



Antioxidative Analysis and Antagonistic Activity of *Apis* and *Trigona* Propolis Collected from Different Geo Locations

H.R. Bhargava, S. Elakkiya, K. Mathumitha, E. Keerthika, Uma Rani and Varsha Uma Eswaran

School of Chemical and Biotechnology,
SASTRA University, Tirumalaisamudram, Thanjavur 613401, Tamilnadu, India

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Abstract: Propolis is a resin like substance collected from various botanical sources. It is commonly used as a bee product for the enhancement of human health. It is rich in antioxidants due to which their therapeutic value is enhanced. It is well known for the treatment of inflammation, minor burns, wounds, ulcers and certain cancers and also has an excellent anti-microbial activity against different pathogens. These properties majorly are dependent on the floral sources of different geographical areas. Present work investigate on antioxidative and antagonistic activity of ethanol extracted propolis collected from *Apis* and *Trigona* species. The anti-oxidative potential of propolis samples were determined using the Ferric Reducing Antioxidant Power (FRAP), Total Phenolic content (TPC) and antiradical scavenging activity. The results revealed that the 5 and 10% *Apis mellifera* propolis samples had maximum antioxidative and antagonistic property compared to that of *Trigona* propolis. From this it is evident that samples collected from Karnataka have the medicinal property; can be used as an alternative medicine for treating the selected pathogens and promoting the use of natural bee products.

Key words: Ethanol extracted Propolis • *Apis mellifera* • *Trigona* • Antioxidative and antagonistic activity

INTRODUCTION

Propolis also known as bee glue is a resin which is collected by honey bees from different plant sources [1]. It is used as a building material by the honey bees but it is also chemical weapon of bees against pathogens [2]. It is rich in flavonoids [3- 5]. This dark brown substance is collected and used by the bees to seal the hive and provides protection against microbial and arthropod intruders and maintains a healthy environment in the hive [2]. It is a complex mixture of phenols, tannins, polysaccharides, terpenes, aromatic acids and aldehydes along with many other compounds [6, 7]. The chemical composition, colour and aroma of propolis depend upon the plants growing in different geographical zones [8]. Source of collection and age affect its colour ranging from yellowish green to dark brown [9]. Propolis is also been used as a medicine by humans since ancient times.

Propolis is the most powerful antioxidant bee product considering the presence of phenolics in high concentrations [10]. Studies showed that the antioxidative property of propolis belonging from different

geographical and botanical regions showed a strong relation with total polyphenolic concentration [11]. Propolis is known to have antagonistic activity against pathogens and shows high anti-oxidative and anti-inflammatory properties [3]. Its application does not inhibit or resists the growth of microflora which is inhabitants to human body [12]. It is an antihypertensive agent and used as a stimulant to the immune system [9]. It acts as a very powerful natural antibiotic [13] and is used to combat respiratory infections like common cold and influenza virus [14]. Propolis is also known as an efficient fungicide which has shown its potency towards *Candida albicans*, *Aspergillus niger*, *Botrytis cinerea* and other fungi species [15]. Lalitha *et al.* [16] reported antimicrobial activities of *Solanum torvum* Swart against pathogenic bacteria; also, Zurida *et al.* [17] demonstrated antifungal activity of *cinnamomum iners* wood due to its phenolic content and radical scavenging activity which is used for the medical purposes.

Although many research work have been reported on the biochemical and antagonistic activity of propolis collected from different locations of the world but the

Corresponding Author: H.R. Bhargava, School of Chemical and Biotechnology, SASTRA University, Tirumalaisamudram, Thanjavur 613401, Tamilnadu, India.

research on Indian propolis that too from Karnataka are still limited. The main objective of the present study was to investigate the biochemical and antagonistic activity of *Apis* and *Trigona* propolis samples from different locations of Karnataka.

MATERIALS AND METHODS

Propolis Collection: The raw propolis samples of *Apis* and *Trigona* species were collected from the University of Agricultural Sciences beekeepers, Bangalore and also from the commercial beekeepers, Karnataka. Later the collected propolis samples were stored at room temperature in sterilized polythene bags till processing.

Ethanollic Extraction of Propolis (EEP): The collected propolis samples of *Apis* and *Trigona* species were further processed to obtain the Ethanollic extraction of propolis. The propolis was extracted at 37°C with 95% v/v ethyl alcohol, in a conical flask for four days, using a shaker. The ethanollic extract was then filtered thrice through a Whatman filter paper No. 4 and evaporated on a rotary evaporator, under reduced pressure at 60°C. A 5 and 10 % propolis ethanollic extract (PEE) from the propolis stock sample was prepared for further investigation. The samples were sealed air tight in air-dried and sterile containers and were stored in dark condition at room temperature (37°C).

Biochemical Analysis of EEP (Ethanollic Extraction of Propolis)

Estimation of Protein Content: Estimation of protein content present in the EEP was carried out using Lowry's method, based on the formation of a copper-protein complex and the reduction of phospho-molybdate and phospho-tungstate present in Folin-Ciocalteu reagent to hetero-polymolybdenum blue and tungsten blue, respectively. Bovine serum albumin (0-100 mg/ml) was used as a standard for preparing the calibration curve.

Total Phenolic Content (TPC): The total phenolic content was determined by Folin-Ciocalteu method [18]. A 30 µL of each propolis sample (0.1 g/mL) was mixed with 2.37 mL of distilled water taken in test tubes and 150 µL of 0.2 N F-C reagents was added to the mixture. The mixture was thoroughly mixed by vortex and incubated for 2 minutes at room temperature. A 450 µL of sodium carbonate solution (0.2 g/mL) was added to the reaction mixture then incubated for 2 hours at room temperature; then the absorbance was determined in spectrophotometer at

wavelength of 765 nm. Gallic acid was used as reference. The test was repeated for each propolis sample in triplicate.

Anti-oxidative Analysis of EEP (Ethanollic Extraction of Propolis)

Ferric Reducing Anti-oxidant Power (FRAP): The Ferric reducing anti oxidant power was assessed according to [21]. To 1 mL of propolis sample 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferric cyanide (1%) was added and incubated it for 20 minutes at room temperature. Then 2.5 mL of trichloro acetic acid (10%) was added and centrifuged at 3000 rpm for 10 minutes. Later 2.5 mL of supernatant was taken after centrifugation. Then, added 2.5 mL of distilled water and 0.5 mL of ferric chloride; the absorbance was recorded at 700 nm. Ascorbic acid was used as reference standard. The test was carried out in triplicate for each propolis sample.

Antiradical Scavenging Activity by DPPH: The reaction mixture contained 1.5 mL of ethanol, 0.5 mM DPPH and propolis samples. After 1 hour of incubation at room temperature in a dark place, the absorbance was read at 517 nm. Control solution had ethanol and DPPH. Results were expressed as percentage decrease with respect to control values [22].

Antimicrobial Activity of Propolis Samples

Preparation of Test Organisms: Antimicrobial activity was conducted by obtaining microorganisms from the Department of Microbiology, School of Chemical and Biotechnology, SASTRA University. *Bacillus subtilis* and *Escherichia coli* were taken as test organisms. The isolates were identified based on standard microbiological techniques and cultured in nutrient agar media at 37°C for 24 hours. Colonies of fresh cultures of the different microorganisms from the overnight growth were picked with sterile inoculating loop and suspended it in 3-4 mL nutrient broth contained in sterile test tubes and incubated it for about 18 hours at 37°C.

Antimicrobial Assay Test by Well-Diffusion

Technique: Antagonistic activity of different dilutions of propolis samples was carried out by well diffusion method against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis*. The test organism was spread plated taking 100 µL of culture and allowed to dry at room temperature for 30 minutes. An equidistant well of mm in diameter were punched

using a sterile 1000 μL pipette tips at different sites on the plates. A 100 μL of the different concentrations (25, 50, 75 and 100% v/v) of the ethanolic extracted propolis samples were separately placed in the different punched wells with 100 μL pipette. Kanamycin (50 $\mu\text{g}/\mu\text{L}$ concentration) was used as a standard antibiotic and the control was autoclaved distilled water (10 μL). The plates were kept for incubation at optimum conditions (37°C) for 24 hours. Clear zones of inhibition around the wells indicated the presence of antimicrobial activity. The zone diameters of inhibition (ZDI) were measured in millimeter, including the diameter (10 mm) and depth (6 mm) of the well were also recorded. The antimicrobial test was carried out for each propolis sample in triplicates and the mean, standard deviation was calculated and graphically represented.

RESULTS

Biochemical Analysis of EEP (Ethanolic Extraction of Propolis)

Protein Content: The protein content ranged from 4983.33 \pm 1.51 mg/g in 5% EEP of *Apis mellifera* propolis to 6120 \pm 1.66 mg/g in 10% *Apis mellifera* propolis. The 5% EEP of *Trigona* propolis was 5280 \pm 1.57 mg/g and in 10% EEP of *Trigona* propolis was 5630 \pm 2.39 mg/g (Table 1 and Figure 1). BSA (Bovine Serum Albumin) was used as standard for plotting the graph. Propolis mainly consists of plant resins, exudates that they gather, wax, secretions and pollen. The propolis is recorded to contain 1g of protein content in 100g of propolis [21].

Total Phenolic Content (TPC): The total phenolic content of the EEP samples ranged from 1.24 \pm 0.04 to 2.35 \pm 0.58 mg/L. The highest was recorded in 5% EEP of *Apis mellifera* propolis with 2.35 \pm 0.58 mg/L (Table 1 and Figure 2). The standard calibration curve was obtained by plotting different concentrations of Gallic acid against absorbance that was read at 765 nm. The results were compared with Turkish propolis where 48.7 (mg GA/g propolis) and 9.2 (mg GA/g propolis) are present in dimethyl sulfoxide (DMSO) extracted and water extracted propolis, respectively [22].

Anti-oxidative Analysis of EEP (Ethanolic Extraction of Propolis)

Ferric Reducing Anti-oxidant Power (FRAP): The ferric reducing/antioxidant power (FRAP) assay for the 5 and 10% EEP samples ranged from 0.49 to 3.75 mg/mL. Propolis samples that showed high reducing power had a high absorbance value at 700nm. This indicates that if the

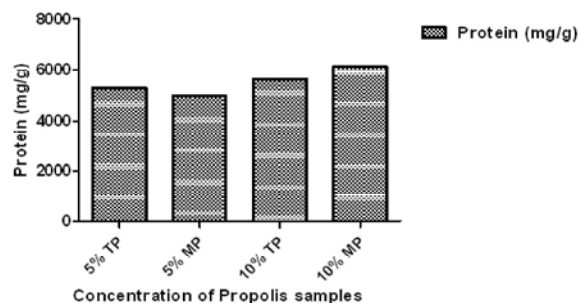


Fig. 1: Protein content present in different concentrations

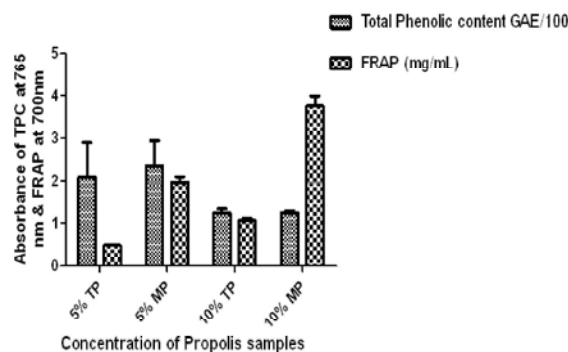


Fig. 2: Total phenolic content and FRAP content present in different concentrations of propolis samples

Table 1: Biochemical analysis of EEP (Ethanolic extraction of propolis)

Sl No.	Sample	Protein (mg/g)	Total Phenolic content GAE/100gm
1	5% TP	5280 \pm 1.57	2.09 \pm 0.80
2	5% MP	4983.33 \pm 1.51	2.35 \pm 0.58
3	10% TP	5630 \pm 2.39	1.24 \pm 0.10
4	10% MP	6120 \pm 1.66	1.24 \pm 0.04

Table 2: Anti-oxidative analysis of EEP (Ethanolic extraction of propolis)

Sl. No.	Sample	FRAP (mg/mL)	DPPH (g/mL)
1	5% TP	0.49 \pm 0.01	79.6 \pm 2.07
2	5% MP	1.97 \pm 0.13	81.4 \pm 1.47
3	10% TP	1.07 \pm 0.05	59.3 \pm 2.4
4	10% MP	3.75 \pm 0.23	59.3 \pm 2.4

absorbance value is high; then there is more reduction of ferric ions to ferrous ions. The 10% *Apis mellifera* propolis showed the highest reducing power which is 3.75 mg/mL (Table 2 and Figure 2). The results were compared with the Turkish propolis where 59.5mg (Tro/g propolis) and 24.1mg (Tro/g propolis) are present in DMSO extracted and water extracted propolis, respectively [22].

Anti-radical Scavenging Activity (DPPH Assay): The free radical scavenging activity of 5 and 10% EEP samples were done by the DPPH (2,2-diphenyl-1-picrylhydrazyl) which is a stable nitrogen centred radical. For high DPPH

scavenging activity, high is the antioxidant capacity of the sample. The DPPH scavenging activity percentage of the propolis samples ranged from 59.3 ± 2.4 to 81.4 g/mL and the percentage was high in the 5% *Apis mellifera* propolis followed by 5% *Trigona* propolis ie, 79.6 g/mL (Table 2 and Figure 3). All samples had antiradical scavenging activity greater than 50%.

Antimicrobial Activity of Propolis Samples: The antibacterial activity of EEP samples was tested against *Escheriachia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis*. The 75% dilution of 5%

Trigona propolis sample revealed that the maximum inhibitory zone with 26.33 ± 3.78 mm was recorded for *Pseudomonas aeruginosa* (Table 3 and Figure 4) and 75% dilution of 5% *Apis mellifera* propolis sample revealed that the maximum inhibitory zone with 25.33 ± 4.73 mm was recorded for *Bacillus subtilis* (Table 4 and Figure 4). The results revealed that the maximum inhibitory zone with 32 ± 2 mm was recorded for *Escheriachia coli* strain in 10% *Trigona* propolis sample at 100% dilution (Table 5 and Figure 4). The 75% dilution of 10% *Apis mellifera* propolis sample showed maximum inhibitory zone with 32 ± 2.65 mm for *Staphylococcus aureus* (Table 6 and Figure 4).

Table 3: Minimum inhibitory concentrations of Propolis samples of 5% *Trigona* Propolis showing inhibitory zones in 'mm' with control organisms

5% <i>Trigona</i> Propolis sample				
Diameter of zones of inhibition (mm)				
Bacterial Strains				
Propolis % (v/v)	<i>E. coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>
25	8 ± 0.5	8.17 ± 1.61	12.67 ± 0.58	14.33 ± 3.21
50	10.17 ± 2.02	14.17 ± 0.76	17.67 ± 0.58	15.67 ± 1.15
75	10.67 ± 1.04	12.67 ± 2.47	26.33 ± 3.78	21.33 ± 2.73
100	13 ± 2.18	12.83 ± 1.04	20 ± 3.46	23 ± 4.58
Kanamycin	16.67 ± 1.53	16.67 ± 2.31	18.33 ± 1.53	14 ± 1

Table 4: Minimum inhibitory concentrations of Propolis samples of 5% *Apis mellifera* Propolis showing inhibitory zones in 'mm' with control organisms

5% <i>Apis mellifera</i> Propolis sample				
Diameter of zones of inhibition (mm)				
Bacterial Strains				
Propolis % (v/v)	<i>E. coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>
25	8.5 ± 1.32	8.83 ± 1.44	9.5 ± 1.5	7.33 ± 0.58
50	10.17 ± 1.53	10.5 ± 1.32	9.83 ± 3.17	9 ± 1
75	15.67 ± 1.15	12.5 ± 0.5	13.33 ± 1.04	14 ± 0.5
100	13.33 ± 2.02	10.67 ± 1.04	12.83 ± 2.52	13.83 ± 0.29
Kanamycin	7.83 ± 1.04	19 ± 1.73	18.33 ± 0.58	15.33 ± 1.15

Table 5: Minimum inhibitory concentrations of Propolis samples of 10% *Trigona* Propolis showing inhibitory zones in 'mm' with control organisms

10% <i>Trigona</i> Propolis sample				
Diameter of zones of inhibition (mm)				
Bacterial Strains				
Propolis % (v/v)	<i>E. coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>
25	13.33 ± 1.15	15.33 ± 2.08	17 ± 1	13.67 ± 1.15
50	21.67 ± 1.53	20 ± 3.61	17.33 ± 1.53	15 ± 1
75	26 ± 4	21.33 ± 7.64	20.33 ± 2.89	19.33 ± 2.89
100	32 ± 2	23.33 ± 5.69	20 ± 3.60	20.67 ± 4.04
Kanamycin	13 ± 1.73	18 ± 1	15.33 ± 2.08	14 ± 1.73

Table 6: Minimum inhibitory concentrations of Propolis samples of 10% *Apis mellifera* Propolis showing inhibitory zones in 'mm' with control organisms

10% <i>Apis mellifera</i> Propolis sample				
Diameter of zones of inhibition (mm)				
Bacterial Strains				
Propolis % (v/v)	<i>E. coli</i>	<i>S.aureus</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>
25	11.17±3.40	16.67±2.52	10.17±0.76	9.5± 2
50	12.33±2.52	15.33±2.08	12.83±1.04	9±1.80
75	19.33±3.06	32±2.65	15.83±0.76	14±1
100	18±4.36	26.67±4.73	17.5±0.5	16.67± 1.53
Kanamycin	15.67±4.04	15.33±2.08	17.67±2.52	14.67±2.08

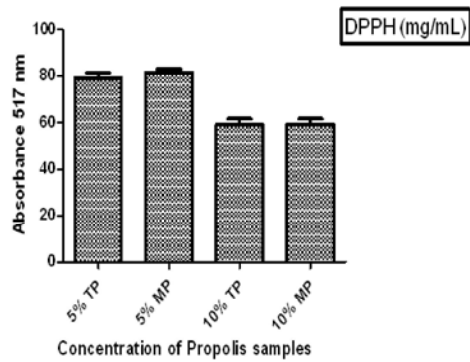


Fig. 3: DPPH antiradical scavenging activity of different concentrations of propolis samples

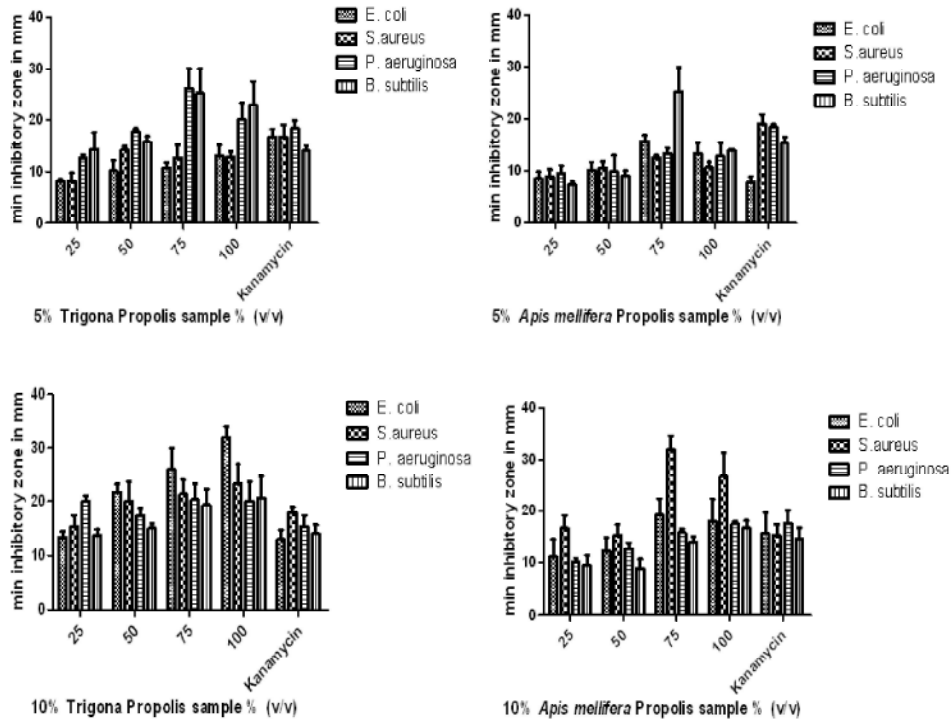


Fig. 4: Antimicrobial activity of different concentrations of propolis samples against selected bacterial strains

DISCUSSION

Biochemical Analysis of Propolis Samples: One of the main components of propolis is phenolics which include flavones, flavanones, phenolic acids and their esters [21]. These polyphenols and flavonoids have major contributions to the activities such as antimicrobial, antioxidative, anti-tumor, anti-inflammatory and anti-allergic activities [10]. The results revealed that 10% *Mellifera* propolis, obtained from *Apis* species, had more protein concentration than that of the 10% *Trigona* propolis from *Trigona* species. On the other hand, 5% *Trigona* propolis showed more protein concentration than that of *Mellifera* propolis. Factors such as geographical and botanical origin account to the varied composition of propolis [21].

Anti-oxidative Activity of Propolis Samples: Propolis is one of the natural products with strong potency as an antioxidant. As a result of the biochemical reactions taking place in body, free radicals and reactive oxygen species (ROS) are formed continuously in cells [23, 24]. Free radicals such as superoxide, hydroxyl radical, hydrogen peroxide and singlet oxygen [5-29] induce oxidative stress that cause damage to biomolecules such as carbohydrates, lipids, amino acids and nucleic acids [30-32]. It is believed that oxidative stress plays a vital role in the development of degenerative and chronic diseases [33]. The present study revealed that the FRAP value of 10% *Apis mellifera* propolis was high. 5% *Apis mellifera* propolis showed better result than that of the Propolis from *Trigona* species and was observed to have the highest anti-radical scavenging activity followed by 5% *Trigona* propolis. The anti-radical scavenging activity of the propolis samples were greater than 50%.

Anti-microbial Activity of Propolis Samples: The anti-microbial activity was highly potent in propolis among all the bee products [21]. There are several factors which account to the antagonistic activity of propolis. These factors include its physicochemical properties, botanical origin, entomological origin and symbioses with beneficial bacteria. The antimicrobial effect on *Apis mellifera* and *Trigona* propolis samples agreed to the reports of the results published by other authors. The antimicrobial activity mainly depends upon the high content of flavonoids and polyphenols in the ethanolic extracts of propolis. It was also reported that all propolis samples will have similar antimicrobial activity [12, 34, 35].

CONCLUSION

In the present work the antimicrobial activity of different concentration of propolis samples against selected pathogens showed good zones of inhibition and established that these samples too are having satisfactory antioxidative and antagonistic property. From this it is concluded that samples collected from Karnataka can also be used as an alternative medicine for treating these pathogens and promoting the use of natural bee products.

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Persian Abstract

چکیده

پروپولیس ماده ای شبیه رزین حاصل منابع گیاهی است. این رزین مثل مومی که زنبور عسل سنتز می کند برای بهبود سلامت انسان استفاده می شود. این ماده غنی از ترکیبات آنتی اکسیدان است که ارزش دارویی داشته و برای درمان استفاده می شود. این ماده خاصیت ضد التهاب داشته و برای درمان زخم و سوختگی و زخم معده و سرطان معده استفاده می شود. بعلاوه این ماده از خاصیت ضد میکروبی و ضد باکتریهای پاتوژن بسیار مطلوبی برخوردار است. خواص این ماده بستگی به در شرایط جغرافیائی متفاوت و منابع فلور گیاهی دارد. این مقاله بر خواص آنتی اکسیدانی و فعالیت آنتی گونستی محلول استخراج شده اتانولی پروپولیس حاصل از گونه های *Apis & Trigona* تاکید دارد. خاصیت آنتی اکسیدانی پروپولیس با استفاده از روند کاهش توان آنتی اکسیدانی فریک و محتوی فنول کل و خاصیت آنتی رادیکالی ماده تعیین می گردد. نتایج با نمونه های پروپولیس با غلظت ۵ و ۱۰٪ از *Apis mellifera* حداکثر خاصیت آنتی اکسیدانی و آنتی گونستی ماده را در مقایسه با پروپولیس حاصل از *Trigona* نشان میدهد. براساس شواهد موجود نمونه های بدست آمده از *Karnataka* از نظر خواص دارویی و درمان برای درمان باکتریهای پاتوژن انتخاب شده است که خود مصرف موم از زنبور عسل را تشویق می نماید.
