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In Vitro Biological Profiles and Chemical Contents of Ethanolic Nest Entrance Extracts of Thai Stingless Bees *Tetrigona apicalis*

[1,†]Touchkanin Jongjitvimol, [2,†]Sathirapong Kraikongjit, [3]Pussadee Paensuwan and [4]Jirapas Jongjitwimol

[1]*Biology, Faculty of Science and Technology, Pibulsongkram Rajabhat University, Phitsanulok, Thailand*
[2]*Biomedical Sciences, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, Thailand*
[3]*Department of Optometry, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, Thailand*
[4]*Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, Thailand*

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Corresponding Author:
Jirapas Jongjitwimol
Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, Thailand
Email: jirapasj@nu.ac.th

[†]These authors contributed equally to this work

Abstract: *Tetrigona apicalis* is a species of stingless bees found in Lower Northern Thailand. They normally construct their nest entrances as an external structure for protecting themselves from enemies. Their biological properties have not well characterized yet. The objectives of this study were to evaluate the antibacterial, antifungal and anti-proliferative activities of their nest entrances. Samples were collected from four provinces and then extracted using 70% ethanol, finally called as ethanolic Nest Entrances Extracts (eNEEs). Broth microdilution method was applied to determine their antimicrobial effects. The cytotoxicity was examined to evaluate their anti-proliferative effects. The total phenolic and flavonoid concentrations were determined using colorimetric methods. Overall, the MICs were between 6.25 and >12.5 mg/mL against the bacteria tested whereas were between 1.56 and >12.5 mg/mL for the yeasts tested. The IC_{50} of HeLa cells was lower than that of LEP cells. TPC (19.3-24.1 mg PGE/g dried eNEEs) and TFC (2.4-4.8 mg QE/g dried eNEEs) contained in the eNEEs with the major substances of hydroquinin and quercetin. In conclusion, the Thai ethanolic nest entrance extracts possess the antibacterial, antifungal and anti-proliferative activities.

Keywords: Antimicrobial Activity, Anti-Proliferative Activity, Natural Product

Introduction

Stingless bees are eusocial insects, which are normally found in tropical and subtropical countries including Thailand (Michener, 2007). There are about 35 species of stingless bees, which have been reported in Thailand (Jongjitvimol and Petchsri, 2015). *Tetrigona apicalis* is one of the common species found in Lower Northern Thailand (Jongjitvimol, 2014; Jongjitvimol and Petchsri, 2015). They usually collect plant resin to construct their nest structure including propolis and nest entrances to prevent themselves from their enemies (Roubik, 2006).

The natural products from bees, e.g., honey and propolis, have been used as food supplements and alternative medicines because of having extensive biological activities, including antibacterial activity and antifungal activity as well as anti-proliferative activity (Pratsinis *et al.*, 2010; Rattanawannee and Chanchao, 2011). For example, honey of stingless bees from different sources has been reported that it could inhibit the growth of both bacteria and fungus e.g., *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Aspergillus niger* etc. (Chanchao, 2009; Chan-Rodríguez *et al.*, 2012). Stingless bees' propolis from different countries has been characterized to have antimicrobial activities against *S. aureus*, *E. coli*, *C. albicans*, *Cryptococcus neoformans* and several *Candida*. (Lakshmi *et al.*, 2014; Freires *et al.*, 2016; Shehu *et al.*, 2016). Some propolis and honey affect the proliferation of human cell lines (Pratsinis *et al.*, 2010; Ahmed and Othman, 2013). The reason why they had

various biological activities was theirs chemical composition, especially phenolic and flavonoid compounds (Cushnie and Lamb, 2005; Daglia, 2012; Spatafora and Tringali, 2012). However, a wide range of biological properties of honey and propolis depends on several factors e.g., plant sources, geographical variation, habitat and species of stingless bees (Sforcin and Bankova, 2011; Huang *et al*., 2014).

Regarding the well-defined knowledge of honey and propolis, they possessed a wide range of biological activities such as antimicrobial, anti-inflammatory and anti-proliferative properties. However, there have been only a few studies on the biological activities of the nest entrance although the nest entrances might be another potential candidate to observe their biological activities. Therefore, this study aimed to examine both antimicrobial and anti-proliferative activities of the nest entrances of stingless bees *T. apicalis* in Lower Northern Thailand and to determine their phenolic and flavonoid substances. We found that Thai ethanolic nest entrance extracts possessed the antibacterial, antifungal and anti-proliferative activities with several potentially chemical substances.

Materials and Methods

Identification of Stingless Bees and Collection of the Nest Entrances

Before collecting the nest entrances, the species of stingless bees were firstly identified as *Tetrigona apicalis* using standard identification keys (Jongjitvimol and Petchsri, 2015). The nest entrances of stingless bees *T. apicalis* were then collected in sterile bottles from four different locations in Lower Northern Thailand as shown in Table 1.

Extraction of the Nest Entrances of Stingless Bees T. apicalis

The nest entrances were extracted according to the previous methods with some modification (Kraikongjit *et al.*, 2018). Briefly, each sample was crushed in small pieces and then macerated in 70% ethanol (one gram per 10 mL of the solvent). The samples were incubated at room temperature for 14 days and followed by vacuum filtration. The filtrated samples were then evaporated to remove all solvent residuals under low pressure and temperature below 40°C using a rotary evaporator (Buchi R-124, Switzerland). The crude extracts from each sample were named as ethanolic Nest Entrance Extracts (eNEEs) and stored at 4°C until further analysis. The eNEEs of each source were re-numbered as shown in Table 1.

The Preparation of eNEEs Concentrations and eNEE Disks

The eNEEs were dissolved in 10% Dimethyl Sulfoxide (DMSO) at the concentration of 500 mg/mL. The solutions of the eNEEs were freshly prepared for each experiment. To prepare eNEE disks (15 mg eNEEs in 6 mm diameter disks), 30 µL of the eNEE solution was added onto empty disks and then dried at 35±2°C for 24 h before used.

Strains of Microorganisms and their Cultivation

The microorganisms used in this study were supplied from ATCC strains. The bacterial strains, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, were cultured on blood agar (HiMedia, India) plates at 35±2°C for 18-24 h prior to further analysis. The yeast strains *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were cultured on Sabouraud dextrose agar (HiMedia, India) plates at 35±2°C for 48 h prior to further analysis.

Disk Diffusion and Broth Microdilution Methods

The antibacterial and antifungal effects of the nest entrance extracts were evaluated by two approaches of disk diffusion and broth microdilution methods. For disk diffusion methods, the antibacterial and antifungal properties of the eNEEs were examined by determining the inhibition zones, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines M02-A11 (CLSI, 2012b) and M44-A2 (CLSI, 2009) with minor modifications, respectively. The experiments were performed in triplicates and the results were shown as mean ± Standard Deviation (SD) in mm. For interpretation, the reading as 6 mm means no inhibitory activity at the concentration tested because it is equal to a disk diameter.

Table 1: Different locations in Lower Northern Thailand where the nest entrance samples were collected

Province	District	Universal Transverse Mercator (UTM) Coordinate	Name[a]
Phitsanulok (PLK)	Wang Thong	47Q 0660845E 1865406N	eNEE1
Phetchabun (PCB)	Khao Kho	47Q 0706787E 1839503N	eNEE2
Sukhothai (SKT)	Khiri Mat	47Q 0573280E 1866562N	eNEE3
Kamphaengphet (KPP)	Khlong Lan	47Q 0528036E 1790434N	eNEE4

[a]The samples were extracted and named as the ethanolic nest entrances (eNEEs)

For broth microdilution methods, the CLSI guidelines, M07-A9 (CLSI, 2012a) and M27- A3 (CLSI, 2008) were followed with some modification for determining the Minimum Inhibitory Concentrations (MICs) of the eNEEs in order to refer antibacterial and antifungal activities, respectively. The Minimum Bactericidal Concentrations (MBCs) or Minimum Fungicidal Concentrations (MFCs) were subsequently examined by pipetting 10 µL of the eNEE-treated conditions on Mueller-Hinton Agar (MHA) and Sabouraud dextrose agar plates, respectively. The agar plates were then incubated at 35±2°C for 24 h (for bacteria) and 48 h (for yeasts) in order to observe the number of colonies. None of the colonies on the agar plates was defined as the MBCs or MFCs. The experiments were performed in the triplicates and the results were then shown as a median in the unit of mg/mL.

Human Cell Lines and Cell Culture

Human cervical cancer cells (HeLa cells) provided by Dr. Felicity Watts (University of Sussex, UK) and human Lens Epithelial cells (LEP cells) provided by Dr. Pussadee Paensuwan (Naresuan University, Thailand) were supplied by ATCC. The cells were cultured as monolayers in Dulbecco's Modified Eagle Medium (Gibco Laboratories, NY, USA), supplemented with sodium bicarbonate (3.7 g/L), fetal bovine serum (10% v/v), penicillin (100 U/mL) and streptomycin (100 µg/mL). Cultured cells were incubated at 37°C under a 5% CO_2 atmosphere for few days to reach 60-80% confluence.

Cell Viability Assay

HeLa and LEP cells at the density of 1×10^4 cells per 100 µL were seeded into 96-well plates for 24 h at 37°C under a 5% CO_2 atmosphere before treatment with the eNEEs. The cells were then incubated with various concentrations of the eNEEs (32, 64, 128, 256, 512, 1,024 2,048 and 4,096 µg/mL). Untreated cells (0 µg/mL of the eNEEs) were used as a negative control. The treated and untreated cells were then re-incubated for 48 h before determining the cell viability. The cell viability was assayed by a cell counting kit-8 (Boster Biological Technology, CA, USA), according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (EnSpire, PerkinElmer, MA, USA). All the tests were performed in triplicate experiments and the results were then calculated, representing in the percentage of cell viability over their negative controls.

Determination of Total Phenolic Contents (TPC)

TPC in the eNEEs was evaluated by the Folin-Ciocalteu method with some modification (Singleton *et al.*, 1999; Zongo *et al.*, 2010). Briefly, 50 µL of Folin-Ciocalteu reagent was mixed (1:10) with either 10 µL of each eNEE (25 mg/mL) in 50% methanol in 96-well plates. The plates were then incubated in the dark at room temperature for 5 min and then 40 µL of 4% sodium carbonate was added to the wells. The plates were then incubated in the dark at room temperature for 2 h. After the incubation, the absorbance of the reaction was measured at 740 nm using a microplate reader (EnSpire, PerkinElmer, MA, USA). Pyrogallol was used as the standard. The TPC of the eNEEs was presented as mg of pyrogallol equivalent/g of the dry extracts. Evaluation of the TPC of the eNEEs was analyzed in triplicates.

Determination of Total Flavonoid Contents (TFC)

TFC in the extracts was determined using a colorimetric method with some modification (Zongo *et al.*, 2010). In brief, 50 µL of 2% aluminium chloride was mixed into 50 µL of each extract (25 mg/mL) in 75% ethanol in 96-well plates. The plates were then incubated in the dark at room temperature for 15 min. The absorbance was determined at 435 nm using the same microplate reader (EnSpire, PerkinElmer, MA, USA). Quercetin was used as the standard. The TFC of the eNEEs was presented as mg of quercetin equivalent/g of the dry extracts. Evaluation of the TFC of the eNEEs was analyzed in triplicates.

Measurement of Polyphenolic Substances

The polyphenolic contents in the eNEEs were determined using High Performance Liquid Chromatography (HPLC) with a Diode Array Detector (DAD) and Mass Spectrometry Detection (MSD) from Agilent Technologies (Waldbronn, Germany). A Zorbax SB C18 column (CA, USA) was used and the diode array detector recorded at 270, 330, 350 and 370 nm. The procedure was described in the previous work (Duangjai *et al.*, 2016; Kraikongjit *et al.*, 2018).

Statistical Analysis

Inhibition zones from disk diffusion methods were shown as mean ± SD, then one-way ANOVA and Post Hoc tests were used to analyze the mean differences between the disks containing the eNEEs and DMSO vehicle control. The same statistics were tested to show the mean differences of the chemical concentrations among the eNEEs using IBM SPSS statistics version 23 (Armonk, NY, USA). The 50% inhibitory concentration (IC_{50}) values were analyzed and the graphs of the eNEE concentrations vs the percentage of cell viability were generated using the GraphPad Prism version 8.2.0 (San Diego, CA, USA). MIC and MBC/MFC values from broth microdilution methods were presented as median. For all analyses, the significant differences were statistically considered at P value < 0.05.

Results and Discussion

All the eNEEs Only Presented a Narrow Diameter of Inhibition Zones Against S. aureus

Disk diffusion methods were used to preliminarily screen the antibacterial and anti-yeast activities of the four eNEEs. The results presented in Table 2, which the mean of inhibition zones of the Quality Controls (QC1, QC2, QC3 and QC4) was in the reference ranges according to the CLSI guidelines. It was ensured that the overall analytical systems in this study were excellent. The disks containing 15 mg of the eNEEs gave the means of inhibition zones against *S. aureus* ATCC 25923 at 7.0-7.5 mm, whereas those against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were at 6 mm.

The screening results suggested that all the eNEEs could inhibit only *S. aureus* ATCC 25923. The anti-*S. aureus* effect may argue whether DMSO used as a solvent affects the activity or not. We had the evidence that the inhibition zones of DMSO (vehicle control) disks were 6 mm (same with a 6-mm disk), demonstrating that DMSO did not involve in the antimicrobial properties. These results were likely to be consistent with previous studies (Velikova *et al.*, 2000; Kraikongjit *et al.*, 2018). For instance, in 2018, the ethanolic resin extracts from stingless bees' nest entrances gave the inhibition zone against *S. aureus*, but not against *E. coli* and *P. aeruginosa* (Kraikongjit *et al.*, 2018). For another example, the Brazilian propolis showed a strong inhibition against *S. aureus* whereas there was a weak or no inhibition against *E. coli* and *C. albicans* (Velikova *et al.*, 2000). It might suggest that the eNEEs have an *in vitro* inhibitory effect against gram-positive bacteria than gram-negative bacteria. This should be further studied. However, the sightlessness of the anti-gram negative bacterial and anti-yeast effects possibly occurred since the amount of the eNEEs applied into the disks may not be enough to show inhibition zones. It would also be more interesting if the antimicrobial activities of the eNEEs would be done against a wide range of gram positive and gram negative bacteria as well as clinical fungal isolates.

The eNEEs Obviously Demonstrated Both Antibacterial and Anti-Yeast Properties as well as Showed the Killing Effect Against S. aureus

The eNEEs concentrations were finally diluted from 0.05 to 12.5 mg/mL in order to determine the antibacterial and antifungal activities as the second approach. All the eNEEs from the different sources against the five microbial strains gave a wide range of the MICs (between 1.56 and >12.5 mg/mL) and MBCs (between 6.25 and >12.5 mg/mL) values as shown in Table 3. For the eNEEs against *S. aureus*, it showed that they could obviously inhibit bacterial growth with the MICs from 6.25 to 12.5 mg/mL and some of them also showed the killing effect such as eNEE1, eNEE3 and eNEE4 with the MBC between 6.25 and 12.5 mg/mL.

For the anti-*E. coli* effect, the MICs were at either 6.25 or 12.5 mg/mL whereas the MBCs were more than 12.5 mg/mL. This could indicate that the extracts could inhibit *E. coli* but did not show the killing effect to it yet in this study. For the anti-*P. aeruginosa* effect, the MICs were at 12.5 and >12.5 mg/mL whereas the MBCs of the eNEEs were higher than 12.5 mg/mL. The results could imply that the eNEEs still had inhibitory effects on *P. aeruginosa* without showing the killing effect yet. Under considering the antibacterial results, the eNEEs from the four different sources were likely to inhibit all the bacterial strains tested by broth microdilution method but only show the killing property against *S. aureus* ATCC 25922. It implied that the eNEEs might kill *S. aureus* (gram positive bacteria) more sensitively than *E. coli* or *P. aeruginosa* (gram-negative bacteria). This result was similar to other works (Ahmad and Beg, 2001; Ibrahim *et al.*, 2016; Kraikongjit *et al.*, 2018), demonstrating that using the ethanolic natural extracts at a particular concentration could kill some gram positive bacteria but might not kill some gram negative bacteria. Although the results from the disk diffusion methods did not exhibit the inhibition effect of the eNEEs against the gram-negative bacteria tested. Interpreting of the broth microdilution results was the strong evidence, confirming that the eNEEs possessed antibacterial property against both the gram-positive and gram-negative bacterial strains tested in this study.

Next, an anti-yeast property of the eNEEs was also seen in this study. The MICs values of the eNEEs against *C. albicans* and *C. parapsilosis* were from 1.56 to 3.125 mg/mL whereas the MFCs values were more than 12.5 mg/mL. These results showed that the eNEEs could inhibit both of the yeasts tested with narrow ranges of the concentrations but did not exhibit the killing effect of the eNEEs. It is consistent with the antifungal activity of some natural extracts, showing that it could inhibit yeast e.g., *C. albicans* (Freires *et al.*, 2016; Shehu *et al.*, 2016). Even though the disk diffusion method could not present any effect, the results from broth dilution methods is confidently reported that the eNEEs possessed the antifungal property, especially the anti-yeast effect.

The eNEEs Possessed the Anti-Proliferative Effect on Human Cervical Cancer Cells and Human Lens Epithelial Cells in Dose-Dependent Manners

According to the post-treatment of HeLa and LEP cells with the eNEEs (eNEE1-4), the morphology of HeLa and LEP cells were abnormally changed e.g., both cells were floating and rounded, comparing to the untreated cells. The cytotoxicity of the eNEEs to both cell lines was evaluated using CCK-8 assay kit. Cell viability percentages of HeLa and LEP cells after

treatment with the eNEE1, eNEE2, eNEE3 and eNEE4 (Fig. 1a to 1d, respectively). The 50% inhibitory concentration (IC_{50}) of the eNEE1-4 on HeLa cells was 525.4, 516.2, 461.8 and 477.4 µg/mL, respectively, whereas the IC_{50} values of the eNEE1-4 on LEP cells were 851.2, 798.7, 825.3 and 917.5 µg/mL, respectively.

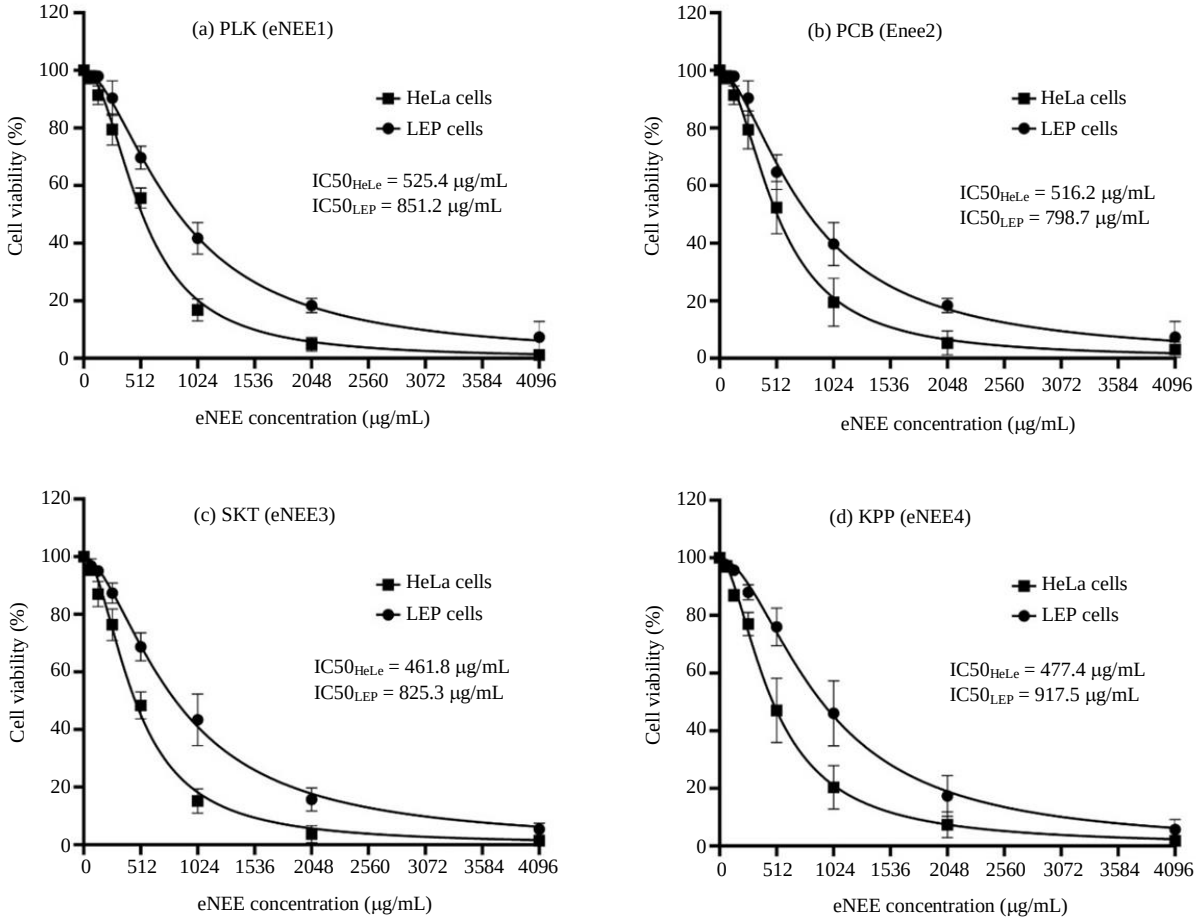

Fig. 1: Percentage of cell viability of human cervical cancer cells (HeLa) and human Lens Epithelial Cells (LEP) after 48 h treatment with the various concentrations of the eNEEs from (a) Phitsanulok (PLK, eNEE1); (b) Phetchabun (PCB, eNEE2); (c) Sukhothai (SKT, eNEE3); and (d) Kamphaengphet (KPP, eNEE4) and the 50% inhibitory concentrations of the eNEEs on both cell lines

Table 2: Inhibition zone (mm) against bacterial and yeast strains using disk diffusion methods at the 15-mg eNEEs

	Mean ± SD of inhibition zone (mm)				
Tested disk	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 90028	*C. parapsilosis* ATCC 22019
15 mg eNEE1	7.1±0.1*	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
15 mg eNEE2	7.0±0.0*	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
15 mg eNEE3	7.5±0.2*	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
15 mg eNEE4	7.2±0.2*	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
DMSO (vehicle control)	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Blank (sterility control)	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Cefotaxime 30 µg (QC1)	26.9±0.4*	29.8±2.2*	18.3±2.4*	ND	ND
Gentamicin 10 µg (QC2)	24.8±0.8*	20.5±1.3*	19.0±0.8*	ND	ND
Ciprofloxacin 5 µg (QC3)	27.2±2.6*	31.8±2.8*	30.4±1.8*	ND	ND
Fluconazole 25 µg (QC4)	ND	ND	ND	34.7±1.2*	27.8±2.8*

* Inhibition zones with asterisk significantly differed from the vehicle and blank controls (P<0.05, one-way ANOVA, Turkey HSD Post Hoc test, analyzed by IBM SPSS statistics version 23). ND means "not determined" as the quality control disks were only preformed with the corresponding microorganisms, which were recommended by the CLSI guidelines

Table 3: Minimum Inhibitory Concentrations (MICs), Minimum Bactericidal Concentrations (MBCs) and Minimum Fungicidal Concentrations (MFCs) of four eNEEs against the microorganisms tested, reported as median in mg/mL

	Median of MIC, MBC/MFC (mg/mL)				
Sample/Drug tested	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 90028	*C. parapsilosis* ATCC 220199
eNEE from					
eNEE1	6.25, 6.25	6.25, >12.5	12.5, >12.5	3.125, >12.5	3.125, >12.5
eNEE2	6.25, >12.5	12.5, >12.5	>12.5, >12.5	1.56, >12.5	1.56, >12.5
eNEE3	12.5, 12.5	12.5, >12.5	>12.5, >12.5	3.125, >12.5	1.56, >12.5
eNEE4	12.5, 12.5	6.25, >12.5	12.5, >12.5	3.125, >12.5	3.125, >12.5
Ciprofloxacin (QC1)[a]	ND	0.004, NA	0.5, NA	ND	ND
Itraconazole (QC2)[b]	ND	ND	ND	ND	0.06, NA
DMSO (vehicle control)[c]	>5%, >5%	>5%, >5%	>5%, >5%	>5%, >5%	>5%, >5%

[a]Ciprofloxacin was used against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 as referring to the CLSI guideline, reported as µg/mL. [b]Itraconazole was used against *C. parapsilosis* ATCC 220199 as referring to the CLSI guideline, reported as µg/mL. [c]DMSO was used as a diluent in this study, demonstrating that low doses (1-5%v/v) of DMSO was not toxic to the microorganisms tested. ND means "not determined" as the quality control drugs were only performed with the corresponding microorganisms, which were recommended by the CLSI guidelines. NA means “not applicable” in terms of quality control values

It suggested that the viabilities of both HeLa and LEP cells were variously inhibited after 48 h treatment with each eNEEs sample in dose-dependent manners. Overall, the results showed that the IC_{50} values of the LEP cells treated with the eNEEs were more than the IC_{50} values of the HeLa cells. Thus, the cytotoxicity of the eNEEs on the HeLa cancer cells was more than the non-cancerous LEP cells.

The IC_{50} values of our ethanolic natural extracts against both human cell lines were very high (more than 450 µg/mL) comparing to other related reports. Most of them have demonstrated that the IC_{50} values were under 50-100 µg/mL, which was lower than our results by approximately 4-10 times (Khacha-Ananda *et al.*, 2013; Borawska *et al.*, 2016; Aru *et al.*, 2019). According to the regulation of American National Cancers of Institute (NCI), the IC_{50} of an extracted compound should be below 30 µg/mL as the acceptable limits of the cytotoxic activity (Suffness and Pezzuto, 1991). Even though our extracts might not be accepted as an anti-tumor compound, a deeper investigation of the eNEEs should be performed to evaluate and discover the active compounds as anti-proliferative agents. In addition, the Selectivity Index (SI) of the active compound should be also investigated, comparing to commercially available drugs.

TPC and TFC were Discovered in the eNEEs as well as Quercetin and Hydroquinin were Mainly Detected in the Samples

We also chemically screened the eNEEs whether they contained phenolic and flavonoid contents. It was found that the TPC concentrations of the eNEE1-4 were between 19.3 and 24.1 mg Pyrogallol Equivalent (PGE) per g of the dried extracts, whereas the TFC concentrations of the eNEE1-4 were from 2.4 to 4.8 mg Quercetin Equivalent (QE) per g of the dried extracts. The amount of both TPC and TFC showed statistical differences using one-way ANOVA and Post Hoc test (P values < 0.05) as shown in Fig. 2. The reasons why the chemical contents differed each eNEE might depend on several factors, such as geographical location, sources of the nest entrances, harvesting season, etc. (Sforcin and Bankova, 2011; Huang *et al.*, 2014), which might subsequently affect the composition of the nest entrances. We also detected phenolic and flavonoid substances in the eNEEs that there were the presence of catechin, eriodictyol, gallic acid, hydroquinin, isoquercetin, quercetin, rutin and tannic acid, but not show apigenin and kaempferol (Table 4). Quercetin and hydroquinin, however, were the main phenolic substances in the eNEEs at 215.2-304.4 mg/kg and 209.6-377.4 mg/kg, respectively. The results were consistent with the previous work (Kraikongjit *et al.*, 2018) that they found hydroquinin as the major content of polyphenolic compounds in the resin of the nest entrance extracts.

Having the chemical compounds of phenolic and flavonoids was related to the inhibitory effects on the bacterial and yeast strains (Velikova *et al.*, 2000; Cushnie and Lamb, 2005; Daglia, 2012; Kraikongjit *et al.*, 2018). For example, some groups of phenolic compounds (e.g., quercetin) effect on nucleic acid synthesis in bacterial cells by disrupting the DNA synthesis-associated enzymes (Cushnie and Lamb, 2005; Daglia, 2012; Rempe *et al.*, 2017). Catechins are a group of flavonoids that can act on and damage bacterial cytoplasmic membrane function (Cushnie and Lamb, 2005; Daglia, 2012). In addition, some phenolic compound could interfere with the activity of 1,3-β-glucan synthase that effect on inhibition of cell wall synthesis of yeast cells, leading to cell wall damage. Some phenolic compounds could also affect in the diverse mechanisms e.g., interfering the synthesis of ergosterol and cell membrane in *Candida* species, inducing apoptosis of yeast cells through the increased ROS levels (Daglia, 2012; Liu *et al.*, 2017).

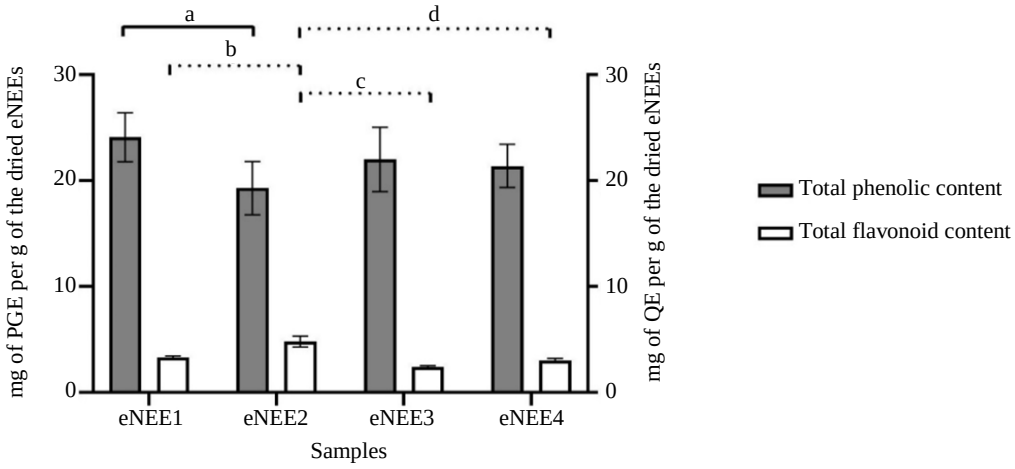

Fig. 2: Total Phenolic Contents (TPC) and Total Flavonoid Contents (TFC) of the eNEE samples from four different sources. The significant difference of TPC between two groups was shown in the solid line "a" (*P* value < 0.05) and the significant differences of TFC between two particular groups were shown in the dashed lines "b, c and d" (*P* values < 0.05). PGE; Pyrogallol Equivalent, QE; Quercetin Equivalent

Table 4: Amount of phenolic substances in each sample of the eNEEs

	Amount (mg/kg of the dried eNEEs)			
Phenolic substances	eNEE1	eNEE2	eNEE3	eNEE4
Apigenin	ND	ND	ND	ND
Catechin	63.8	47.0	26.4	54.2
Eriodictyol	29.4	15.2	23.4	36.4
Gallic acid	38.2	45.0	68.2	25.4
Hydroquinin	377.4	318.6	209.6	269.0
Isoquercetin	19.6	39.2	33.0	27.6
Kaempferol	ND	ND	ND	ND
Quercetin	263.8	252.8	215.2	304.4
Rutin	27.2	70.4	56.6	39.0
Tannic acid	96.2	122.6	86.4	143.6

ND means "not detectable" as the limit of detection

In addition, the phenolic and flavonoid contents in natural extracts have been reported that may cause more cytotoxic effects on human cells (Khacha-Ananda *et al.*, 2013; Žižić *et al.*, 2013). For example, the related natural products e.g., propolis, honey have been demonstrated that they contained both the phenolic and flavonoid contents (Cushnie and Lamb, 2005; Daglia, 2012; Spatafora and Tringali, 2012), which could affect the proliferation of various cell lines such as breast, colon, lung, oral, leukemic cancer cell lines (Barbarić *et al.*, 2011; Kaewmuangmoon *et al.*, 2012; Khacha-Ananda *et al.*, 2013; Žižić *et al.*, 2013). Thus, the eNEEs might be another alternatively natural product as a candidate material for further study in the term of an anti-cancer agent.

Conclusion

The ethanolic extracts of the nest entrances that are constructed by Thai stingless bees *T. apicalis* interestingly possess the antibacterial, antifungal and anti-proliferative activities. The four different sources chemically contained loads of phenolic and flavonoid substances. This study suggests that the nest entrance is a potential natural product in terms of antimicrobial and anti-cancer sources. *In vivo* anti-proliferation study or animal models of these extracts should be further examined.

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Author's Contributions

Touchkanin Jongjitvimol: Conceptualization, methodology, resources.

Sathirapong Kraikongjit: Methodology, data analysis, data curation and writing of the manuscript.

Pussadee Paensuwan: Resources.

Jirapas Jongjitwimol: Conceptualization, methodology, data analysis, resources, data curation and writing the manuscript, project administration and funding acquisition.

All authors have read and agreed to the published version of the manuscript.

Ethics

This manuscript has not been published elsewhere in part or in entirely. The authors declare that there is no conflict of interest.

The human ethics of this study was approved by the Naresuan University Institutional Review Board, Thailand (Exemption review No.851/58). According to the biosafety aspect, this study was also approved by the Naresuan University Institutional Biosafety Committee (NUIBC No.60-11).

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