Antimicrobial Investigation and determination of total phenolic and flavonoid contents of Indian Propolis from Satpuda Hills of Maharashtra

Vijay D Wagh* and Rameshwar D Borkar1

1Department of Pharmaceutics, R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur-425405, Maharashtra. *For correspondence e-mail: drvijaywagh@gmail.com.

ABSTRACT

Propolis is natural resinous and sticky material obtained from honey hives of Apis mellifera L bees. In the present study, we investigated the total phenolic and flavonoid contents and anti-microbial activity. Total phenolic and flavonoid contents of crude ethanolic extract were found to be 25.59 ± 0.48mg/g of Gallic acid equivalent and 4.57 ± 0.46 mg/g of Quercetin equivalent respectively. Ethanolic extract (80%) of Indian propolis shows highest inhibition against P. aeruginosa (18.6 ± 0.58 mm), S. aureus (17.3 ± 0.57 mm), E. coli (18.4 ± 0.45 mm) and C. albicans (24.3 ± 0.57 mm) at 20 mg/ml concentration which are especially responsible for ophthalmic infection and keratitis.

Keywords: Propolis, anti-microbial activity, ophthalmic keratitis

1. INTRODUCTION

Over the last few decades, worldwide increases in the use of natural products for pharmacological purposes have been observed. Propolis is resinous mixture produced by honey bees from substances collected from parts of plants, buds and exudates. Etymologically the word Propolis derived from the Greek pro (for ‘in front of’, ‘at the entrance to’) and Polis (for ‘community’ or ‘city’), meaning that this natural product is used for hive defense. Another name of propolis is honeybee glue. Due to its waxy nature and mechanical properties, bees use propolis in the construction and repair of their hives for sealing openings and cracks and smooth out the internal walls[1-4] and as a protective barrier against external invaders likes snakes, lizards, etc. or against weathering threats like wind and rain. Bees gather propolis from different plants, in the temperate climate zone, mainly from poplar. Propolis is lipophilic in nature, hard and brittle material when cold; however, when the temperature rises it becomes soft, pliable, gummy and very sticky [5]. It possesses a characteristic and pleasant aromatic smell and varies in color from yellow green to red and to be dark brown depending on its source and age [2].

The antibacterial and antifungal activities are the most popular and among the most extensively investigated biological actions of propolis [6]. The numerous authors, who have demonstrated these properties of Propolis, have done their work using propolis from different geographical locations. Nevertheless, bee glue was always active, although it
is known that in its chemical, composition varies due to the distinctive plant sources.

The proportion of the various substances presents in the propolis depends upon its place and time of collection but, in general, raw propolis is composed of around 50% resins, 30% waxes, 10% essential oils, 5% pollen and 5% of various organic compounds [6]. The chemical composition of propolis is complex, flavonoid and (hydroxyl) cinnamic acid derivatives are considered to be the primary biologically active constituents in propolis extract. Furthermore, depending on its geographical origin, its composition is highly variable. More than 300 constituents were identified in different samples [5], and new ones are still being recognized during chemical characterization of new types of Propolis [2, 7].

Although numerous researchers have been reported the biological activities of Propolis collected worldwide, information about Indian Propolis is still very least. The aim of this study is to investigate total phenolic and flavonoid contents, antibacterial and antifungal activity of Propolis samples from Satpuda hills, Maharashtra. Furthermore, we attempted to concentrate on Propolis activity against causative agents of ophthalmic Keratitis.

2. MATERIALS AND METHODS

2.1. Collection of plant material

Folin-Ciocalteu phenol reagent (Qualigen, Mumbai), Gallic acid (Fluka USA), AINO3, CH3COONa, sodium carbonate, the nutrient media used for the bacterial growth nutrient agar (Hi Media), Potato Dextrose Agar (Hi Media), all reagents used were of Analytical grade.

2.2 Microorganism strains

Bacterial and fungal strains were procured from Microbial Test Culture Collection (MTCC) Pseudomonas aeruginosa (MTCC 2453), Staphylococcus aureus (MTCC 1430), Escherichia coli (MTCC 614), and Candida albicans (MTCC 183) strains were used.

2.3 Collection and extraction of Indian propolis

Propolis is natural resinous and sticky material obtained from honey hives of Apis mellifera L bees from Satpuda hills, Maharashtra, India. Collected Propolis sample kept in the deep freezer at -20°C. Harden Propolis sample crushed and ground. Grounded Propolis was extracted with 80% of ethanol by maceration procedure for three days in the dark. Filter the mixture using Whatmann filter paper#41. Filtrate called as ethanolic extract of Propolis (EEP). Ethanol evaporated from filtrate in water bath [8]. Resinous and sticky material was obtained. This material used for the further studies.

2.3 Total phenolic and flavonoid contents

Total phenolic and flavonoid contents of the propolis extract were determined by the method already published [9, 10]. The mean of three readings was used and the total phenol and total flavonoid contents were expressed in milligrams of Gallic acid equivalents/g extract and Quercetin equivalents/g extract respectively.

2.4 Antibacterial and antifungal activity

The antimicrobial activity of the extracts was carried out by an agar well diffusion method [11, 12]. Using 100 µl of suspension containing 108 CFU/ml of bacteria spread on nutrient agar (NA) medium for a bacteria and potato dextrose medium for fungi. On the agar plate, 6mm diameter well was created using borer. The wells were impregnated with 50 µl of the different extracts in the concentration of 01, 02, 04, 05, 10 and 20 mg/ml in DMSO and Gentamycin (gentamycin sulphate IP 40 mg/ 30 ml) and clotrimazole (clotrimazole USP 150 mg/ 15 ml vial, i.e., 10 mg/ ml) used as standard antibiotics for bacteria and fungi respectively. The bacterial inoculated plates incubated as 30°C for 24h and fungi at 30°C for 72h. The antibacterial and antifungal activity measured and calculates the zone of inhibition.

2.5 Minimum inhibitory concentration

For the MIC value of the ethanolic (80%) extract of propolis was initially dissolved in DMSO and tested at the different concentration of 1 to 20 mg/ml. The test microorganisms used are P. aeruginosa, S. aureus, E. coli, and C. albicans. Sterile NA plates and Potato dextrose agar plates for bacteria and fungi were prepared respectively and the inoculums of test microorganisms were spread uniformly. Wells were prepared by using sterile borer having the diameter of 6 mm. Add 100 µl of extract solution. The plates were kept at 4°C for 20 min for the diffusion of the test solution and then place them in the incubator for 24 hrs at 37°C for bacteria and 72 hrs for fungi.

3. RESULTS AND DISCUSSION
There is an urgent need to control antimicrobial resistance by improved antibiotic usage and reduction of hospital cross infection. However, the development of new antibiotics should be continued as they are of prime importance to maintain the effectiveness of antimicrobial treatment. In developing countries, the WHO estimates that about three-quarters of the population relies on plant based preparations used in their traditional medicinal system and as the basic need for human primary health care [13].

3.1 Phenolic and flavonoid contents

Propolis shows varied compositions due to the different geographical region [14]. Total polyphenol contents were estimated with Folin–Ciocalteu spectrophotometer method [15]. Polyphenol including phenolic acids and flavonoids form a blue color complex with phosphomolybdic-phosphotungstic acid reagent (Folin–Ciocalteu reagent) with maximum absorbance at 660 nm. Quercetin was employed as a standard compound for estimation of total polyphenol contents because it is one of the most abundant phenolic acids found in propolis. In aluminum nitrate spectroscopic method, aluminum nitrate forms an acid stable complex with the keto group and either the hydroxyl group in A or C ring of flavonoids, in addition it forms acid labile complexes with orthodihydroxyl groups in the A or B ring of flavonoids. The aluminum nitrate complexes of flavonoid compounds show strong absorbance at 415 nm and flavonoids with more functional groups absorb stronger at 415 nm. We used quercetin as a standard compound because it is one of the widely spread flavonoids in propolis samples and has strong absorbance at concentrations lower than 100 ppm at 415 nm because of its more functional hydroxyl groups.

The total polyphenol contents of crude ethanolic extract was found to be 25.59 ± 0.48 mg/g of Gallic acid equivalent. The total flavonoid content of crude ethanolic extracts of Propolis samples was found to be 4.571 ± 0.46 mg/g of Quercetin equivalent. Propolis from Satpuda hills contained fewer amounts of phenolic and flavonoid contents comparing with Indian propolis from Uttarakhand [16]. Furthermore, Chinese’s propolis contains greater phenolic and flavonoid contents 174.7 ± 3.0 mg/g of Gallic acid equivalent and 45.1 ± 0.2 mg/g of Quercetin equivalent respectively [16] than that of propolis from Maharashtra.

3.2 Determination of zone of inhibition

The mean diameters of microbial growth inhibited by different concentrations of propolis extract are shown in Table 1. Higher concentration required for gram-negative bacteria [17]. Among the bacteria and fungi, C. albicans was the most sensitive to the highest concentration 20mg/ml. E. coli was shown inhibition at 4mg/ml. Propolis is mainly active in Gram-positives. However, it has been reported that EEP is effective on Gram-negative bacteria at higher concentrations [18]. The studies carried out on the antimicrobial activity of Propolis show conflicting results [1]. The variation in the antimicrobial activity of propolis has been attributed to the differences in its chemical components. Present study showed that propolis inhibited the gram-positive bacteria better than gram-negative bacteria. Generally, plant extracts are usually more active in against gram-positive bacteria than gram-negative bacteria [20].

3.3 Minimum inhibitory concentration

The minimum inhibitory concentration of 80% of EEP against S. aureus, P. aeruginosa, E. Coli and C. albicans presented in Tables 2. S. aureus, P. aeruginosa and C. albicans showed inhibition at 1mg/ml concentration. E. coli showed an inhibition zone at a concentration of 4mg/ml. From experimental results; we conclude that EEP from Maharashtra region (India) most sensitive to gram-positive bacteria and fungi. Gram-negative bacteria required a higher concentration than that of gram positive and fungi. These MIC results are different than the MICs found to be active in this study because of different methodologies to determine antibacterial activity [19].

4. CONCLUSION

India, being a vast country for ayurvedic preparations. Propolis differing in chemical compositions and medicinal values. However, unfortunately, it is still to be explored. To our knowledge, this is the first report of its kind describing the antibacterial and anti-fungal activity of (Maharashtra) Indian Propolis extracts. Our results indicate that the extracts of the propolis contain polyphenol and flavonoid compared to Gallic acid and Quercetin, respectively used as reference. This ethanolic (80%) extract was also able inhibit the bacterial and especially fungal growth.
Table 1. Zone of inhibition for 80% ethanolic extract of Propolis

<table>
<thead>
<tr>
<th>Conc. of Propolis</th>
<th>Zone of inhibition (mm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>1mg/ml</td>
<td>6.8 ± 0.29</td>
</tr>
<tr>
<td>2mg/ml</td>
<td>8.3 ± 0.58</td>
</tr>
<tr>
<td>4mg/ml</td>
<td>9.6 ± 1.53</td>
</tr>
<tr>
<td>5mg/ml</td>
<td>16.6 ± 2.52</td>
</tr>
<tr>
<td>10mg/ml</td>
<td>15.7 ± 0.56</td>
</tr>
<tr>
<td>20mg/ml</td>
<td>17.3 ± 0.57</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>22.4 ± 0.78</td>
</tr>
<tr>
<td>Ketokonazole</td>
<td>-</td>
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</tbody>
</table>

Values are in mean± SEM of three separate determinations

Table 2. Minimum inhibitory concentration of ethanolic extract of Propolis

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>S. aureus</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
<th>C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of Propolis</td>
<td>1mg/ml</td>
<td>1mg/ml</td>
<td>2mg/ml</td>
<td>1mg/ml</td>
</tr>
<tr>
<td>Growth inhibition (mm)</td>
<td>6.8 ± 0.29</td>
<td>6.7 ± 0.58</td>
<td>6.7 ± 0.77</td>
<td>6.6 ± 0.55</td>
</tr>
</tbody>
</table>

Values are in mean± SEM of three separate determinations

The spectrum of action of the extracts is broad because it covers the Gram negative and positive bacteria. Based on the results, it can be concluded that the propolis extracts have been great potential as antimicrobial components against microorganisms, and they can be used in the treatment of infectious diseases caused by microorganisms, especially Ophthalmic Keratitis. Further work is needed to isolate the secondary metabolites from the extracts studied in order to test the specific antibacterial activity and the underlying mechanisms.

Conflict of Interest
The authors declare that they have no conflicts of interest.

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