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Primary screening of multipotent therapeutic properties exhibited by Indian propolis

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This study characterizes the antimicrobial, anti-oxidant properties and chemical profile of the propolis collected from the Shahapur region of Maharashtra. Chemical constituents were identified to be flavonoids, alkaloids, phytosterols, triterpenes and glycosides. Total phenolic content was 4.751 mg/g, while the protein content was 1.187 g percent. The anti-oxidant activity accounts to about 4.732 mM of ascorbic units, determined by phospho-molybdenum assay. Antimicrobial activities were tested on selected Gram-positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative (*Escherichia coli* and *Salmonella typhi*) bacteria, as well as on pathogenic fungi *Candida albicans*. Crude extracts in absolute ethanol was tested for *in vitro* anti-angiogenic activity by the CAM assay on fertilized Leghorn eggs. The anti-inflammatory test in terms of inhibition of hypotonicity-induced HRBC membrane lysis was determined to be 39.13 % at 8000 µg/mL. Results indicated that propolis possesses remarkable properties and provide avenues for new chemical entities that could lead to the production of medicine in different therapeutic areas of health science.

Keywords: Anti-angiogenic, Anti-inflammatory, Antimicrobial; Anti-oxidant, Propolis.

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Introduction

Nature has been the cornerstone for many scientific applications for thousands of years. Impressive number of modern drugs have been isolated from natural sources, many based on their use as traditional medicine[1-4]. Even in the era of modern technology among the developed nations; 64 % of the total global population remains dependent on traditional, complementary and alternative medicine for health care provisions[5]. It has been noted that the original source of many important pharmaceuticals such as herbs, honey products, natural gums and alum are used by indigenous people either directly as food or as medicine[6].

Propolis is a complex sticky resinous mixture collected by honeybees composed mainly of plant exudates mixed with hypopharyngeal secretions, bee wax and pollen. The materials available to bees for "manufacturing" of propolis include substances actively secreted by plants as well as substances exuded from wounds in plants: lipophilic materials on leaves and leaf buds, resins, mucilages, gums, lattices[7]. The ecological function of propolis is to offer both physical and chemical protection to the colony from parasites and pathogens, and to ward off predators namely ants[8]. Depending upon the diversity of plants and the geographical locations from where the bees collect it, chemical composition of the propolis may vary[9].

Several different bioactive compounds have been characterized and are used as an antimicrobial, hepatoprotective, anti-inflammatory and antitumor agent[10]. These constituents are directly related to the diverse chemical composition of plant resins collected by bees[11]. Studies documented by Freitas *et al*[12] ascribed the antimicrobial and anti-parasitic effects of propolis to the presence phenolic compounds. Recent studies have demonstrated propolis to be non-irritant and effective in skin diseases, dental decay and different stomatological pathologic conditions[7,8,11,13]. The present study aimed to promote the use of nature-based extract i.e., propolis as potential source of biologically important compounds, investigate its composition as well as activity and thus validate its use.

Materials and Methods

Collection and identification of bee type

Propolis was collected from the forest of Shahapur, Maharashtra during the month of May, 2012 and

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refrigerated at 4 °C until analysis. Based on the size of the bee and the honeycomb structure the bee type was identified.

Extraction of the propolis

The collected propolis was pulverized into coarse powder and subjected to continuous grinding as proposed by Sosnowski Z with 70 % ethanol[14]. Propolis (100 g) was extracted in 10 % w/v of ethanol. The extract was filtered through Whatman filter paper, Grade 1. The filtrate obtained was subjected to dryness under reduced pressure in Rotary evaporator at 55 ºC. This rendered a gummy concentrate of yellowish brown color; designated as the crude extract. The crude extract was dried by freeze drier and preserved at 4 ºC. Dry extracts were reconstituted in ethanol for the chemical analyses and the antimicrobial assays.

Chemical analysis

Crude extracts were subjected to qualitative chemical screening to identify the presence of wax content, alkaloids, flavonoids, carbohydrates, gum, reducing sugars, saponins, steroids, tannins and terpenoids using the established methods according to Brossi A[15] and Shriner R[16].

Assay of total phenolic and protein content

The concentration of total phenolic compounds in the extract was determined using Folin–Ciocalteu reagent and spectrophotometry as described by Julkunen-Tiitto R[17]. Extract (1 mL) was mixed with 1 mL of FC reagent (1:1), incubated for 20 min in the dark and was diluted with 2 mL of distilled water (d/w). Then 0.1 mL of 15 % Na_2CO_3 was added and mixed thoroughly. The mixture was incubated for 30 min and estimated spectrophotometrically at 765 nm. Based on the standard curve, phenolic content was expressed in terms of mg equivalents of phenol per g of raw propolis. The protein content of the extract was measured spectrophotometrically at 660 nm following Folin-Lowry method[18] and was expressed in terms of g percent.

Antimicrobial assay

Earlier studies have demonstrated that propolis exerts various degree of antimicrobial activity[19-21]. Antimicrobial activity in the present study was evaluated by disc diffusion assay. The bacterial strains procured from hospital isolates were used in the investigation. Dried and sterilized filter paper discs (6 mm diam.) were then impregnated with known amounts of the crude extract reconstituted in absolute ethanol using micropipette. Resulting discs were then placed on Mueller Hinton medium seeded with the microbial strains. A positive control disc (impregnated with streptomycin 30 µg/disc and 0.12 % Chlorohexidine 30 µg/disc) and negative control disc (impregnated with extraction solvent) were used to compare antimicrobial effects of the extracts. These plates were incubated for 24 h (bacterial) or 48 h (fungal) at 37 ºC to allow maximum diffusion and zone of inhibition was measured for each disc as the standard protocol (NCCLS, 2006)[22].

Anti-oxidant assay

The radical scavenging activity was determined by phospho-molybdenum assay, based on the ability to reduce Mo(VI) – Mo(V) by antioxidants and subsequent formation of a green phosphate complex [Mo(V)] at an acidic pH, which is measured spectrophotometrically. Extract (0.3 mL) was thoroughly mixed with 0.3 mL of the reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and incubated at 95 °C for 90 min. The mixture was diluted with 2 mL of d/w and absorbance was measured spectrophotometrically at 695 nm. Based on the standard curve for ascorbic acid, anti-oxidant was expressed in terms of mg Molar equivalents of ascorbic acid.

Anti-inflammatory activity

The *in vitro* anti-inflammatory activity of the extract was evaluated by the human red blood cell (HRBC) membrane stabilization assay, established on stabilization of HRBC membrane by hypotonicity-induced membrane lysis in comparison to diclofenac.

Preparation of HRBC suspension

Fresh whole human blood was collected and mixed with equal volume of sterilized Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.05% citric acid and 0.42 % sodium chloride in water). The blood was centrifuged at 3000 rpm for 10 min and packed cells were washed three times with isosaline (0.85 %, pH 7.2). The volume of the blood was measured and reconstituted as 10 % v/v suspension with isosaline. About 0.5 mL of the extract was added to 1 mL phosphate buffer (pH 7.4, 0.15 M), 2 mL hyposaline (0.36 %) and 0.5 mL HRBC suspension (10% v/v). Incubated at 37 °C for 30 min and centrifuged to produce hemolysis. The

hemoglobin content in the suspension was estimated spectrophotometrically at 560 nm. A standard curve for drug- diclofenac sodium (50-2000 µg/mL) and control (d/w instead of hypo saline) were simultaneously carried out. The percentage of hemolysis and protection of HRBC membrane stabilization was calculated using the following formula:

% Hemolysis = (Optical density of Test sample/Optical density of Control) X 100

% Protection = 100 – [(Optical density of Test sample/Optical density of Control) X 100]

Anti-angiogenic activity

The fertilized eggs were kept in a humidified egg incubator at 37 °C. On the 4th day the fertilized leghorn eggs were swabbed with alcohol and candled to check for viability. The shell covering the air sac was punched out and removed by forceps. The shell membrane on the floor of the air sac was peeled off. Sample of each solution (15 µL) was applied to sterile discs. The loaded discs were inverted and applied to the chick chorioallantoic membrane (CAM) surface through the window. The air ends of the embryo shells were sealed with scotch tape. Erythropoietin was used as a positive control whereas heparin was used as a negative control of angiogenesis. The anti-angiogenic response was assessed on the 12th day by checking for the viability of the eggs and measuring the avascular zone of the CAM beneath the discs. An avascular zone of 3 mm or larger diameter was scored as positive response. At least 4 eggs were used for each dose of the agents. Other controls include sterile distilled water impregnated discs and uninoculated embryos.

The most common approach to quantification of angiogenesis involves branch counting. In this approach, the number of vessel branches was counted under a dissecting microscope at a magnification of 7–10X. The branch counting was started by selecting a major vessel and tracing it to the first junction. The selected vessel branch was considered to "create" two new vessels. Each of these was counted. It typically involves branches of similar size, however, within angiogenic tissues a small neovessel will often branch from a much larger vessel. Both the small and the large vessels were nevertheless counted as "new" vessels beyond the branch. This convention increases consistency among counts by different observers. This process was repeated following the initial vessel as it continued to branch until it became impossible to observe further branches or the vessel exited the CAM tissue above the filter disk. The current vessel was then backtracked to the nearest uncounted branch and followed as above, counting branches, until again no further branching was seen. The approach was repeated in the assigned area of the CAM until all branches were counted.

Statistical analysis

All statistical analysis was computed with Statistica 6 software. Data was expressed in terms of mean ± SEM (Standard error of mean).

Results and Discussion

The investigated propolis was of *Apis florae* based on the dwarf size and based on the nature of the comb hung from slender branch less than 8 m away from ground[23]. Chemical constituents play a major role that steers the pharmacological effects of the extract[24,25]. Preliminary screening of the propolis extract revealed the presence of bioactive components of which alkaloid, flavonoids, glycosides, reducing sugars and gums, carbohydrates were the most prominent (Table 1). The total phenolic content of extract was found to be 4.751 mg/g, whereas the protein content was 1.187 g%. There is a positive correlation between phenolic content and free radical scavenging activity. This represents propolis as a health-improving protein

Table 1-Preliminary chemical screening

Tests	Inferences
Mollisch's alpha-naphthol test	Presence of carbohydrates
Ninhydrin test	Presence of free amino acids
Biuret test	Presence of proteins
Saponification	Presence of fixed oils and fats
Mayer's test	Presence of alkaloids
Wage's test	
Hager's test	
Tannic acid test	
Vitalin morin's test	Presence of tropane alkaloids
2,4-DNP test	Presence of ketones
Baeyer's test	Test for active unsaturation
Nitration test	Test for aromaticity
Bromine water test	Test for unsaturation
Jones's test	Presence of primary and secondary alcohols
Lieberman-Buchard test	Presence of phytosterols/ triterpenes
Ferric acid test	Presence of phenolic compounds
Gelatin test	
Lead acetate test	
Alkaline reduction test	Presence of flavonoids
Magnesium and hydrochloric acid reduction	Presence of flavonol glycosides

supplement. Studies conducted by Chen *et al* showed propolis collected from May to June contains high amount of propolins C, D and F[26]. Sforcin *et al* showed that ethanolic extract of the propolis obtained during summer had higher microbial action than the other seasons[27]. Ethanolic extract showed an efficient antibacterial action against both Gram positive and Gram negative strains. *E. coli* seemed to be more susceptible with a zone of inihibition of 17 mm followed by *S. pyogenes* (16±2 mm), *S. aureus* (15 mm) and *S. typhi* (14 mm), respectively. With regards to propolis solvent (70 % ethanol), the inhibitory action was much lower in the range of 6-9 mm zones. Thus, the antibacterial activity in this assay may exclusively be due to propolis components. Conversely, in a study, Massaro *et al* observed propolis to be active only against Gram positive bacteria[28]. Thus, the extent of bioactivities is likely to be linked to the different chemical compositions. Besides, it also showed antifungal activity against *C. albicans*. Similar results were reported by Salas *et al*[29].

Results of anti-oxidant activity followed a concentration-dependent pattern which amounts to 4.732 mM equivalents of ascorbic units. This effect may be due to the high concentration of phenolics and other antioxidant compounds[30]. Guo *et al* observed that anti-oxidant properties were attributed to the polyphenol content[31]. Propolis showed maximum inhibition of hypotonicity-induced HRBC membrane lysis, which indicates stabilization of HRBC membrane of about 39.13 % at 8000 µg/mL concentration. With the increasing concentration, membrane hemolysis seemed to decrease providing maximum stabilization (Fig. 1). Borrelli *et al* reported that the anti-inflammatory effects of propolis were due to the presence of Caffeic acid phenethyl ester[25]. Wagh[32] claimed phenolic compounds to be responsible for the biological activities of propolis.

Angiogenesis refers to the growth of new blood vessels from parent microvessels which are essential for normal placental, embryonic and fetal growth but almost occurs in physiological growth and during the process of wound healing[33]. It plays a central role in a variety of processes like tumor growth, atherosclerosis, psoriasis, inflammatory reactions and rheumatoid arthritis; suggesting that angiogenesis and inflammation are mutually co-dependent[34,35]. Degree of angiogenesis varied among all the four samples - erythropoietin, heparin, extract and un-inoculated control. Heparin known for suppression of neo-vascularisation showed very few branch points and blood vessels around the filter disc, although normal angiogenesis could be seen in areas away from the disc. Erythropoietin, which is known to enhance angiogenesis served as a positive control with increased branch points around the disc compared to the uninoculated control. Propolis inoculated eggs showed a decreased amount of angiogenesis (Table 2). This is indicated by few branch points in the area of the disc as in Plate 1. Thus, propolis was found to exhibit similar properties to that of heparin and be conferred as an anti-angiogenic compound. Since variations have been reported, further studies are needed to explain the actual targets in cell system involved in different biological properties.

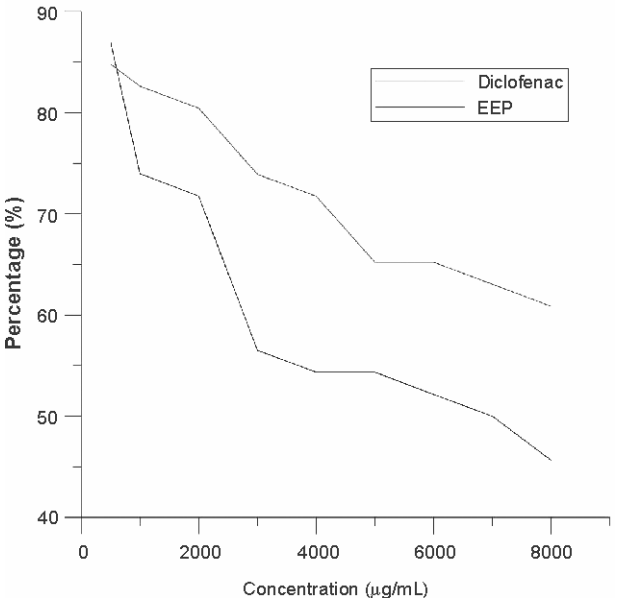

Fig. 1-Anti-inflammatory activity exhibited by EEP in comparison to diclofenac.

Table 2-Semi-quantitative evaluation of angiogenesis by counting branch points

Test agent	Average number of branch points in 20 mm area around the inoculated filter paper disc*
Saline	153
Erythropoietin (positive control)	374
Heparin (negative control)	10
Propolis	25

* rounded off to the nearest decimal

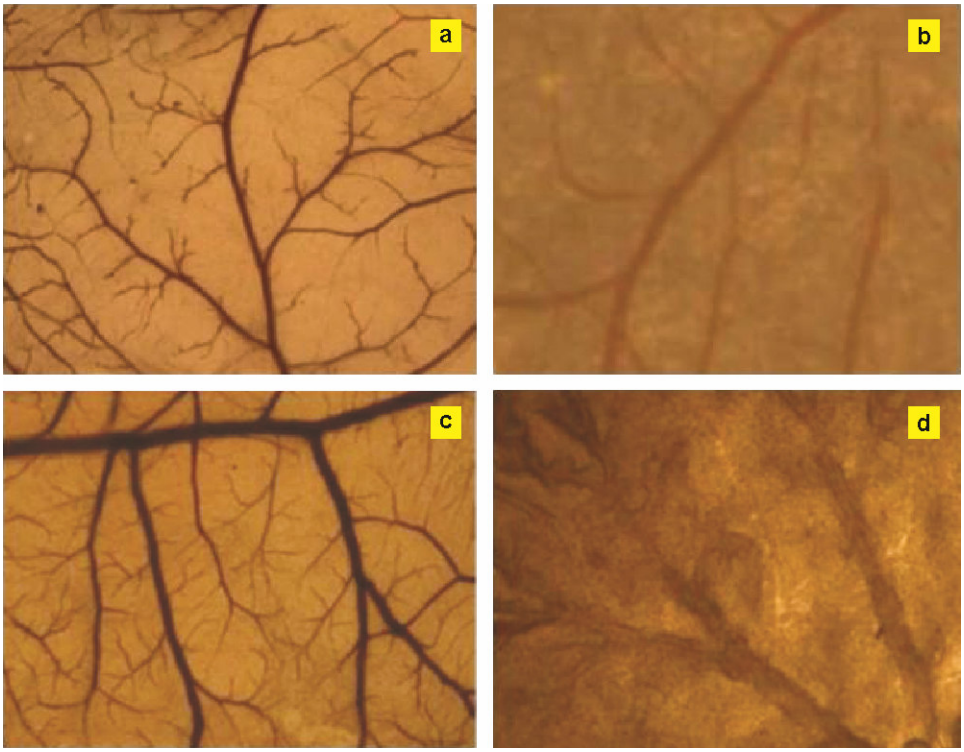

Plate 1-Effect of propolis on blood vessel density on the topical application of CAM assay a) uninoculated, b) propolis, c) erythropoietin and d) heparin

Conclusion

The results of the present study add support to the potential use of ethanolic extracts of propolis to develop therapeutic products with anti-inflammatory, antioxidant and antimicrobial properties. Further research is needed to establish variation in the single bioactive constituents and associated traditional medicine practices, thereby creating a synergy between traditional medicine and modern medicine.

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