

Antibacterial Effect of Plant Resin Collected from *Tetrigona apicalis* (Smith, 1857) in Thung Salaeng Luang National Park, Phitsanulok

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Abstract

Tetrigona apicalis (Smith, 1857) is a common species of stingless bee found in lower northern Thailand. In previous studies, the propolis of stingless bees has been shown to have antibacterial properties, due to its chemically contained phenolic contents. The major component of propolis is resin. The purpose of this study, therefore, was to evaluate the antibacterial activities of crude resin extracts by disk diffusion and broth microdilution methods. We also determined the total phenolic contents using the Folin-Ciocalteu method and, to detect individual polyphenolic contents, we used the high performance liquid chromatographic method. Two samples of resin were collected from Thung Salaeng Luang National Park, Phitsanulok. The first sample was from fresh plants, which stingless bees used for nest construction. The second sample was taken from entrances of the bee's nest. All samples were macerated in 30 % ethanol and incubated at room temperature for 14 days. The supernatants were filtered and ethanol residues then removed as ethanolic resin extracts (eREs). The antibacterial activity of the extracted resins against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 was examined. The disks containing 9 and 14 mg of eREs produced obvious inhibition zones against *S. aureus*, but did not show zones against *E. coli* and *P. aeruginosa*. The minimum inhibitory concentrations (MICs) of the eREs against the bacterial strains tested were variously between 6 and 48 mg/ml, whereas the minimum bactericidal concentrations (MBCs) were from 12 to 48 mg/ml. The amount of the total phenolic compounds in the eREs from the fresh resin was 9,908 mg of pyrogallol equivalent (PGE) per kg of eREs, and from the nest entrances, 14,740 mg per kg. We also found that hydroquinin had the highest concentration in both extracts. In conclusion, the crude resin extracts demonstrated antibacterial properties against the *S. aureus*, *E. coli*, and *P. aeruginosa* strains tested. They also contained phenolic compounds which were active antibacterial agents. We have identified new and novel knowledge which can be used as preliminary data, leading to further, more detailed, investigation of the mechanistic action of the resin against bacterial cells.

Keywords: Antibacterial activity, nest entrances, resin extracts, stingless bees, *Tetrigona apicalis*

Introduction

Stingless bees are a group of native eusocial pollinators, and are commonly found in tropical and subtropical countries [1,2], including Thailand, where stingless bees are endemic to all the regions of the country [3]. Stingless bees have been identified over 5 genera and 35 species, including *Tetrigona apicalis* (Smith, 1857), which is the most abundant species found in lower northern Thailand [4], especially in Thung Salaeng Luang National Park [3]. They normally collect plant resin as a major product for mixing with their biological materials (e.g., enzymes) and other components (e.g., pollen, soil) in order to form propolis and to build their nest entrance [5,6]. These structures are used for protection against their enemies [6].

Natural products (e.g., propolis, honey) from stingless bees and honey bees have been characterized as a food supplement, and are used in traditional medicines due to their having various biological activity, including antimicrobial activity, antiproliferative activity, and antioxidant activity [7]. However, the level of these properties vary depending on plant sources, geographical areas, and bee species [8,9].

Propolis is a resinous material produced by bees and used for sealing their nests. It consists of 50 % plant resin, 30 % wax, 10 % oil, 5 % pollen, and 5 % other organic compounds [8]. The chemical composition of propolis has been characterized as usually containing a group of phenolic compounds, fatty acids, amino acids, polysaccharides, and other compounds in trace amounts [10,11]. Antibacterial activity is the most studied aspect of the propolis produced by stingless bees [12,13]. However, the similarities and differences in the antibacterial activity of fresh plant resin and of the resin surrounding nest entrances have not yet been characterized, which led us to characterize both the former, which is collected by the bees and is a major component of the propolis, and the latter, used in the nest entrances.

Our assumption was that the antibacterial activity of the natural product could depend on the different sources of wood resin in each area, giving rise to the question of whether or not the resin from the fresh plant and the resin surrounding the nest entrances have the same antibacterial effects against certain pathogenic bacteria strains, in particular, the bacterial strains of the gram positive *Staphylococcus aureus* and the gram negative *Escherichia coli* and *Pseudomonas aeruginosa*. These 3 strains are important causes of diseases in several human body systems, e.g., the blood system [14,15]. Given that *T. apicalis* is commonly found in Thung Salaeng Luang National Park, Phitsanulok, which is close to our facilities, we were able to conveniently use the species to guide us to the particular plant types from which they collected the resin. Additionally, there was an ample availability of nest entrances from which to collect that resin.

Samples of both resins were extracted *in vitro* and investigated to determine the antibacterial effects of the ethanolic resin extracts (eREs) from the 2 sources; the resin collected by the stingless bees, referred to as eREs-1, and the resin surrounding the nest entrances, referred to as eREs-2. Additionally, the expected phenolic contents of those extracts were also analyzed.

Materials and methods

Collection and species identification of stingless bees

Stingless bees were collected from Thung Salaeng Luang National Park, Phitsanulok Province and, following correct identification of them as being *T. apicalis*, were preserved in 70 % ethanol solution. Species identification was made according to the key characteristics stated in Rasmussen *et al* [16]. The preserved specimens were then kept in the Entomology Laboratory at Pibulsongkram Rajabhat University in Phitsanulok.

Plant resin extraction

Two samples of plant resin were collected from different locations in Thung Salaeng Luang National Park. The first sample was from plant resin (known from *Dipterocarpus turbinatus*) which was known to be collected by *T. apicalis* (no. 1). The second sample was from the nest entrances of *T. apicalis* (no. 2). A sample of 100 g was ground and then added into 100 ml of 30 % ethanol. The mixtures were left at room temperature and shaken once a day for 14 days, after which time they were filtrated. The

solvents were then totally evaporated using a rotary evaporator (Buchi R-124, Switzerland) at 40 °C. Finally, the ethanolic resin extract from the plant resin, which we named eREs-1, and from the nest entrance sample, eREs-2, were kept at 4 °C for further analysis. The eREs were then dissolved in dimethyl sulfoxide (DMSO) before use.

Bacterial strains and identification

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were obtained from Dr. Noppadon Jumroon, Naresuan University, Thailand, for use in this study. All strains were cultured on blood agar (HIMEDIA, India) at 37 °C for 18 h, and then the species identified using phenotypically biochemical media (HIMEDIA, India) according to the manual of clinical microbiology [17].

Preparation of inoculations

The bacterial strains were inoculated onto 2 ml of trypticase soy broth (TSB, HIMEDIA, India) at 37 °C for 3 - 4 h. The cultured strains were then suspended in sterile saline solution to adjust the turbidity by comparison with a No. 0.5 McFarland standard. The final number of bacteria was approximately 1.5×10^8 CFU/ml.

Antimicrobial susceptibility test using disk diffusion agar

The surfaces of Müller Hinton agar plates (MHA, OXOID, Thermo Fisher Scientific, USA) were totally inoculated using a sterile swab containing the prepared inoculations of each stain and allowed to dry for a few minutes. Paper disks (6 mm in diameter) containing 9 and 14 mg of the eREs were placed on the MHA plates. Antibiotic disks (30 µg cefotaxime or 10 µg imipenem) were also placed on the plates as positive controls, and blank disks placed as negative controls (OXOID, Thermo Fisher Scientific, USA). The plates were incubated at 37 °C for 16 - 20 h, and the inhibition zones were then measured using a vernier caliper. All tests were independently performed in triplicate.

Antimicrobial susceptibility test using broth microdilution method

After preparation of the inoculated bacterial strains, 100 µl of the bacterial suspension was diluted into 1.9 ml of sterile saline (dilution as 1:20) to obtain about 5×10^6 CFU/ml. To determine the minimum inhibitory concentrations (MIC) of the eREs, 96-well plates (flat bottom type) were used. Two-fold serial dilutions of the eREs were performed on a 100 µl final volume of Müller Hinton broth (MHB, HIMEDIA, India) in order to contain 48, 24, 12, and 6 mg/ml concentrations of the eREs. Then, 10 µl of the 1:20 bacterial suspension was added into each well. The plates were incubated at 37 °C for 16 - 20 h to observe the turbidity as optical density (OD) using a microplate reader (EnSpire, PerkinElmer, USA) at a wavelength of 620 nm by comparison with the OD values of positive (only MHB without antibiotics or eREs) and negative (saline solution) controls. The minimum bactericidal concentration (MBC) was also determined by subculturing 10 µl of the MHB mixture from the previous MIC tests on MHA plates in order to observe bacterial growth after 24 h incubation at 37 °C. Having no growth on the plates indicated the MBC values of the eREs. The samples for these tests were independently performed in triplicate.

Measurement of total phenolic compounds and polyphenolic contents

The total phenolic compounds of the eREs were determined using the Folin-Ciocalteu method [18]. Briefly, 4 µl of the eREs and standard solutions were diluted with 100 µL of deionized water. Then, 20 µl of the mixtures were added into 100 µL of the diluted (1:10) Folin-Ciocalteu reagent. The reaction tubes were incubated at room temperature for 5 min. Eighty µL of 4 % sodium carbonate solution was added and incubated at room temperature for 2 h. The ODs of the eREs samples and pyrogallol standards were then measured at 740 nm using the microplate reader. The absorbance of the samples was compared with the curve of the standard concentrations, and the total phenolic compounds of eREs were recorded in mg pyrogallol equivalent/mg of dry extracts. For the measurement of the polyphenolic contents, the eREs were analyzed using high performance liquid chromatography (HPLC) with a diode array detector and

mass spectrometry method (HPLC-DAD/MSD) from Agilent Technologies (1100 series, Waldbronn, Germany). The procedure was described in previous work [19].

Results and discussion

Disk diffusion demonstrated inhibition zones against *S. aureus*

In our study, we used the disk diffusion method as a screening tool for detecting the antibacterial activity of the ethanolic resin extracts. The results showed that the representative sample of the gram positive microorganism *S. aureus* ATCC 25923 was narrowly inhibited by both the fresh plant resin (eRES-1) and the resin material from nest entrances (eRES-2) (**Figure 1** and **Table 1**).

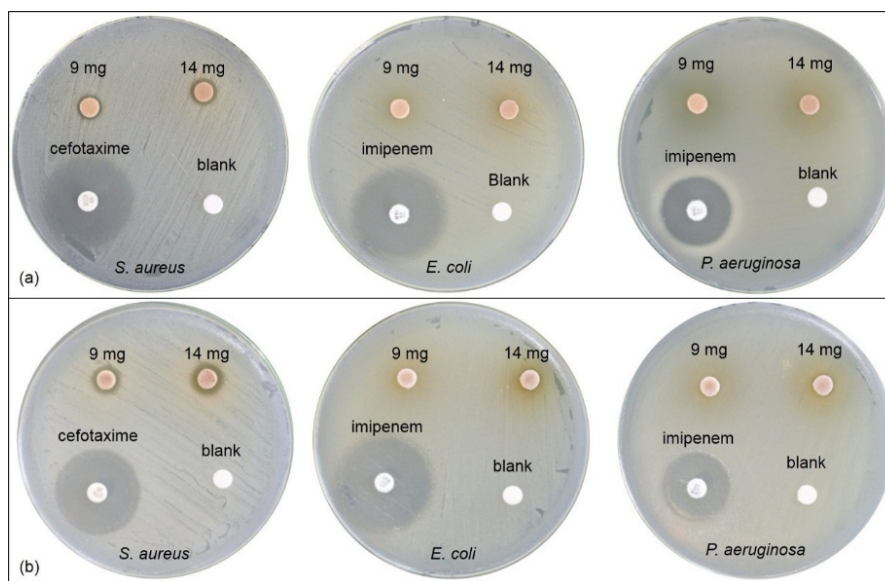


Figure 1 The antibacterial effect of the eREs was shown by disk diffusion agar. There were 2 samples of eREs, namely (a) eREs from plant resin, and (b) eREs from the nest entrance, which showed inhibition zones (mm) against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853. Note: “9 mg” and “14 mg” represented the disks containing 9 mg and 14 mg of eREs, respectively. “cefotaxime” and “imipenem” represented the disks containing 30 µg cefotaxime and 10 µg imipenem, respectively, as positive controls whereas “blank” represented empty disks as negative controls.

Unlike the result of *S. aureus*, neither extract sample obviously showed a clear inhibition zone around the paper disk against *E. coli* ATCC 25922 or *P. aeruginosa* ATCC 27853. These results suggest that, while the eREs-1 and eREs-2 both manifested similar bacterial inhibition against the *S. aureus* strain, neither resin type fully inhibited *E. coli* ATCC 25922 or *P. aeruginosa* ATCC 27853. Therefore, it is suggested that the gram positive bacteria is more sensitive to the resin extracts than the gram negative bacteria. However, while the eREs appear to have an ability to interrupt the synthesis of the bacterial cell wall, their roles in this effect are still unknown [20].

It is possible that the amount of the eREs used (9 and 14 mg) in our tests was not enough for them to present antibacterial activity against the 2 gram negative strains when tested under the disk diffusion method. Previous studies have shown that a higher amount of natural products (e.g., propolis) had a greater inhibitory effect on the bacteria, demonstrating a dose-dependent effect [21]. However, we were not able to increase the amount of the eREs used in our tests because we had a limited amount of the extracts available. Our intention to do further analysis by the antimicrobial susceptibility technique using

the broth microdilution method required our available extract. The inhibition zone occurring on the plate means only bacterial growth inhibition, not mean bacterial death, and it was necessary to apply the broth microdilution method to further distinguish the bacteriostatic and bactericidal effects [22].

Table 1 The antibacterial results of the eREs determined by disk diffusion method, demonstrating inhibition zones (mm) against the 3 strains tested.

eREs or control disks	Inhibition zone of bacteria (mm)					
	<i>S. aureus</i>		<i>E. coli</i>		<i>P. aeruginosa</i>	
	9 mg	14 mg	9 mg	14 mg	9 mg	14 mg
eREs-1	8.1±0.1	8.8±0.2	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
eREs-2	9.4±0.6	10.4±0.3	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Positive control (antibiotics)	27.0±2.0 (cefotaxime)		29.0±3.0 (imipenem)		22.0±2.6 (imipenem)	
Negative control (blank disk)	6.0±0.0		6.0±0.0		6.0±0.0	

Note: *S. aureus* used was *Staphylococcus aureus* ATCC 25923, *E. coli* used was *Escherichia coli* ATCC 25922, and *P. aeruginosa* used was *Pseudomonas aeruginosa* ATCC 27853.

The ethanolic resin extracts obviously inhibited the 3 bacterial strains using broth microdilution methods

The antibacterial properties of the ethanolic samples were also analyzed using broth microdilution methods, modified from the CLSI guidelines. It was found that there were a wide range of antibacterial activities against *S. aureus*, *E. coli* and *P. aeruginosa* strains (**Table 2**). The overall MIC values of the 2 eREs showed a range between 6 and 48 mg/ml (**Table 2**), while the MBC values ranged from 12 to 48 mg/ml (**Table 2** and **Figure 2**) in the 3 bacterial strains.

Specifically, the MIC values of eREs-1 were higher against the *S. aureus* (12 mg/ml) and *E. coli* (24 mg/ml) strains than against those of the eREs-2 (6 and 12 mg/ml, respectively), whereas the MIC value of the eREs-1 (48 mg/ml) against the *P. aeruginosa* was observed to be higher than that of the eREs-2 (24 mg/ml). This suggests that the eREs from fresh plant resin had a lesser bacterial inhibition effect than the eREs from nest entrances. Similar to the MIC values, the MBC values of the eREs-1 were higher than those of the eREs-2. This may be because the nest entrances do not only contain the resin, but also contain other organic and inorganic components, e.g., soil, pollen, etc. [6], which may affect the antibacterial activities of hive entrances in terms of both the inhibition and killing effects against the microorganisms.

The MIC values of the eREs-1 and eREs-2 against *S. aureus* were observed at 12 and 6 mg/ml, whereas the MBC values were observed at 24 and 12 mg/ml. For the antibacterial effect against *E. coli*, the MIC and MBC values of the eREs-1 were both 24 mg/ml, while the MIC and MBC values of the eREs-2 were equal at 12 mg/ml. The MIC and MBC values against the *P. aeruginosa* tested were 48 mg/ml for eREs-1, and 24 mg/ml for eREs-2. This means that both samples were observed to show greater inhibition of the growth of *S. aureus* ATCC 25923 than that of both *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. It was found that the ethanolic resin extracts could kill *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 more effectively than killing *P. aeruginosa* ATCC 27853. In summary, the results indicate that both eREs inhibit and kill all organisms tested and, further, that the resin extracts inhibit and kill gram positive bacteria more effectively than gram negative bacteria, *in vitro*. This is consistent with other research, demonstrating that these natural products inhibit gram positive bacteria more effectively than gram negative bacteria [21,23]. The mechanism of this is not yet known; the possible reasons may involve the inhibition of cell wall synthesis in gram positive bacteria or the penetration of some molecules in eREs through the outer membrane of gram negative bacteria.

Table 2 The antibacterial results of the eREs determined by the broth microdilution method, demonstrating MIC and MBC (mg/ml) against the 3 strains tested.

Bacterial strains	Resin collected by <i>T. apicalis</i> (eREs-1)		Resin from the nest entrance of <i>T. apicalis</i> (eREs-2)	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
<i>S. aureus</i> ATCC 25923	12	24	6	12
<i>E. coli</i> ATCC 25922	24	24	12	12
<i>P. aeruginosa</i> ATCC 27853	48	48	24	24

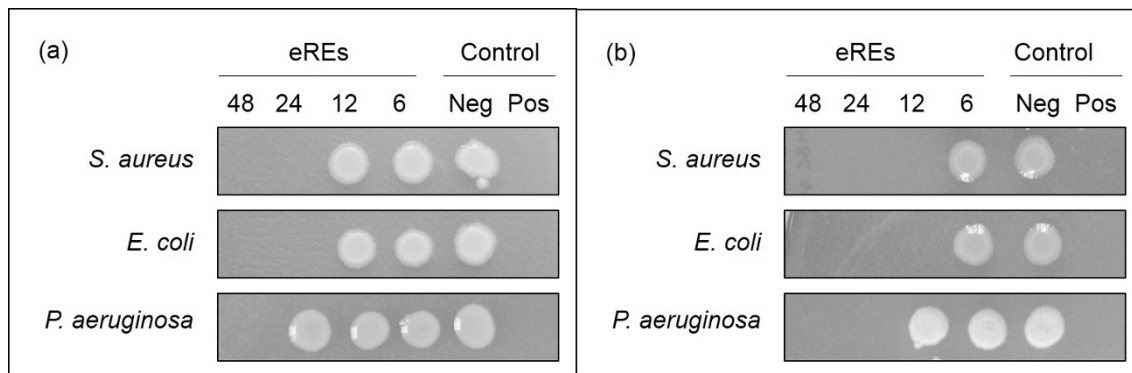


Figure 2 The MBC analysis of the eREs from (a) plant resin, collected by *T. apicalis*, and (b) the nest entrance, showing as a spot of colonies grown on MHA.

Phenolic compounds and the specific compositions of the eREs presented

We also investigated the chemical components of the eREs, particularly the total phenolic compounds (TPCs) and the specific chemicals related to the phenolic compounds. The results are shown in **Table 3**. The amount of the TPCs in the eREs from the fresh resin was 9,908 mg of PGE per kg of eREs and, from the nest entrances, 14,740 mg of PGE per kg. As we expected, phenolic compounds were detected in both resin extracts, because the compounds are normally found in most plants and plant-related products [21,24]. Hydroquinin was the major phenolic content found in both of the eREs, while quercetin and tannic acid were the second and third most abundant phenolic compounds. In addition, gallic acid, eriodictyol, isoquercetin, and catechin were also detected in both samples, but in quantities of 68 mg/kg of dried eREs or less, whereas apigenin and kaempferol were not detectable. Rutin was only found in the eREs from the nest entrances. These results are similar to other reports on the antimicrobial properties of propolis and honey, but are different in terms of the component variety [8,21,25-27]. The results suggest that phenolic compounds might be active molecules which have bacteriostatic and bactericidal properties. However, to fully demonstrate this, it will be necessary to perform further work, whereby the specific substances would be purified and the antibacterial activities of the purified chemicals re-evaluated.

Table 3 The total phenolic compounds and specific compositions in the eREs

Chemical contents	Amount of phenolic compound	
	Resin collected by <i>T. apicalis</i> (eREs-1)	Resin from the nest entrance of <i>T. apicalis</i> (eREs-2)
Total phenolic compounds (mg of PGE/kg of the eREs)	9,908	14,740
Polyphenolic content (mg/kg of dried eREs)		
Gallic acid	25	36
Eriodictyol	42	68
Apigenin	Not detected	Not detected
Isoquercetin	12	30
Kaempferol	Not detected	Not detected
Quercetin	181	321
Hydroquinin	205	352
Rutin	Not detected	27
Catechin	35	48
Tannic acid	69	191

Conclusions

The ethanolic resin extracts in this study were proven to have antibacterial properties, containing as they did phenolic compounds which may be actively antibacterial. This previously untested information is useful as preliminary data, and should lead to a deeper investigation of these resins. We suggest that these resins can also be used in alternative medical applications. However, it will be necessary to further determine the antibacterial activities of the specific substances in the resins, and also to investigate their mechanistic actions against extended pathogenic bacterial cells.

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