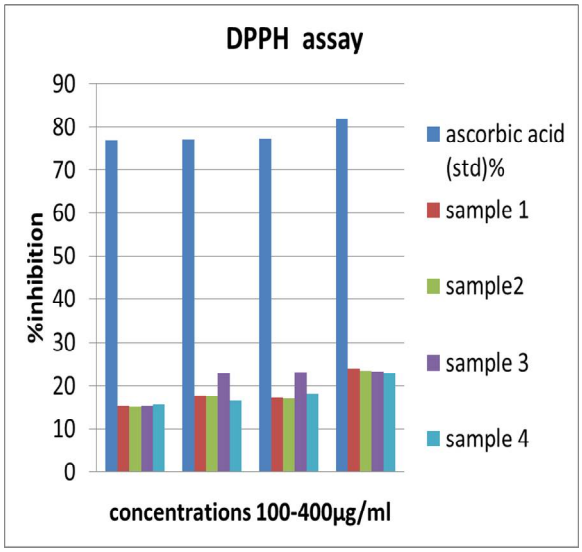


Fig 3. Antioxidant activity

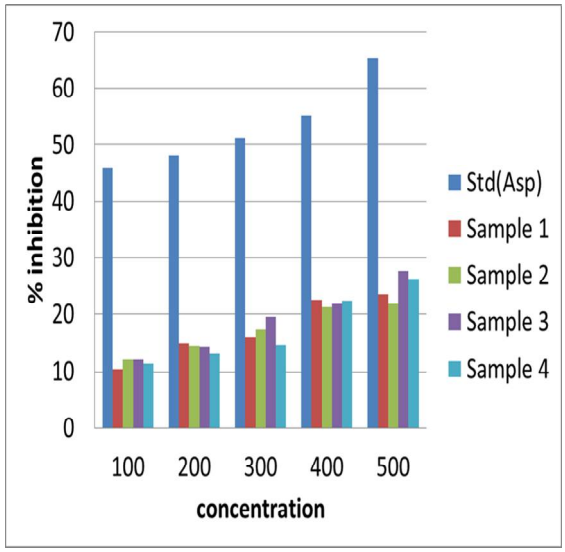


Fig 4. Anti-inflammatory activity

Table 4 .In vitro anti-inflammatory activity of Propolis

Sample	Concentration of Propolis extracts / % anti-inflammatory activity (µg/ml)				
	100	200	300	400	500
1	10	15	20	22	25
2	12	16	20	24	25
3	12	18	20	24	28
4	8	14	22	25	29
Asprin	41	45	50	55	65

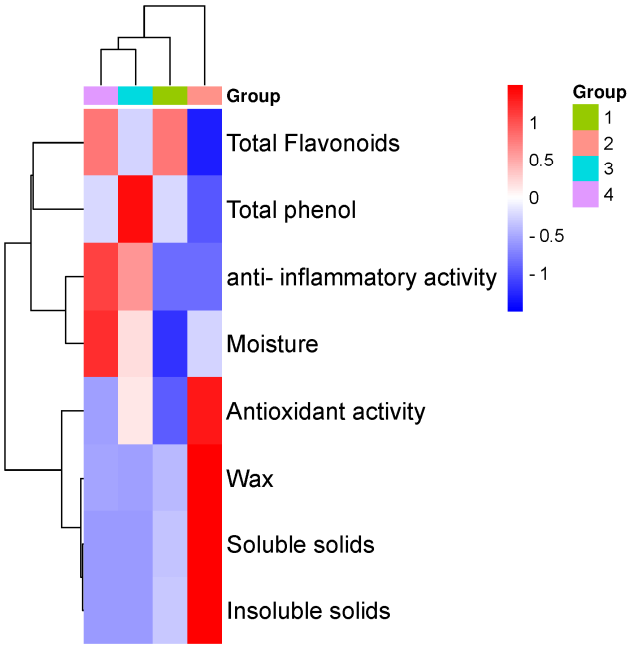
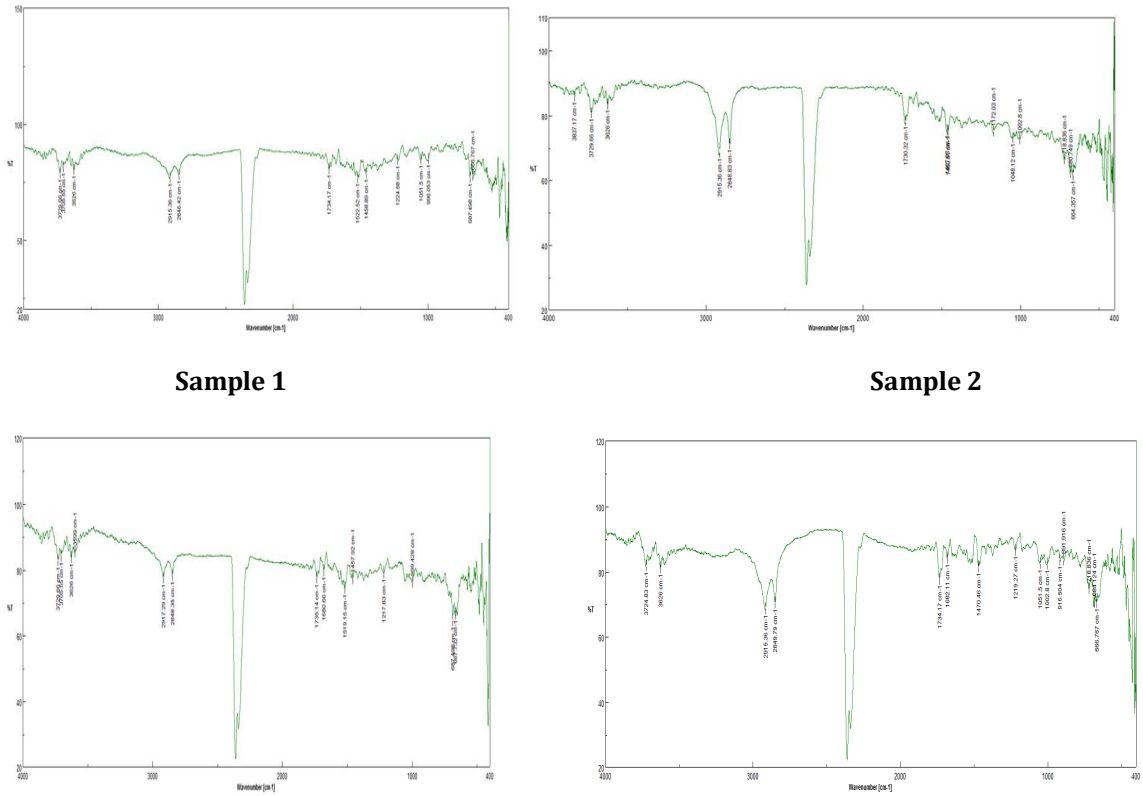


Fig.5. Cluster Heat Map of the propolis samples and physicochemical parameters



Sample 1 **Sample 2**

Sample 3 **Sample 4**

Fig 6. Fourier transform infrared (FT-IR) spectra of propolis

DISCUSSION

The values of moisture content, soluble and insoluble solid were determined in our propolis were lower then those found by Luis G. Dias et. al [8]) in propolis from different Portuguese sample. Result reported values between 3.4- 5.4 %moisture content and 60.7-71.1 % in soluble and insoluble solids.

The content of phenolic compound was located between 3- 4.9% in our sample and flavonoid was in between 3.6 - 3.8 %, these are lower than those found by Garima Agarwal et.al [2] in Chinese propolis ,they reported values of Phenol 19.44% and Flavonoid was18.79%. The free radical scavenging effect of our EEP of all samples was 15.4, 15.2, 15.3, 15.7 respectively at concentration of 100µg/ml and compared

with std vit c which showed 77.05% . The scavenging effect of EEP was 70.96% at 100µg/ml, which is very high as compare to our EEP activity at 100µg/ml [11-13]. The Anti-inflammatory activity of EEP of all samples at conc. of 500µg/ml showed moderate activity compared with the results were showing maximum activity at 500 µg/ml [20-27]. The antimicrobial activity of ethanolic extract of propolis obtain from honey institute and kuruduwadi displayed activity on almost all tested microorganisms in that the sample from honey institute showed more antimicrobial activity than sample from kuruduwadi. The Gram + ve bacterium, *staphylococcus aureus, ATCC 6538* was most susceptible microorganism to the extracts [19].

CONCLUSION

Thus, it was concluded that the EEP was the most active of all the four extracts showing the maximum zone of inhibition at the concentration of 200 mg/ml. Even in case of the free radical scavenging activity EEP showed the good activity. Further studies can be done for the identification of the chemical compounds responsible for the antimicrobial activity and its isolation along with its characterization. The exact mode of physiological or biochemical mechanisms responsible for the antibacterial effect is yet to be studied.

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