ANTIBACTERIAL AND ANTITUMOR ACTIVITY OF METHANOLIC EXTRACT OF PROPOLIS FROM MEGHALAYA

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ABSTRACT

Background: Propolis is a beehive product with a very complex chemical composition, widely used in folk medicine because of its several pharmacological activities. Propolis is used by bees to block holes and cracks, to repair combs, to strengthen thin borders of the comb, and for making the entrance of the hive weather tight. Propolis has attracted much attention in recent years as a useful substance having application in medicine and cosmetics. Aims: To evaluate the antibacterial and antitumor activity of methanolic extract of propolis from Meghalaya (India). Results: The antibacterial activity of methanolic extract of propolis against four bacterial strains Bacillus cereus MTCC430, Staphylococcus aureus MTCC96, Salmonella enterica MTCC735 and Escherichia coli MTCC730 revealed that propolis is more effective against gram positive bacteria as compared to gram negative. The antitumor activity against murine ascites Dalton’s lymphoma tumor was also studied and the results showed that propolis induces apoptosis in tumor cells in a time-dependent manner. Conclusion: From the results obtained it can be concluded that propolis could be useful as an antibacterial as well as anticancer chemotherapeutic agent.

KEY WORDS: Propolis, Dalton’s lymphoma, antibacterial, antitumor, apoptosis.
INTRODUCTION
Propolis is a strongly adhesive resinous substance, collected, transformed and used by worker bees to seal holes in their honeycombs to smooth out the internal walls and protect the entrance against intruders.[1] The name is derived from Greek word pro-, for defense, and polis-, the city, that is, defense of the city (or the hive). It was used by human beings since ancient times for its pharmaceutical properties. Propolis has a complex chemical composition[2-3] and has been the subject of many studies due to its diverse biological activities such as antibacterial, antiviral, anti-inflammatory, anticarcinogenic and immunomodulatory activities.[4-12] Fernandes et al[13] demonstrated the antibacterial and antifungal activities of propolis, comparing the susceptibility of microorganisms to different concentrations. Despite its many uses, scientific research on propolis is still limited. Research are not exactly sure how it works, but the bee product does appear to provide protection from some bacteria, viruses and fungi. The antitumor activity has also been investigated and the main mechanisms by which propolis affects tumor cells are related to the inhibition of cell growth and to apoptosis.[14] Though the antibacterial and antitumor activity of propolis has been extensively reported[15-16] the pharmacological properties of propolis from Meghalaya have not been studied. Thus, the present work was undertaken to examine the antibacterial and antitumor activity of methanolic extract of propolis collected from Ngunraw village of South West Khasi Hills district of Meghalaya, India. The antibacterial and antitumor activities were evaluated against different bacteria isolates (Gram positive and Gram negative) and murine ascites Dalton’s lymphoma respectively.

MATERIALS AND METHODS
Chemicals
Acridine orange and ethidium bromide were purchased from Sigma Chemical Co., St. Louis, Mo, USA. Methanol and other chemicals used in the experiments were of analytical grade and purchased from SRL Pvt. Ltd., Mumbai, India.

Propolis collection and preparation of methanolic extract
Raw propolis was collected from Ngunraw village, South West Khasi Hills district of Meghalaya during spring and winter seasons of 2014. It was cleaned, freed from wax and its methanolic extract was prepared following the method of Nagai et al[17] with slight modifications. Thirty grams of raw propolis was taken and Dissolved in 300 ml of 70% methanol with continuous stirring for 24 hours, followed by filtration (Whitman filter paper)
and evaporation. The crude extract obtained was dissolved in phosphate buffer saline (pH 7.4). Stock solutions were used to prepare the requisite dilutions.

**Animals and tumor maintenance**
Mice colony was maintained under conventional laboratory conditions at room temperature of 22±2°C keeping 5-6 animals per propylene cage using paddy husk as bed with food pellets (Amrut Laboratory animal feeds, New Delhi) and drinking water *ad libitum*. Inbred Swiss albino male mice in the age group of about 10-12 weeks weighing about 25-28 g were used for the experiments.

Ascites Dalton’s lymphoma (DL) is being maintained *in vivo* in mice of both sexes by serial intraperitoneal (i.p.) transplantations of approximately $1 \times 10^7$ viable tumor cells per animal (0.25 ml in phosphate-buffered saline (PBS), pH 7.4). Tumor transplanted hosts usually survive for 19-21 days. The maintenance, use of the animals and the experimental protocol of the present study was approved by the Institutional ethical committee, North-Eastern Hill University, Shillong.

**Drug treatment**
Different doses of methanolic crude extract of propolis (i.e., 20, 50 and 100μg/ml) were used for the assessment of antibacterial activity.

Depending on the preliminary screening results for antitumor activity using different doses of methanolic crude extract of propolis (i.e., 10, 20, 50, 75 and 100 mg/kg body weight) in DL-bearing mice, the most potent dose of the propolis extract (i.e., 50 mg/kg body weight) showing maximum survival was selected for the apoptosis study.

**Assessment of antibacterial activity**
Antibacterial activity of propolis was examined using agar well diffusion method\textsuperscript{[18]} against pathogenic bacterial strains, *Bacillus cereus* MTCC430, *Staphylococcus aureus* MTCC96, *Salmonella enterica* MTCC735 and *Escherichia coli* MTCC730. The test organisms were spread on the surface of Müeller-Hinton Agar (MHA) (Himedia, India) using a sterile swap stick. Wells of 3 mm in diameter were cut into MHA plates by using a sterilized tip and 70μl of the extract was placed into each well. The plates were pre-incubated for two hours for diffusion and incubated aerobically overnight at 37°C. These assays were performed in...
triplicates. Zones of inhibition were measured and the mean value was obtained.\textsuperscript{19} Positive control was carried out under similar conditions by using chloramphenicol 30 mcg.

**Apoptosis study using fluorescence microscopy**

Fluorescence-based determination of apoptosis in DL cells was done using acridine orange and ethidium bromide (AO/EtBr) staining method as described by Baskic et al\textsuperscript{20} and also used earlier in our laboratory.\textsuperscript{21} After treatment of tumor-bearing mice with propolis (50mg/kg body weight) \textit{in vivo} for five consecutive days, DL cells were collected after 24, 48, 72 and 96 h, washed with PBS and treated with AO/EtBr (100 μg/ml PBS of each dye). The cells were thoroughly examined under a fluorescence microscope (Leica), photographed and compared with that of control. Viable cells’ nuclei stain green due to permeability of only acridine orange whereas, apoptotic cells appear red/orange due to co-staining of both the fluorescent dyes. Based on the scores of apoptotic and viable cells under the microscope, apoptotic index was determined.

**Statistical analysis**

The results were expressed as mean±S.D. The data were statistically analyzed using one-way analysis of variance (ANOVA) with Tukey’s multiple comparison post hoc tests to compare the level of significance between control and experimental groups. A $P$-value $<$ 0.05 was considered as statistically significant in all cases.

**RESULTS**

**Antibacterial activity**

The antibacterial potential of methanolic extract of propolis is evaluated according to their zone of inhibition against various test organisms. The zones of inhibition were calculated and compared with the activity of the standard, (positive control). In the wells containing propolis extract the zone of inhibition was seen to be maximum (18.33 mm) against \textit{B. cereus} MTCC430 at a concentration of 100µg/ml (Fig. 1Ab) and the minimum zone of inhibition was seen against \textit{S. enterica} MTCC735 at 20µg/ml concentration (Table 1, graph not shown as zero value is neglected).

As compared with the control of each bacterial isolates (Gram positive and Gram negative bacteria) it is observed that the Gram negative bacteria, \textit{S. enterica} and \textit{E. coli} are less susceptible to propolis and was only inhibited at a higher propolis concentrations (Fig. 1B). The zone of inhibition decreases with decrease propolis concentration i.e. at 50µg/ml and
20µg/ml respectively. On the other hand, it is seen that in gram positive bacteria *S. aureus* and *B. cereus*, the zone of inhibition shows a slight decrease as compared to the positive control indicating that they are highly vulnerable to propolis.

**Table 1:** *In vitro* antibacterial activities of different concentrations of methanolic extract of propolis on gram positive and gram negative bacteria by agar well diffusion method

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Bacterial isolates</th>
<th>Control 30 mcg</th>
<th>Propolis 100 µg/ml</th>
<th>Propolis 50 µg/ml</th>
<th>Propolis 20 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>B. cereus</em> MTCC430</td>
<td>19.55 ± 0.5</td>
<td>18.33 ± 0.5</td>
<td>15.33 ± 0.57</td>
<td>13.33 ± 0.57</td>
</tr>
<tr>
<td>2</td>
<td><em>S. aureus</em> MTCC96</td>
<td>16.66 ± 0.57</td>
<td>13.33 ± 0.57</td>
<td>13.33 ± 0.57</td>
<td>10 ± 0.00</td>
</tr>
<tr>
<td>3</td>
<td><em>S. enterica</em> MTCC735</td>
<td>25.33 ± 0.28</td>
<td>12.33 ± 0.57</td>
<td>10 ± 0.00</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td><em>E. coli</em> MTCC730</td>
<td>23.66 ± 0.57</td>
<td>16.33 ± 0.57</td>
<td>14 ± 0.57</td>
<td>10 ± 0.00</td>
</tr>
</tbody>
</table>

Values represent mean ±SD., (n=3) (control= chloramphenicol 30µg )

Fig. 1A: Zone of inhibition around the wells with methanol extract of propolis (100µg/ml, 50µg/ml & 20µg/ml, (a) *S. aureus* MTCC96, (b) *B. cereus* MTCC430, (c) *S. enterica* MTCC735, (d) *E. coli* MTCC 730.
Fig. 1B: Antibacterial activity of propolis showing maximum and minimum zone of inhibition (bar not shown for zero value).

Apoptosis study
As illustrated in Fig. 2A, light green nuclei (viable cells) with normal membrane structure were seen in the control group (Fig. 2Aa). Different apoptotic features were observed in DL cells at different time intervals after propolis treatment and these include membrane blebbing, nuclear condensation (Fig. 2Ab) and fragmentation (Figs. 2Ac-e), membrane disintegration and appearance of cytoplasmic vacuoles (Figs. 2Cc-d). A time-dependent increase in apoptotic features were observed in DL cells during 24 to 96 h of propolis (50 mg/kg body weight) treatment. Determination of apoptotic index revealed that as compared to control, the number of apoptotic cells significantly increased in propolis treated group during 24 to 96 h of treatment (Fig. 2B).
Fig. 2A: Acridine orange-ethidium bromide staining of Dalton’s lymphoma (DL) cells under different treatment conditions in vivo. Control DL cells (a), propolis treatment (50 mg/kg body weight) for 24 h (b), 48 h (c), 72 h (d) and 96 h (e).

Fig. 2B: Apoptotic index (percent apoptotic cells) of Dalton’s lymphoma (DL) cells from control and treated mice. Results are expressed as mean±S.D. n = 3, Tukey test, # P < 0.05 as compared to control.
DISCUSSION

As a natural honeybee hive product, propolis extracts have been used both internally and externally for thousands of years as a healing agent in traditional medicine. People may take in some propolis when they eat honey. The composition and biological properties of propolis can vary depending on the bee species and what trees and flowers they have access to.\[^{11}\] In the present study, raw propolis was collected from Ngunraw, South West Khasi Hills district of Meghalaya, India. The village is approximately 135km away from the Main city Shillong and covers about 10,000 square metres with 400-450 houses and agriculture is their main source of livelihood. The most common bee species that the people in this area are rearing is *Apis cerena indica*. It was studied and observed that bees collect nectar from the surrounding area which mainly consists of citrus flowers and a yellow flowering plant named *Inula cappa* from the family Asteraceae.

There are some reports on the antibacterial activity of propolis.\[^{22-26}\] Grange and Davey,\[^{24}\] observed a marked action of propolis against Gram-positive bacteria and limited activity against Gram-negative bacteria. Dobrowolski et al\[^{25}\] and Woisky et al\[^{26}\] also obtained similar results to support the hypothesis that propolis is active mainly against Gram-positive bacteria. In the present study, it was observed that propolis exhibited antibacterial activity against four bacterial isolates *Bacillus cereus* MTCC430, *Staphylococcus aureus* MTCC96, *Salmonella enterica* MTCC735 and *Escherichia coli* MTCC730. Further, our findings also support that the antibacterial effect was more pronounced on gram positive bacteria (*Staphylococcus aureus and Bacillus cereus*) than on gram negative bacteria (*Salmonella enterica and Escherichia coli*). Many studies have shown that fatty acid esters, phenolic compounds and cinnamic acid were the main propolis constituents and some of them were shown to possess antibacterial activity.\[^{22, 27, 28}\] Most of the antibacterial, antifungal and anti-inflammatory activity of propolis is due to the presence of polar compounds, mainly phenols (flavonoids, phenolic acids and their esters) and aromatic acids (caffeic acid and p-coumaric acid).\[^{27, 29}\]

Variations in the susceptibility to propolis among several microorganisms have been reported, but not their mechanisms of action, further studies are needed to explain whether the cell structure and permeability to such compounds or even specific targets in the cell enzymatic systems are involved in the microbial susceptibility. The most possible explanation for the low sensitivity of Gram negative bacteria to propolis extract is that, their outer
membrane inhibits and/or retards the penetration of propolis. Another reason is their possession of multidrug resistance (MDR) pumps, which extrude amphiphatic toxin across the outer membrane.\[30\] It is evident that the mechanism of action of propolis on bacterial cell is complex and in simple comparison cannot be made to the mode of action of any classic antibiotic components.

Majority of treatment regimens for cancers produce side effects, hence extremely unpleasant for patients. Scientists have spent therefore efforts to develop new therapies for the treatment of cancer so as to reduce the side effects. Propolis has been a subject of intense research, especially in the areas of anticancer research.\[31-33\] Murine ascites Dalton's lymphoma has been used to evaluate the anticancer effects of different drugs.\[21, 34, 35\] Methanolic extract of propolis caused the development of apoptotic features in murine ascites DL cells at different time intervals of treatment and these include membrane blebbing, nuclear condensation (Fig.2Ab) and fragmentation (Figs.2 Ac-e), membrane disintegration and appearance of cytoplasmic vacuoles (Figs.2Ac-d). A time-dependent increase in apoptotic features were observed in DL cells during 24 to 96 h after propolis treatment. Determination of apoptotic index revealed that as compared to control, the number of apoptotic cells significantly increased in propolis treated group of mice. The study by Motomura et al\[36\] indicated that methanol extracts of propolis increased apoptosis in U937 cells due to activation of caspase-3 and down-regulation of Bcl-2 protein. The over activity of caspase-6 induced by EEP is stronger than the induced activity of caspase-8 and -9 in MCF-7 cells, which confirms the involvement of intrinsic caspase pathway of apoptosis and the antitumor activity of propolis.\[37\] The ethanolic extract of propolis (EEP) inhibited the growth and proliferation of AGS human gastric cancer cell line. The antiproliferative effects were revealed in a dose and time-dependent manner.\[38\]

The detailed molecular mechanism(s) involved in the antitumor activity of propolis against Dalton’s lymphoma cells may be elucidated.

CONCLUSION

It may be concluded that the methanol extract of propolis was active against gram positive bacterial isolates showing the maximum zone of inhibition of 18.33 mm at 100µg/ml. *B. cereus* was more sensitive to propolis extract as compared to others. The antitumor activity of propolis (50 mg/kg body weight) showed more apoptotic activities at 96 h of treatment which suggests that propolis may have efficient cytotoxic effect on cancer cells.
ACKNOWLEDGMENTS
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REFERENCES


