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A method for the extraction and analysis of Taiwanese green propolis

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TITLE:

Extraction and Analysis of Taiwanese Green Propolis

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KEYWORDS:

Antibacterial activity, ethanol, extraction, propolins, Taiwan, green propolis

SHORT ABSTRACT:

We present a protocol for using ethanol as a solvent to extract and characterize Taiwanese green propolis that exhibits antibacterial activity.

LONG ABSTRACT:

Taiwanese green propolis is rich in prenylated flavonoids and exhibits a broad range of biological activities, such as antioxidant, antibacterial, and anticancer ones. The bioactive compounds of Taiwanese green propolis are propolins, namely C, D, F, and G. The concentration of propolins in Taiwanese green propolis varies depending on the season and geographic location. Thus, it is critical to establish a standard and repeatable procedure for determining the quality of Taiwanese green propolis. Here, we present a protocol that uses ethanol-based extraction, high-performance liquid chromatography, and an antibacterial activity analysis to characterize Taiwanese green propolis quality. This method indicates that 95% and 99.5% ethanol extractions achieve the maximum dry matter yields from Taiwanese green propolis, thereby yielding the highest concentrations of propolins that have antibacterial properties. According to these findings, the present protocol is deemed reliable and repeatable for determining the quality of Taiwanese green propolis.

INTRODUCTION:

Propolis is a natural resinous mixture produced by the bee species *Apis mellifera*. Propolis has been widely used since ancient times in folk medicines. A study recently reported that propolis

is beneficial for preventing microbial infections and inflammation[1]. Numerous studies have demonstrated that the main bioactive compounds in propolis are flavonoids, phenolic acid esters, prenylated *p*-coumaric acids, and diterpenic acids[2,3]. To date, 10 prenylated flavanone derivatives from Taiwanese green propolis have been identified through high-performance liquid chromatography (HPLC)[4-7]. The most abundant among these are propolins C, D, F, and G[5,7]. The considerable biological effects of Taiwanese green propolis are correlated with its high content of propolins[8].

The concentrations of bioactive compounds in propolis vary greatly depending on the season and geographic location from which the propolis is obtained. European propolis mainly contains the flavonoid aglycone and phenolic acids[9]. The major bioactive compounds in propolis from Brazil are prenylated *p*-coumaric acids, such as artepillin C[10]. A study demonstrated that the season is a critical factor for determining the total propolin content in Taiwanese green propolis[11]. The propolin content in Taiwanese green propolis is highest in summer (May - July) and lowest in winter[11]. The antibacterial property of propolis has been widely considered to be an indicator of biological activity. Generally, the samples of propolis collected from various regions have exhibited a similar antibacterial property; for example, it is generally effective against almost all gram-positive bacteria and exhibits a limited antibacterial effect against gram-negative bacteria[10,12,13]. Synergistic interactions between the flavonoids in propolis were demonstrated to have an antibacterial effect[14]. Similarly, Taiwanese green propolis was reported to have an antimicrobial effect against gram-positive bacteria[15]. Furthermore, a study also identified an antimicrobial effect from the interactions of propolins in Taiwanese green propolis[8].

The characterization of the bioactive compounds in propolis is difficult because its chemical composition can vary according to its source of origin. Therefore, it is necessary to establish a feasible and repeatable method for determining the quality of the Taiwanese green propolis. However, no standard procedure has been established for Taiwanese green propolis extraction and subsequent functional analysis. Several methods that variously apply organic and inorganic solvents have been proposed for propolis extraction[16-20]. Because propolis is a lipophilic mixture, studies have demonstrated that organic extraction is better than inorganic extraction[8,18,19]. The total propolin concentration in Taiwanese green propolis and its antibacterial properties are key indicators of the quality of Taiwanese green propolis. Thus, the purpose of this study is to present a protocol for using ethanol as a solvent to extract and characterize the antibacterial properties of Taiwanese green propolis.

PROTOCOL:

1. Preparation of Ethanol-extracted Compounds

1.1. Weigh 10 g of frozen Taiwanese green propolis, which was collected from beehives in Taiwan from May to July, and grind it using the spice grinder. Confirm that whole pieces of Taiwanese green propolis are ground into a fine powder without any large particles.

1.2. Add 100 mL of various concentrations of ethanol (60%, 70%, 80%, 95%, and 99.5%) and water

to separate flasks and mix each concentration with 10 g of ground propolis.

1.3. Incubate at 25 °C and shake the flask at 250 rpm for 48 h.

1.4. Filter the ethanol extracts through filter paper with a 25 µm pore size.

1.5. Reconstitute the filtrates to their original volume (100 mL) with 95% ethanol using a volumetric flask.

1.6. Store the ethanol extracts at -20 °C.

NOTE: The protocol can be paused here.

2. Preparation of Ethanol Extracts for HPLC

2.1. Concentrate 10 mL of ethanol extracts by vacuum evaporation at 40 °C for 15 min.

2.2. Bake the dry matter at 45 °C for 24 h.

2.3. Reconstitute the dry matter with 10 mL of 95% ethanol.

2.4. Filter 1 mL of ethanol extracts using a sterile syringe filter with a 0.45 µm pore size.

2.5. Refilter the ethanol extracts using a sterile syringe filter with a 0.22 µm pore size. The filtrate is collected and can be directly analyzed using HPLC.

3. Analysis of the Propolin Content Using HPLC

3.1. Establishment of standard curves of propolins

3.1.1. Prepare 1 L of the mobile phase of 88.8:11.2 (v/v) methanol:water solution.

3.1.2. Prepare serial dilutions of propolin standard (C, D, F, and G) concentrations (15.625 mg/mL, 31.25 mg/mL, 62.5 mg/mL, and 125 mg/mL, respectively) using the mobile phase solution as a solvent.

3.1.3. Inject 20 µL of propolin standard concentrations into the reverse-phase column, sequentially from the low concentration to the high concentration.

3.1.4. Set the HPLC column at 30 °C and the flow rate to 1 mL/min.

3.1.5. Set the wavelength of the UV detector to 280 nm and the recorder time to 20 min.

3.1.6. Analyze the standards at least 3x.

3.1.7. Plot the measurement response (y-axis) against the concentration (x-axis) using calculation sheet software, and create a standard curve with the equation and R-square value.

3.2. Analysis of ethanol extracts

3.2.1. Inject 20 µL of the ethanol extracts obtained from step 2.5 into the reverse-phase column.

3.2.2. Set the HPLC column at 30 °C and the flow rate to 1 mL/min.

3.2.3. Set the wavelength of the UV detector to 280 nm and the recorder time to 20 min.

3.2.4. Analyze the standards at least 3x.

3.2.5. Calculate the concentration of propolin in the ethanol extract, using the equation for the standard curve obtained from step 3.1.7 that uses the peak area for each propolin.

4. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Analysis

NOTE: The microdilution method is used to evaluate the antibacterial efficacy of ethanol-extracted propolins from Taiwanese green propolis.

4.1. Preparation of test organisms

4.1.1. Thaw the bacterial strains, *Staphylococcus aureus* and *Escherichia coli,* and then, culture them in tryptic soy broth and nutrient broth, respectively, at 37 °C for 24 h.

4.1.2. Passage the *S. aureus* using tryptic soy broth and *E. coli* using nutrient broth for two generations and, then, calculate colony-forming units by counting individual colonies on an agar plate.

4.2. Preparation of ethanol extracts for antibacterial activity testing

4.2.1. Concentrate the ethanol extracts obtained from step 1.5 by vacuum evaporation at 40 °C for 15 min.

4.2.2. Reconstitute the dry matter with dimethyl sulfoxide (DMSO) and adjust the concentration of the extract to 12.8 mg/mL.

4.2.3. Make a serial dilution (concentrations: 5, 10, 20, 40, 80, 160, 320, and 640 µg/mL) of ethanol extracts using the broth.

4.3. Minimum inhibitory concentration tests

4.3.1. Add 10 µL of diluted ethanol extracts ranging from 0.156 to 640.0 µg/mL into a 96-well plate.

4.3.2. Using broth, adjust the volume to 100 µL, and maintain 5% DMSO in all the dilutions.

4.3.3. Inoculate 100 µL of bacterial culture (1 x 10^6/mL) into the 96-well plate.

4.3.4. Culture inoculum containing various concentrations of ethanol extracts at 37 °C for 48 h.

4.3.5. Analyze the bacterial growth according to turbidity and using optical density (microplate reader) at 590 nm to determine the minimum inhibitory concentration (MIC).

4.4. Minimum bactericidal concentration tests

4.4.1. Inoculate 10 µL of liquid culture from each well of the MIC test that exhibited no growth onto an agar plate.

4.4.2. Incubate at 37 °C for 24 h.

4.4.3. Determine the bactericidal activity by identifying the lowest concentration that revealed no visible bacterial growth. The concentration that completely eliminates cell growth is considered to be the minimum bacterial concentration (MBC).

REPRESENTATIVE RESULTS:

Positive representative data for the ethanol extraction are presented in **Table 1**. The dry matter yield from Taiwanese green propolis was positively associated with the concentration of ethanol. The 95% and 99.5% ethanol extracts had the highest dry matter yield from Taiwanese green propolis. The lowest dry matter yield from Taiwanese green propolis occurred when water was used as the extraction solvent. These results indicate that an organic solvent, such as ethanol, performs best for Taiwanese green propolis extraction. The signal of standard propolins C, D, F, and G was identified and quantified using HPLC (**Figure 1a**). The signal of propolins in the ethanol extracts was characterized using individual propolin standards and HPLC (**Figure 1b**). Normally, the concentration of propolins (C, D, F, and G) in Taiwanese green propolis is positively associated with the ethanol concentration during extraction (**Table 1**). The highest yield of propolins in Taiwanese green propolis was produced in the 95% and 99.5% ethanol extracts.

Positive representative data for the antibacterial effect of ethanol extracts are presented in **Table 2**. Antibacterial activity against *S. aureus* and *E. coli* in the ethanol extracts were examined. The average MIC and MBC of ethanol extracts for *S. aureus* were 10 - 20 µg/mL and 20 µg/mL, respectively (**Table 2**). Water extracts did not have antibacterial effects against *S. aureus* (**Table 2**). No antibacterial effect on *E. coli* was observed with either the ethanol or water extracts (**Table 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: Identification of propolins in Taiwanese green propolis. These panels show (**a**) standards of propolins and (**b**) the measurement of propolins in Taiwanese green propolis using HPLC. This figure has been modified from Chen *et al.*[8].

Table 1: Dry matter yield (%) and propolin content (mg/mL) in Taiwanese green propolis extracted using various solvents. * 10 g of propolis was extracted using 100 mL of solvent, and extracts were finally reconstituted to 100 mL. ** The values are the mean ± the standard deviation (SD). [a-e] Means within a column that have no common superscript are significantly different ($P < 0.05$). *** ND = not detected. This table has been modified from Chen *et al.*[8].

Table 2: MIC and MBC (μg/mL) of various extracts against *S. aureus* and *E. coli*. This table has been modified from Chen *et al.*[8].

DISCUSSION:

A study reported that maceration, using various concentrations of ethanol, could be used for Brazilian propolis extraction[17]; however, the process was time-consuming[17]. It took at least 10 days to extract the bioactive compounds, such as phenolic content, from Brazilian propolis[17]. Alternatively, ethanol extraction in combination with heating at 37 °C, 50 °C, or 70 °C for 30 min has been proposed for extracting Brazilian propolis[19,21,22]. It was determined that bioactive compounds in Brazilian propolis could be differentially extracted depending on the percentage of ethanol[19]. The extraction efficiency of bioactive compounds in propolis may vary because of the different chemical compositions of Brazilian propolis and Taiwanese green propolis. Here, we provide a reliable and repeatable protocol for extracting Taiwanese green propolis. The bioactive compounds in Taiwanese green propolis could be harvested within 2 days, using ethanol as a solvent. A similar finding also supports the protocol presented here and determined that propolins (C, D, F, and G) from Taiwanese green propolis could be extracted using 95% ethanol in combination with a 3 day extraction[23]. Another study demonstrated that 95% ethanol in combination with a 21 h extraction was able to harvest propolins from Taiwanese green propolis[24]. However, the exact concentration of propolins after the 21 h ethanol extraction must be verified. Researchers have proposed that the heating process increases the extraction efficiency for Brazilian propolis[19,21,22]. Whether propolins from Taiwanese green propolis can be extracted by heating must be investigated.

The ethanol extracts from Taiwanese green propolis have antibacterial effects against gram-positive pathogens. Because propolis is hydrophobic, studies have demonstrated that an organic solvent such as ethanol is a suitable solvent for propolis extraction[8,16-20]. Studies have also demonstrated that an increased ethanol concentration leads to more extracted bioactive compounds[8,17,18,20]. In the present protocol, the maximum dry matter yields of Taiwanese green propolis are observed in the 95% and 99.5% ethanol extracts, as well as the highest concentration of propolins and antibacterial activity.

The present protocol is designed for Taiwanese green propolis extraction using ethanol, and the quality assessment is made on the basis of the contents of propolins. However, some ethanol-

insoluble bioactive compounds in Taiwanese green propolis cannot properly be isolated using the present protocol.

The most crucial aspect of the method with respect to existing methods is that this method saves time relative to methods proposed in other studies[17,23]. Moreover, the 95% and 99.5% ethanol extracts of Taiwanese green propolis exhibited a high concentration of propolins and antibacterial activity.

This approach has direct applications for characterizing other unknown ethanol-soluble bioactive compounds in Taiwanese green propolis. Additionally, ethanol extracts containing propolins could be used for determining other bioactivities.

One of the key experimental procedures is ensuring the uniformity of the Taiwanese green propolis during grinding (protocol step 1.1). Inappropriate grinding of Taiwanese green propolis can lead to low dry matter yields and propolin content. It is essential to use a proper grinding apparatus, such as a spice grinder, tissue grinder, or homogenizer, and to take care that the propolis becomes a fine powder without any large particles remaining after grinding.

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DISCLOSURES:
The authors have nothing to declare.

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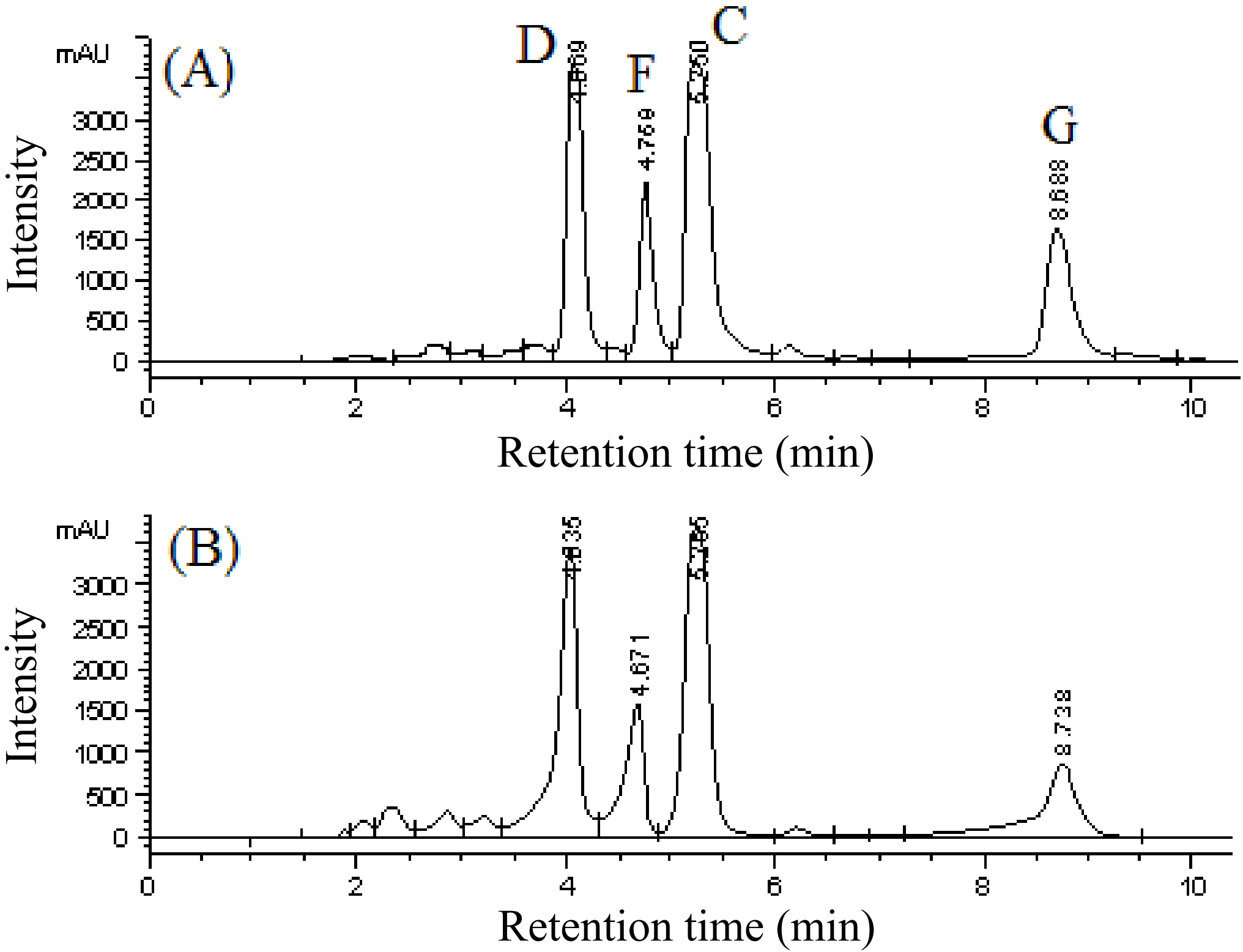

Table 1.

Solvent*	Yield (%)	Propolin (C+D+F+G) (mg/mL)
99.5% EtOH	66.75 ± 0.5[a]**	36.73 ± 0.80[a]
95% EtOH	66.25 ± 0.50[a]	37.55 ± 1.29[a]
80% EtOH	64.75 ± 0.96[b]	34.25 ± 0.71[b]
70% EtOH	63.25 ± 0.96[c]	31.53 ± 0.31[c]
60% EtOH	59.00 ± 1.41[d]	29.34 ± 1.59[d]
Water	7.00 ± 0.82[e]	ND***

Table 2.

Extract	*Staphylococcus aureus*		*Escherichia coli*
	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)
99.5% EtOH	10	20	＞640
95% EtOH	10	20	＞640
80% EtOH	20	20	＞640
70% EtOH	10	20	＞640
60% EtOH	20	20	＞640
Water	ND	ND	ND

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
ethanol	Sigma-Aldrich	E7023	
Whatman no. 4 filter paper	Sigma-Aldrich	WHA1004125	
methanol	Sigma-Aldrich	34860	
reverse phase RP-18 column	Phenomenex Inc.	00G-0234-E0	
Staphylococcus aureus	ATCC	BCRC 10780	
Escherichia coli	ATCC	BCRC 10675	
tryptic soy broth	Sigma-Aldrich	22092	
nutrient broth	Sigma-Aldrich	70122	
dimethyl sulfoxide	Sigma-Aldrich	D2650	
spice grinder	Waring	WSG60K	
microplate Reader	Molecular Devices	EMAX PLUS	
HPLC system	Agilent	1200 Series	
vacuum evaporation	BÜCHI	Rotavapor R-215	

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