Research Article

Antibacterial Activity and Phenolic Content of Propolis From Four Different Areas of Thailand

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ABSTRACT

Propolis from stingless bees is known for its therapeutic activity but the information dealing with antibacterial property of stingless bee propolis in Thailand is still limited. This research aimed to determine the antibacterial activity together with its polyphenol and flavonoid contents of ethanolic extracts of stingless bee, *Tetragonula pagdeni*, propolis collected from 4 different geographical areas of Thailand. Propolis samples were extracted with ethanol by the maceration method and ethanolic extracts of propolis samples (EEP 1-4) were tested against gram-positive *Staphylococcus aureus* ATCC 25923 and gram-negative *Escherichia coli* ATCC 25922 bacteria by the paper disc diffusion assay. Their polyphenol and flavonoid contents were analyzed. The results indicated that ethanolic extract of propolis from community forest in Mueang Rayong District, Rayong Province (EEP 4) showed the best antibacterial effect against *E. coli* followed by ethanolic extract of propolis from mixed fruit orchard in Klaeng District, Rayong Province (EEP 3). The EEP 3 extract was rich in total polyphenol (70.04 mg Gallic acid equivalent, GAE/g EEP 3) while the highest flavonoid content (8.99 mg Catechin equivalent, CE/g EEP) was detected in EEP 4. These results indicated that EEP 4 and EEP 3 extracts from this study have potential for antibacterial activity in relation to their polyphenol and flavonoid contents. The geography is one of the factors influencing the quality of stingless bee propolis in Thailand.

Keywords: Thailand propolis, Stingless bee, Antibacterial activity, Polyphenol, Flavonoid.

INTRODUCTION

Stingless bee, so called meliponiculture, is one of the honey bees belonging to the family Apidae.[1] It has no functional sting so that it is not danger to its intruders such as spiders, flies, wasps, ants and lizards. Consequently, it must protect food resources such as honey or pollen by sealing holes of the hive with resinous substance called propolis obtained by mixture of its own body secretion from the salivary glands and resins collected from various parts of plant sources.[2-4] Propolis is a sticky and dark brown resinous material.[5] Because of its waxy nature and mechanical activities, propolis was used by bees for building and repairing its hive, coating the internal wall of the hive to prevent against wind and rain[6,7] and embalming dead organism inside the hive.[8] Nowadays, propolis has increased more interest by worldwide people because of its therapeutic activities which include antibacterial[9-11], antifungal[12], antiviral[13], antimicrobial[14,15], antiproliferation[16], antioxidant[17-19], anti-diabetic[20], anti-inflammatory[21,22], anti-herpes[23], antiulcer[24] and antitumor.[25,26] Moreover, propolis can be used by humans both internally or externally and showed various properties such as a local anesthetic, reducing sparms, healing gastric ulcers and strengthening capillaries.[27,28]

Flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones, steroids, coumarins, amino acids and inorganic compounds are the chemical compounds contained in propolis.[29] The antimicrobial activity and chemical constituents found in the propolis vary depending on the honey bees and plants species presenting in different temperature, season, collection site, harvesting periods, year and other factors.[27,30-33] Therefore, the objective of this study is to investigate and compare the antibacterial activity and their chemical contents of ethanolic extract of *Tetragonula pagdeni* propolis samples harvested from different locations of Thailand on *Staphylococus aureus* and *Escherichia coli* bacteria. The knowledge gained from this study not only provides an insight on antibacterial property of selected propolis extracts, but also exploites as an alternative way to control such bacteria for natural and safe antibacterial agent.

MATERIALS AND METHODS

Propolis collection

Propolis samples from stingless bees, *Tetragonula pagdeni* (Schwarz) were collected from 4 different environment locations of Thailand in 2015 (Table 1). All raw propolis samples were also gathered from different parts of the hives and stored in various temperatures until extraction process.

Propolis extraction

Each raw propolis sample (100 g) (Figure 1) was cut into small pieces, ground and macerated in 300 mL of 95% ethanol (w/v) at room temperature for 3 days. The suspension was filtered to remove rough particles under Whatman filter paper No.1. Extraction procedure was repeated three times. The filtrate was evaporated to dryness under reduced pressure using a rotary evaporator to remove the solvent and obtain the ethanol

extract of propolis (EEP). The dry extracts were then weighted for calculating the yields of extracts (Table 2) before they were kept in the refrigerator at 10 °C until used for antibacterial activity and chemical determination.

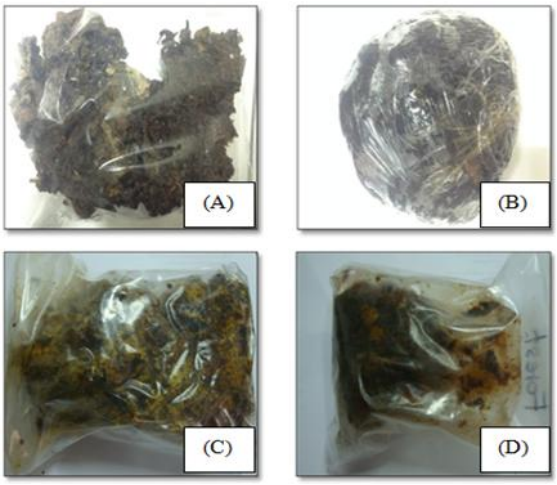

Figure 1 (A-D): Raw propolis from stingless bees **(A)** propolis 1 for EEP 1, **(B)** propolis 2 for EEP 2, **(C)** propolis 3 for EEP 3 and **(D)** propolis 4 for EEP 4

Antibacterial activity

Bacterial cultures

Gram positive (*Staphylococcus aureus* (ATCC 25923, DMST 8840) and Gram negative (*Escherichia coli* (ATCC 25922, DMST 4212) bacteria were tested for the antibacterial activity of EEP. Both bacteria were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand.

Glycerol stocks of *S. aureus* and *E. coli* were streaked on nutrient agar (5 g of peptone from meat, 3 g of meat extract and 12 g of agar). All cultures were incubated aerobically at 37 °C for 24 h. Then, 5 mL of nutrient broth (5 g of peptone from meat, 3 g of meat extract) was inoculated with a randomly selected single colony of each bacterial isolate. The bacterial suspensions were then incubated aerobically on a shaker (n-Biotex, INC) at 200 rpm, 37 °C for 12 h. Each bacterial suspensions was adjusted with fresh medium to obtain a 0.5 McFarland standard turbidity using a spectrophotometer (GeneQuant 1300) at 625 nm.

Paper disc diffusion assay

For each bacterial culture, 1 mL of 10^8 colony forming units (CFU) were spread on the surface of nutrient agar plate using sterile cell spreader and left to completely dry at room temperature. EEP was prepared in 10, 20 and 50% (w/v) concentrations by dissolving in DMSO. Then, 20 µL of each test concentration or DMSO (negative control) or streptomycin sulphate (200 µg/mL) (positive control) was dropped on each sterile paper disk (6 mm diameter) and the permeated test disc was then placed onto the nutrient agar plate containing one of the mentioned bacteria. The plates were incubated aerobically at 37 °C for 24 h. Each concentration was performed in 10 replications. Antibacterial activity was evaluated by measuring the diameters of the clear zone (inhibition zoon) developed around the bacterial colony. The mean of inhibition zoon was compared using Analysis of variance (ANOVA) and Duncan's Multiple Rang Test was considered as the criterion for statistically significant by SPSS program version 19 (SPSS Inc.) at $p<0.05$.

Determination of total polyphenol and flavonoid

The total polyphenol and flavonoid contents in EEP prepared according to the Folin-Ciocalteu colorimetric method.[34] For the total polyphenol, 125 µL of EEP was mixed with 500 µL of water and 125 µL of Folin reagent. After 6 min 1,250 µL of 7% sodium carbonate and 1,000 µL of water were added to the mixture. The mixture was then allowed to stand for 90 min and the absorbance was measured at 760 nm using spectrophotometer. The same process was repeated for the standard gallic acid solution (20-200 µg/mL) to produce a calibration graph. The total phenolic content was presented as average of triplicates and expressed as the mg of gallic acid equivalents (GAE) per g of the EEP.

For flavonoid content, 125 µL of EEP was mixed with 1,250 µL of water and 75 µL of 5% sodium nitrite. The mixture was allowed to stand for 5 min. Then it was added to 150 µl of 10% aluminium chloride and allowed to stand for 6 min. Five hundred µL of 1 M sodium hydroxide and 275 µL of distilled water were added to the mixture. Then, the mixture solution was immediately measured for its absorbance at 510 nm. A standard calibration graph obtained by repeating the same procedure for catechin solution (30-300 µg/mL). The flavonoid content, presented as average of 3 readings, was expressed as the mg of catechin equivalents (CE) per g of the EEP.

RESULTS AND DISCUSSION

Antibacterial activity

The results of the inhibitory effect of ethanolic extract of propolis 1-4 samples (EEP 1-4) on *S. aureus* and *E. coli* were shown in Table 3. All ethanolic extracts of *T. pagdeni* propolis at 10% concentration were unable to induce clear zone on both bacteria. When the concentration of EEP samples was increased, all tested propolis demonstrated only a weak activity on both bacteria. Though inhibition zone of streptomycin sulphate (positive control) was the highest on both *S. aureus* and *E. coli*, but EEP 3 and EEP 4 at 20% concentration expressed more efficacy on antibacterial activity as compared to EEP 1 and EEP 2 which did not show any inhibitory effect against both bacteria. However, EEP 3 and EEP 4 were not significantly different when compared to each other.

Propolis extracted by ethanol was effective against *E. coli* in higher concentration.[35] Our result seems to confirm their information due to the inhibition zones of EEP 3 (2.35 mm) and EEP 4 (3.05 mm) at 50% concentration

higher than those of EEP 3 (2.00 mm) and EEP 4 (2.25 mm) at 20% concentration. Nevertheless, they were not significantly different in inhibition of *E. coli*. The origin, harvesting and storing of propolis 1 (EEP 1) and propolis 2 (EEP 2) were not suitable to use as antibacterial activity against bacteria tested in this study. EEP 3 and EEP 4 showed possible antibacterial activity against *E. coli* in this study if they were induced with higher concentrations. However, these concentrations failed to induce the inhibition activity on *S. aureus*. According to the results, it can be inferred that type of propolis, concentration, type of bacteria tested and method to evaluate antibacterial effect may be related to antibacterial activity.[36]

Determination of total polyphenol and flavonoid

Among EEP samples, EEP 3 and EEP 4 contain the highest polyphenol and flavonoid, respectively while the lowest amount of both chemical contents were found in EEP 2 (Table 4). The highest polyphenol and flavonoid concentrations were 70.04 mg GAE/g of EEP 3 and 8.99 mg CE/g of EEP 4, respectively. This result seems to be related to that of previous antibacterial activity where EEP 3 and EEP 4 showed the greater inhibitory zone on *S. aureus* and *E. coli* than EEP 1 and EEP 2. It is possible to infer that extracts with higher polyphenol and flavonoid contents relate to the ability to inhibit the growth of bacteria. Our assumption can be supported by work indicated that flavonoids and aromatic compounds in propolis affect for antibacterial activity.[37] Furthermore, flavonoid content in propolis is significantly correlated with the MIC (Minimal Inhibitory Concentration).[38,39] In this study the efficacy to inhibit the growth of *S. aureus* and *E. coli* was found in EEP 4 and EEP 3 which have the highest flavonoid and polyphenol contents, respectively. The mechanism of flavanoid for inhibit growth of bacteria must be occurred from permeability to bacterial cell wall, microsomes and lysosomes damaging due to interaction between flavonoids with bacteria DNA.[40] Flavonoid is also well known as chemical compounds to inhibit viral enzyme and avoid free radicals.[41] The chemical compounds found in the propolis depend on the honey bees, botanical sources and seasonal collection presenting in different geography.[27,30,31] Accordingly, all EEP samples under this investigation contain different polyphenol and flavonoid contents due to their raw propolis samples collected and stored in different conditions before extraction process. In this study, raw propolis materials (propolis 4 and propolis 3) were collected from stingless bee hives located in the appropriate environment, harvested in proper part of the nest and maintained in suitable temperature after collection. Consequently, EEP 4 and EEP 3 showed possibility for antibacterial activity which relates to their polyphenol and flavonoid contents.

CONCLUSION

The result of this study indicated that the ethanolic extracts of propolis samples collected in different geographic coordinate of Thailand contain different amount of polyphenol and flavonoid contents. The antibacterial activity of these extracts also varied accordingly to the location where the propolis samples were collected. Ethanolic extracts of propolis collected from community forest at Mueang Rayong District, Rayong Province (EEP 4) and mixed fruit orchard in Klaeng District, Rayong Province (EEP 3) showed the highest inhibitory activity on gram negative bacteria, *E. coli* when concentration increased but activity against gram positive, *S. aureus* seemed to decline gradually with increasing concentration. More research is recommended to investigate the antibacterial activity of active compounds in EEP 3 and EEP 4 which the total polyphenol and flavonoid contents in EEP 3 and EEP 4 are quite good when compared with those in EEP 1 and EEP 2. The Geographic coordinate, collected site, plant species diversity, stored temperature and other factors affected on antibacterial activity of stingless bee propolis samples in this study. Therefore, it is very essential to study the suitable environment, method for rearing the stingless bees and how to store their propolis for possible use in pharmacological activity in the future.

Table 1: Geographic coordinate and description of propolis samples from stingless bees.

Location	Geographic Coordinate		Description		
	Latitude	Longitude	Habitat of stingless bee hives	Propolis collection location	Temperature of propolis storaged
Amphawa District, Samut Songkhram Province (propolis 1) = EEP 1	N13°40.362′	E99°99.732′	Mixed fruit orchard[1/]	from whole nest structure	25-30°C
Na Yai Am District, Chanthaburi Province (propolis 2) = EEP 2	N12°68.776′	E101°86.369′	Mixed fruit orchard[2/]	from honey pot	0-5°C
Klaeng District, Rayong Province (propolis 3) = EEP 3	N 12°40.198′	E 101°34.323′	Mixed fruit orchard[3/]	from top of nest	-10°C
Mueang Rayong District, Rayong Province (propolis 4) = EEP 4	N12°40.734′	E 101°24.035′	Community forest[4/]	from top of nest	-10°C

[1/] Pomelo (*Citrus maxima* (Burm.) Merrill), mango (*Mangifera indica* L.), lychee (*Litchi chinensis* Sonn.) and banana (*Musa sapientum* Linn.)
[2/] rambutan (*Nephelium lappaceum* Linn.), mangosteen (*Garcinia mangostana* L.), durian (*Durio zibethinus* Murray.) and long kong (*Lansium domesticum* Corr.)

[3/] rambutan (*Nephelium lappaceum* Linn.), mangosteen (*Garcinia mangostana* L.), durian (*Durio zibethinus* Murray.), long kong (*Lansium domesticum* Corr.) and para rubber (*Hevea brasiliensis* Muell. Arg.) plantation
[4/] mixed deciduous forest and para rubber (*Hevea brasiliensis* Muell. Arg.) plantation

Table 2: Extraction rate and characteristic of extracts.

Ethanol extract of propolis (EEP)	Extraction rate	Characteristic of extract
EEP 1	40.88	Pale brown, sticky solid
EEP 2	34.18	Dark brown, sticky solid
EEP 3	28.86	Pale brown, sticky solid
EEP 4	40.73	Dark brown, sticky solid

Table 3: The mean of the diameter (mm)[1/] of bacterial growth inhibited by different concentrations of ethanolic extract of EEP samples on tested bacteria.

Concentration (%, w/v)	EEP samples	Tested bacteria	
		S. aureus	*E. coli*
10	EEP 1	-	-
	EEP 2	-	-
	EEP 3	-	-
	EEP 4	-	-
	Positive control (Streptomycin)	7.05 ± 0.12 a	7.20 ± 0.11 ab
	Negative control (DMSO)	-	-
20	EEP 1	-	-
	EEP 2	-	-
	EEP 3	0.40 ± 0.27 c	2.00 ± 0.29 de
	EEP 4	0.60 ± 0.31 c	2.25 ± 0.15 cde
	Positive control (Streptomycin)	7.60 ± 0.18 a	7.85 ± 0.15 a
	Negative control (DMSO)	-	-
50	EEP 1	-	0.65 ± 0.27 f
	EEP 2	0.10± 0.10 c	1.45 ± 0.42 e
	EEP 3	-	2.35 ± 0.30 cd
	EEP 4	0.25 ± 0.25 c	3.05 ± 0.40 c
	Positive control (Streptomycin)	6.15 ± 0.42 b	6.80 ± 0.29 b
	Negative control (DMSO)	-	-

[1/] Values are mean ± SE (n=10). Values within each column followed by the same letters are not significantly different at P>0.05.

The symbol "-" means no zone of inhibition.

Table 4: Total polyphenol (mg GAE/ g EEP) and flavonoid (mg CE/ g EEP) contents[1/] in EEP.

Chemical content	EEP samples			
	EEP 1	EEP 2	EEP 3	EEP 4
Total polyphenol	52.45 ± 1.45	12.54 ± 0.87	70.04 ± 1.66	40.85 ± 0.67
Total flavonoid	3.30 ± 0.09	1.13 ± 0.16	4.95 ± 0.16	8.99 ± 0.09

[1/] Values are mean ± SE of triplicate determinations

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REFERENCES

1. Kumar MS, Singh AJAR, Alagumuthu G, Traditional beekeeping of stingless bee (*Trigon* sp.) by *Kani* tribes of Western Ghats, Tamilnadu, India, Indian Journal of Traditional Knowledge, 11, 2012, 342-345.
2. Ghisalberti EL, Propolis A review, Bee World, 60, 1979, 59-84.
3. Yaghoubi SMJ, Ghorbani GR, Soleimanian ZS, Satari R, Antimicrobial activity of Iranian propolis and its chemical composition, DARU Journal of Pharmaceutical Sciences, 15, 2007, 45-48.
4. Bankova V, Recent trends and important developments in propolis research, Evidence-Based Complementary and Alternative Medicine, 2, 2005, 29-32.
5. Wolfe D, Superfoods: The food and medicine of the future, North Atlantic Books, California, USA, 2009, 313 PP.
6. Toreti VC, Sato HH, Pastore GM, Park YK, Recent progress of propolis for its biological and chemical compositions and its botanical origin, Evidence-Based Complementary and Alternative Medicine, 2013, 2013, DOI.org/10.1155/2013/697390.
7. Marghitas LAL, Dezmirean DS, Bobis O, Important developments in Romanian propolis research, Evidence-Based Complementary and Alternative Medicine, 2013, 2013, DOI.org/10.1155/2013/159392.
8. Sanpa S, Popova M, Bankova V, Tunkasiri T, Eitssayeam S, Chantawannakul P, Antibacterial compounds from propolis of *Tetragonula laeviceps* and *Tetrigona melanoleuca* (Hymenoptera: Apidae) from Thailand, PLOS ONE, 10, 2015, DOI:10.1371/journal.pone.0126886.
9. Pujirahayu N, Ritonga H, Agustina L S, Uslinawaty Z, Antibacterial activity of oil extract of Trigona propolis, International Journal of Pharmacy and Pharmaceutical Sciences, 7, 2015, 419-422.
10. Segueni N, Benlabed K, Hassane B, Moussaoui F, Zellagui A, Lahouel M, Rhouat S, Antibacterial activity of two Algerians propolis, International Journal of Pharmaceutical Sciences Review and Research, 25, 2014, 106-110.
11. Raghukumar R, Vali L, Watson D, Fearnley J, Seidel V, Antimethicillin-resistant *Staphylococcus aureus* (MRSA) activity of 'Pacific propolis' and isolated prenylflavanones, Phytotherapy Research, 24, 2010, 1181-1187.
12. Quiroga EN, Sampietro DA, Soberón JR, Sgariglia MA, Vattuone M, Propolis from the northwest of Argentina as a source of antifungal principles, Journal of Applied Microbiology, 101, 2006, 103-1010.
13. Schnitzler P, Neuner A, Nolkempor S, Zundel C, Nowack H, Sensch KH, Reichling J, Antiviral activity and mode of action of propolis extracts and selected compounds, Phytotherapy Research, 24, 2010, S20-S28.
14. Surendra NS, Bhushanam M, Ravikumar H, Antimicrobial activity of propolis of *trigona* sp. and *Apis mellifera* of Karnataka, India, Prime Journal of Microbiology Research, 2, 2012, 80-85.
15. Kosalec I, Pepeljnjak S, Bakmaz M, Vladimir-Knezević S, Flavonoid analysis and antimicrobial activity of commercially available propolis products, Acta Pharmaceutica, 55, 2005, 423-430.
16. Kouidhi B, Zmantar T, Bakhrouf A, Anti-cariogenic and anti-biofilms activity of Tunisian propolis extract and its potential protective effect against cancer cells proliferation, Anaerobe, 16, 2010, 566-571.
17. Laskar RA, Ismail SK, Roy N, Begum NA, Antioxidant activity of Indian propolis and its chemical constituents, Food Chemistry, 122, 2010, 233-237.
18. Alexandra CH, Juliana C, Leticia C, Ivan PA, Ademilson E, Patricaia V, Composition and antioxidant activity of propolis from three species of Scaptotrigona stingless bees, Journal of Apiproduct and Apimedical Sciences, 2, 2009, 37-42.
19. Kumazawa S, Hamasaka T, Nakayama T, Antioxidant activity of propolis of various geographic origins, Food Chemistry, 84, 2004, 329-339.
20. Kang LJ, Lee HB, Bae HJ, Lee SG, Antidiabetic effect of propolis: Reduction of expression of glucose-6-phosphatase through inhibition of Y279 and Y216 autophosphorylation of GSK-3α/β in HepG2 cells, Phytotherapy Research, 24, 2010, 1554-1561.
21. McLennan SV, Bonner J, Milne S, Lo L, Charlton A, Kurup S, Jia J, Yue DK, Twigg SM, The anti-inflammatory agent propolis improves wound healing in a rodent model of experimental diabetes, Wound Repair Regeneration, 16, 2008, 706-713.
22. Paulino N, Lemos Abreu SRL, Uto Y, Koyama D, Nagasawa H, Hori H, Dirsch VM, Vollmar AM, Scremin A, Bretz WA, Anti-inflammatory effects of a bioavailable compound, Artepillin C, in Brazilian propolis, European Journal of Pharmacology, 587, 2008, 296-301.
23. Vynograd N, Vynograd I, Sosnowski Z, A comparative multi-centre study of the efficacy of propolis, acyclovir and placeo in the treatment of genital herpes (HSV), Phytomedicine, 7, 2000, 1-6.
24. Iyyampillai S, Kandaswamy M, Subramanian S, Antiulcerogenic and ulcer healing effects of Indian propolis in experimental rat ulcer models, Journal of ApiProduct and Apimedical Science, 1, 2010, 21-28.
25. Choudhari MK, Haghniaz R, Rajwade JM, Paknikar KM, Anticancer activity of Indian stingless bee propolis: An *in vitro* study, Evidence-Based Complementary and Alternative Medicine, 2013, 2013, 1-10.
26. Franchai Jr GC, Moraes CS, Toreti VC, Daugsch A, Nowill AE, Park YK, Comparison of effects of the ethanolic extracts of Brazilian propolis on human leukemic cell as assessed with the MTT assay, Evidence-Based Complementary and Alternative Medicine, 2012, 2012, DOI:10.1155/2012/918956.
27. Selvan AK, Prabhu T, Extraction of propolis from beehives and characterization of its constituents and medicinal properties: A review, International Journal of Advanced Engineering Technology, 1, 2010, 50-53.
28. Barros MP, Souza JPB, Bastos JK, Andrarde SF, Effect of Brazilian green propolis on experimental gastric ulcers in rats, Journal of Ethnopharmacology, 110, 2007, 567-571.

29. Bankova VS, De Castro SL, Marcucci MC, Propolis: Recent advances in chemistry and plant origin, Apidologie, 31, 2000, 3-15.

30. Kalogeropoulos N, Konteles SJ, Troullidou E, Mourtzinos I, Karathanos VT, Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus, Food Chemistry, 116, 2009, 452-461.

31. Tylkowski B, Trusheva B, Bankova V, Giamberini M, Georgi P, Nikolova A, Extraction of biologically active compounds from propolis and concentration of extract by nanofiltration, Journal of Membrane Science, 348, 2010, 124-130.

32. Trusheva B, Popova M, Bankova V, Simova S, Marcucci MC, Miorin PL, Pasin FR, Tsvetkova I, Bioactive constituents of Brazilian red propolis, Evidence Based Complementary and Alternative Medicine, 3, 2006, 249-254.

33. Moreira L, Dias LG, Pereira JA, Estevenho L, Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal, Food Chemical Toxicology, 46, 2008, 3482-3485.

34. Wolfe K, Wu X, Liu RH, Antioxidant activity of apple peels, Journal of Agricultural and Food Chemistry, 51, 2003, 609-614.

35. Sforcin JM, Fernandes JA, Lopes CAM, Bankova V, Funari SRC, Seasonal effect on Brazilian propolis antibacterial activity, Journal of Ethnopharmacology, 73, 2000, 243-249.

36. Garedew A, Schmolz E, Lamprecht I, Microbiological and calorimetric investigations on the antimicrobial actions of different propolis extracts: An *in vitro* approach, Thermochimica Acta, 422, 2004, 115-124.

37. Ivancajic S, Mileusnic I, Cenic-Milosevic D, *In vitro* antibacterial activity of propolis extracts on 12 different bacteria in condition of 3 various pH values, Archives of Biological Sciences, 62, 2010, 915-934.

38. Agarwal G, Vemanaradhya GG, Mehta DS, Evaluation of chemical composition and efficacy of Chinese propolis extract on *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*: An *in vitro* study, Contemporary Clinical Dentistry, 3, 2012, 256-261.

39. Popova M, Bankova V, Butovska D, Petkov V, Nikolova-Damyanova B, Sabatini AG, Marcazzan GL, Bogdanov S, Validated methods for the quantification of biologically active constituents of poplar-typepropolis, Phytochemical Analysis, 15, 2004, 235-240.

40. Sabir A, Dental pulp inflammation response in mice after application of propolis ethanol extract (EEP), Dental Journal, 38, 2005, 77-83.

41. Havsteen BH, The biochemistry and medical significance of the flavonoids, Pharmacology and Therapeutics, 96, 2002, 67-202.

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