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Research Article

Antioxidant and Antimicrobial Activities of *Pogostemon cablin* (Blanco) Benth.

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Pogostemon cablin (Lamiaceae) has been widely used in traditional medicine. In this study, the antioxidant and antimicrobial activities of leaves from *P. cablin* extracts were investigated. The water extracts had the highest total phenolic content 116.88±0.48 mg gallic acid equivalent/g of dry plant extract. Nevertheless, high levels of total flavonoid content were found in ethanolic extracts 280.12 ± 2.04 mg quercetin equivalent/g of dry plant extract. The highest antioxidant activities were found for the ethanolic extract (IC_{50} = 18 ± 0.90, 20 ± 0.24 μg/mL) by DPPH and ABTS scavenging assays, respectively. Both extracts showed moderate inhibition of superoxide inhibition ($O_2^{\bullet-}$) and nitric oxide (NO) production in concentration-dependent manner. Antibacterial activity was calculated by disk diffusion, minimum inhibitory concentration (MIC), and minimum bacterial concentration (MBC). The ethanolic extract had the greatest activity against methicillin resistant *Staphylococcus aureus*, methicillin sensitive *S. aureus*, and *Streptococcus pyogenes* with zone diameters of 11.67 ± 1.53, 10.33 ± 2.52, and 10.33 ± 1.15 mm, respectively. The corresponding MIC and MBCs were 5, 0.625, and 0.039 mg/mL. *P. cablin* extracts contain antioxidant and antibacterial properties that should be exploited for possible clinical application.

1. Introduction

Fever is the regulated rise in body temperature above the normal range; it has been recognized as a sign of infection since ancient times [1]. The most common cause of fever is bacterial infections [2] which are also one of the most common causes of the inflammation response [3]. Acute inflammation is characterized by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes, and inflammatory mediators such as cytokines [4]. Inflammation leads to the increased expression of nitric oxide (NO) which influences many aspects of the inflammatory cascade and may play a critical role in the pathophysiology of acute and chronic inflammatory diseases [5]. High concentrations of NO rapidly react with superoxide radical ($O_2^{\bullet-}$) to form peroxynitrite ($ONOO^-$), a highly reactive and toxic reactive nitrogen species (RNS). Indeed, a combination of reactive oxygen species (ROS) and NO production has been implicated in the pathogenesis of atherosclerosis, cancer, cardiovascular, and neurological diseases [6]. Therefore, inhibition of ROS and NO is a potential way to reduce oxidative stress and subsequent inflammation as well as prevent these chronic diseases in the future. However, currently used antimicrobials are failing to bring an end to many bacterial infections because of drug resistance and some antioxidants like butylated hydroxytoluene (BHT) cause carcinogen [7]. For this reason research is ongoing for natural antioxidant and antimicrobial activities.

Pogostemon cablin belongs to the family Lamiaceae and grows wild in Southeast Asia [8]. This plant has been widely used in traditional medicinal practice in Southeast Asia. In Thai medical scripture, the whole plant is used as a component herb in ailments and approximately 27 formulae are reported. The most common method of preparation is decoction and maceration with whisky for 4 formulae. Typically, folk medicine used *P. cablin* to reduce fevers and chronic fatigue, restore function, and improve the digestion [9].

There are reports detailing individual properties of this plant's essential oils such as antimicrobial [10, 11], antioxidant [12], analgesic [13], anti-inflammatory [14], antimutagenic [15], antithrombotic [16], antiemetic [17], and cytotoxic activities [18, 19]. However, there are no reports which have studied the relationship between the antimicrobial and antioxidant activities of leaf extracts by using traditional extraction methods.

2. Material and Methods

The leaves of *Pogostemon cablin* were collected in March 2016 in Ratchaburi, Thailand. The leaves were dried at 45°C for 12 hours (h) and then underwent maceration and decoction to obtain the extracts.

2.1. Preparation of Extracts

2.1.1. Maceration Method. 30 g of dry powder leaf was soaked in 500 mL of 95% ethanol and allowed to stand at room temperature for 74 hours in a well-lit room. The extract was stirred every 24 hours by using a sterile glass rod and filtered through a Whatman No. 1 filter paper and was concentrated by evaporator to obtain the ethanolic extracts. Then, the extract was stored at −20°C.

2.1.2. Decoction Method. 30 g of dry powder leaf was boiled in 500 mL of distilled water for 15 minutes. Then, the concentrated extract was filtered and dried by lyophilization to obtain the aqueous extract. Then, the extract was stored at −20°C.

2.2. Determination of Bioactive Compounds Content

2.2.1. Determination of Total Phenolics Content [20]. Total phenolic contents in the extracts were determined by the modified Folin-Ciocalteu method using microplate readers. First, 20 μL of the extract was mixed with 100 μL Folin-Ciocalteu's reagent in 96-well microplate. After waiting for 5 minutes, 80 μL of sodium carbonate was added, and the plate was allowed to stand for 30 minutes. Absorbance values of samples were measured at 765 nm. Gallic acid (GA) was used as the standard phenolic substance to compare with extracts. Total phenolic content was expressed as milligram of gallic acid equivalent per gram of dry weight of each extract (mg GAE/g). All measurements were performed in triplicate.

2.2.2. Determination of Total Flavonoid Content [21]. Total flavonoid content was performed by the aluminum chloride colorimetric method. 500 μL of each extract was mixed with 75 μL of 5% (w/v) sodium nitrite after which 150 μL of 10% (w/v) aluminum chloride complex had been added and left for 5 minutes (m). 500 μL of 1 M sodium hydroxide was added, and the volume was supplemented to 1500 μL with distilled water. Then, the reaction was incubated for 30 m at room temperature (25°C). The absorbance was measured at 510 nm. Total flavonoid content was expressed as milligram of quercetin equivalents per gram of dry weight of each extract (mg QE/g).

2.3. Determination of Antioxidant Activity Using the DPPH Radical Scavenging Assay [22]. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radial. The assay is based on the reduction of DPPH with antioxidant. In brief, 100 μL of 6×10^{-5} M DPPH solution in absolute ethanol was mixed with 100 μL of different concentrations of the extract and incubated for 30 m in a dark at room temperature. The absorbance was measured at 520 nm using microplate readers. Trolox and BHT were used as positive controls. Initially, DPPH IC_{50} was determined and defined as the antioxidant concentration needed to reduce DPPH activity by 50%, a parameter widely used to measure the antioxidant activity. The antioxidant scavenging activity was calculated as a percentage inhibition in the formulae below:

$$\text{The Percentage of Inhibition} = \left(\frac{\text{Abs. Control} - \text{Abs. Sample}}{\text{Abs. Control}}\right) \times 100. \quad (1)$$

2.4. Determination of Antioxidant Activity Using ABTS Radical Cation Decolorization Assay [23]. The ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] assay has two steps, the preparation of ABTS radical solution followed by the discoloration assay. $ABTS^{\bullet+}$ radical solution is prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate. The reaction mixture was allowed to stand in the dark at room temperature for 12–16 hours to complete radical generation. Then, the ABTS radical solution was diluted with deionized water to reach an absorbance of 0.68–0.72 at 734 nm before being used. The discoloration assay was assessed by mixing 180 μL of ABTS radical solution with 20 μL of various dilutions of each extract in 96-well microplates. The reaction mixture was allowed to stand for 6 minutes, and the absorbance was measured at 734 nm by using a microplate reader. The scavenging activity of extracts against ABTS radicals was calculated by the percentage inhibition of scavenging activity using the formula below and expressed as IC_{50} (μg/mL).

$$\text{The Percentage of Inhibition} = \left(\frac{\text{Abs. Control} - \text{Abs. Sample}}{\text{Abs. Control}}\right). \quad (2)$$

2.5. Determination of Nitric Oxide Production Using the Griess Reaction Assay [24]. Lipopolysaccharide (LPS) is inducible nitric oxide synthase (iNOS) which is the specific enzyme for synthesizing NO from L-arginine. In this assay, RAW 264.7 cells were seeded at 1×10^5 cells/well in 96-well microplates. After that, the medium containing 5 ng/mL lipopolysaccharide (LPS) and the extract at various concentrations were incubated for 48 hours at 37°C in a humidified atmosphere containing 5% CO_2. NO production was determined by measuring the accumulation of nitrite (NO_2^-) in the culture supernatant using the Griess reagent at 570 nm. The percent

inhibition of the extract at each concentration was calculated by the formula below:

$$\text{The Percentage of Inhibition} = \frac{(\text{Ac} - \text{Ab}) - (\text{As} - \text{Ab})}{(\text{Ac} - \text{Ab})} \times 100. \quad (3)$$

Ac and As are the absorbance of a mixture stimulated with LPS. Ab is the absorbance of a mixture nonstimulated with LPS.

The cytotoxic effect of the extract in RAW 264.7 cells was performed using the MTT assay. Briefly, MTT solution had been added to the cells and left for 4 hours. Then the medium was removed, and isopropanol containing 0.04 M HCl was added to dissolve the formazan product in these cells. The absorbance of formazan solution was measured with a microplate reader at 570 nm.

2.6. Determination of Superoxide Production Using Nitroblue Tetrazolium (NBT) Dye Reduction Assay [25]. HL-60 cells had been incubated for 6 days in PRMI 1640 medium supplement with 10% heated fetal bovine serum containing 1.3% DMSO. Intracellular superoxide formation was quantified by the NBT reduction assay. Briefly, 1×10^6 differentiated HL-60 cells were dissolved in 200 μL of Hank's Buffered Salt Solution (HBSS) to which various concentrations of each extract were added. The mixture was incubated at 37°C in 5% CO_2 for 15 minutes after which 250 ng/mL of phorbol-12-myristate-13-acetate (PMA) and 1.25 mg/mL NBT were added and incubated for 60 min. Finally, 2 mL of 1 M HCl was added to the reaction mixture. After centrifugation at 12,000 ×g for 10 minutes, DMSO was added to dissolve the resulting formazan by using sonication. The absorbance was measured with a microplate reader at 560 nm. The percent inhibition of each concentration was calculated by using the equation below:

$$\text{The Percentage of Inhibition} = \frac{(\text{Ac} - \text{Ab}) - (\text{As} - \text{Ab})}{(\text{Ac} - \text{Ab})} \times 100. \quad (4)$$

Ac and As are the absorbance of a mixture stimulated with PMA. Ab is the absorbance of a mixture nonstimulated with PMA.

The cytotoxic effect of extracts on HL-60 cells was assessed with the MTT assay. Briefly, the cells had been immersed in MTT solution for 4 hours. Following the centrifugation for 7 minutes, the mixture was dissolved with DMSO. The absorbance of formazan solution was measured with a microplate reader at 570 nm.

2.7. Evaluation of the Antimicrobial Activity

2.7.1. Microbial Strains. Cultures of the following microorganisms were used in the study: (i) *Staphylococcus aureus* ATCC 25923 methicillin-susceptible, (ii) methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 20651, (iii) *Streptococcus pyogenes* ATCC 19615, (iv) *Pseudomonas aeruginosa* ATCC 9097, and (v) *Candida albicans* ATCC 90028.

2.7.2. Disk Diffusion Method [26]. Disk diffusion was carried out with Mueller-Hinton agar (MHA) for *S. aureus*, MRSA, and *P. aeruginosa*, MHA with 5% sheep blood for *S. pyogenes*, and Sabouraud Dextrose agar (SDA) for *C. albicans*. The colonies were suspended to a 0.5 McFarland standard. 10 μL of the extracts was added at concentration of 500 μg/mL to paper discs (6 mm in diameter). Positive controls in this study were Gentamicin and Amphotericin B (1 mg/mL). These plates had been incubated at 37°C for 24 hours for the bacteria and at 37°C for 48 h for the *Candida*. The zone of inhibition was calculated by measuring the diameter of the inhibition zone. Three different fixed directions were taken in triplicate, and the mean value was calculated.

2.7.3. Determination of Minimum Inhibitory Concentration (MIC) [27]. The MIC of the extracts was determined according to the microbroth dilution technique. In brief, the cultures were inoculated equivalent to a 0.5 McFarland Standard in Mueller-Hinton broth (MHB). The cultures were diluted to 1/200. The plant extracts were diluted by using double fold serial dilution in MHB. Then 50 μL of each culture and extract was mixed to a sterile 96-well and incubated at 37°C for 24 hours for the bacteria and 37°C for 48 hours for the *Candida*. After that, resazurin 10 μL was added in each well and incubated for 2 hrs. The color change was then assessed visually. The growth was indicated by no color change from purple to pink as the MIC value.

2.7.4. Determination of Minimum Bactericidal Concentration (MBC) [28]. The minimum bactericidal concentration (MBC) is defined as the lowest concentration of an antimicrobial agent needed to kill the initial inocula. The MBC was determined by first selecting the well that showed no growth during the MIC determination. 10 μL of each tube was subcultured onto agar plates and incubated for 37°C for 24 hours for the bacteria and at 37°C for 48 hours for the *Candida*.

2.8. Statistical Analysis. All experiments were carried out in triplicate and presented as means ± SEM (standard error of the mean). A dose-response curve was obtained by plotting the percent inhibition values versus extract concentrations, and the IC_{50} values (μg/mL) were determined by cubic spline interpolation using GraphPad Prism (version 4.3).

3. Results and Discussion

The yields of water and ethanolic extracts were, respectively, 12.70% and 7.47% w/w in terms of dried starting material. In case of *P. cablin*, the extract yield increased with the temperature and polarity. The results of this study are in agreement with the extraction yields of some medicinal plants.

The total phenolic content was expressed as mg gallic acid per gram of dry plant extract (mg GAE/g). The highest total phenolic content was in the water extract (Table 1). These results indicate that increasing the temperature, polarity index (PI), and solubility of phenolic compounds in the extraction solvents enhance the content of phenols [29].

Table 1: Percent yield, antioxidant activity, and total phenolic and flavonoid contents of *Pogostemon cablin* leaves extracts ($n = 3$).

Methods	Extract	% Yield	Antioxidant activity (IC_{50} μg/mL)		Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
			DPPH radical scavenging assay	ABTS radical cation decolorization assay		
Decoction	Water	12.70	60 ± 1.18	33 ± 1.80	116.88 ± 0.48	221.38 ± 0.96
Maceration	Ethanol	7.47	18 ± 0.90	20 ± 0.24	99.59 ± 0.54	280.12 ± 2.04
	BHT		14 ± 0.90	4 ± 0.90	—	—
	Trolox		5 ± 0.90	3 ± 0.90	—	—

Table 2: Cell based assay results of nitric oxide formation and superoxide production of *Pogostemon cablin* leaves extracts ($n = 3$).

Extract	Nitric oxide inhibition		Superoxide inhibition	
	(IC_{50} μg/mL)	% Cell survival	(IC_{50} μg/mL)	% Cell survival
Water	>200	>70	195.98 ± 1.00	>70
Ethanol	144 ± 4.90	>70	108 ± 1.10	>70

The solubility of polyphenols was observed to depend mainly on the presence and position of the hydroxyl groups and the molecular sizes and the lengths of constituent hydrocarbon chains [30].

The total flavonoid content was expressed as mg quercetin equivalents per gram of dry plant extract (mg QE/g). According to the results, the effective solvent used to extract flavonoid content appeared on ethanol solvent (Table 1). This may indicate that sequential extraction using ethanol solvent is the most appropriate to use for extractability of flavonoid content from *P. cablin* [31].

The DPPH radical scavenging activity is presented in Table 1. Compared to the water extract, the ethanolic extract had approximately a threefold increase in DPPH radical scavenging activity with $IC_{50} = 18 \pm 0.90$ μg/mL and had ~1.5-fold higher antioxidant activity with $IC_{50} = 20 \pm 0.24$ μg/mL in the ABTS radical cation decolorization antioxidant assay. These results, which are consistent with those of others [32–35], suggest that flavonoids have intrinsically high antioxidant properties and provide most of the antioxidant activity of leaf extracts. The result found flavonoid content which showed positive correlations with the antioxidant activity. Flavonoids, including flavones, flavanols, and condensed tannins, are plant secondary metabolites and their antioxidant activities depend on the presence of free OH groups, especially 3-OH [36], the total number of hydroxyl groups, and the substitution of functional groups on their nuclear structures [37].

In cell-based bioassays, the results showed that ethanolic extract was the most active against NO and $O_2^{\bullet-}$ in a dose-dependent manner ($IC_{50} = 144 \pm 4.90$ and 108 ± 1.10 μg/mL) (Table 2). To ensure that the extracts were not cytotoxic, MTT assay was used to measure cell viability. All the extracts showed more than 70% of the cell viability. The ethanolic extract had higher nitric oxide scavenging and antioxidant activities compared to the water extract but the IC_{50} values were relatively high and the ethanolic to water ratios lower compared to the results of the DPPH and ABTS assays. Those experimental results have demonstrated that the ethanolic extract exhibited $O_2^{\bullet-}$ and NO. Interestingly, the results of this study imply that Thai medicine used *P. cablin* as a component in formulation to relief fever. The mechanism inhibited the generation of ROS and RNS which associated with anti-inflammatory effects. Likewise chemical assays, the flavonoids would be main active compounds that displayed antioxidant properties. In previous report, the active hydroxyl groups of flavonoid are generally glycosylated. Glycosylation increases hydrophilicity of a molecule, which in its turn impairs its interaction with cellular membranes and decreases its rate of absorption [38, 39]. Moreover, the literature showed flavonoids also effective scavengers of peroxyl, superoxide, and peroxynitrite radicals [40].

The MIC and MBC results of the extracts against *S. aureus*, MRSA, *P. aeruginosa*, *S. pyogenes,* and *Candida albicans* are shown in Table 3. The ethanolic extract (5 mg/disk) demonstrated the best activity against MRSA, *S. aureus,* and *S. pyogenes* with zone diameters of 11.67 ± 1.53, 10.33 ± 2.52, and 10.33 ± 1.15 mm, respectively. The MIC and MBC values ranged from 0.039 to 5 mg/mL for all bacteria. The ethanolic extracts were the most effective against *S. pyogenes* (0.039 mg/mL). In earlier report on the antimicrobial activity, Lv et al., 2011, it has been reported that essential oil of *P. cablin* showed moderate antibacterial activity against *S. aureus*, *B. subtilis,* and *S. cerevisiae* [41].

The antibacterial activities are attributed to patchouli alcohol, α-patchoulene (−)-patchouli alcohol, and pogostone that have several modes of action [42]. Yang et al. found that pogostone and (−)-patchouli alcohol show potential against cell wall membranes by molecular docking, and the result proved those chemical compounds have good antibacterial activities by MIC and MBC assay [11].

4. Conclusion

We have shown that ethanolic extract from *Pogostemon cablin* showed moderate antioxidant activity and inhibited bacteria commonly responsible for community and hospital acquired infections. Our results support the ethnotherapeutic claim of *Pogostemon cablin*. Additional work should be done to exploit these properties to see if these extracts might have a clinical application.

Table 3: Antimicrobial activity against five microorganisms of *Pogostemon cablin* leaves extracts ($n = 3$).

Extract	Inhibition zone (mm.), MIC (mg/mL), MBC (mg/mL)				
	S. aureus	MRSA	*P. aeruginosa*	*S. pyogenes*	*C. albicans*
Water (Conc. 1 mg/disc)	0* 0.625† 0.625‡	8.33 ± 1.53 2.5† 2.5‡	0 1.25† 1.25‡	0 >5† >5‡	0 >5† >5‡
Ethanol (Conc. 5 mg/disc)	10.33 ± 2.52* 0.625† 0.625‡	11.67 ± 1.53* 5† 5‡	0* 0.625† 0.625‡	10.33 ± 1.15* 0.039† 0.039‡	0* >5† >5‡
Amoxicillin (Conc. 10 μg/disc)	NT	NT	NT	35 ± 0.00* 0.016† 0.0256‡	NT
Gentamycin (Conc. 10 μg/disc)	15 ± 0.00* 0.195† 0.195‡	10 ± 0.00* >200† >200‡	12 ± 0.00* 0.39† 0.39‡	NT	NT

*Inhibition zone.
†MIC.
‡MBC.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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