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Artikel Asli/*Original Article*

Qualitative Phytochemical Analysis and Antimicrobial Activity of *Piper sarmentosum* Leaves Extract Against Selected Pathogens

(Analisis Kualitatif Fitokimia dan Aktiviti Antimikrobial Ekstrak Daun *Piper sarmentosum* terhadap Patogen Terpilih)

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ABSTRACT

The controversial usage of antiseptic in treating wound infections had become a huge issue over the years due to its minimal effectiveness and high toxicity level that are harmful to humans. Hence, numerous studies had been carried out to determine other possible approaches including herbal remedies. The alarming situation had led us to study on Piper sarmentosum and its antimicrobial activity against selected pathogens. In present study, methanol extract of Piper sarmentosum leaves were prepared to investigate the presence of phytochemical compounds. The in vitro antimicrobial activity of crude methanolic extract was evaluated using disc diffusion and microdilution broth methods. The study demonstrated the antimicrobial activity of leaves extract against Staphylococcus aureus (7 mm) and Escherichia coli (6.5 mm). However, no zone of inhibition was observed against Pseudomonas aeruginosa. Meanwhile, the MIC values for Staphylococcus aureus was 6.25 mg/mL whilst Escherichia coli was 12.5 mg/mL. In addition, the phytochemical screening results revealed that the extract contained glycosides, flavonoids, terpenoids, alkaloids and phenolics. In conclusion, methanolic extract of Piper sarmentosum leaves has the potential as a new, effective alternative towards current drugs that are available for skin-associated infection. The findings from this study are crucial in providing latest information of the plant's additional values that can be incorporated as a baseline for current and future studies as well as in investigating other possible plants that are beneficial for health purposes, particularly for combating skin-associated infection.

Keywords: Piper sarmentosum; antimicrobial; pathogenic; phytochemical; methanol extract

ABSTRAK

Penggunaan antiseptik dalam merawat jangkitan luka telah menjadi satu isu yang besar selama ini kerana keberkesanannya yang minimum dan kandungan toksik tinggi yang merbahaya kepada manusia. Oleh itu, pendekatan lain telah dibuat bagi memperolehi agen antimikrobial semula jadi di mana kajian terhadap aktiviti antimikrobial ekstrak daun Piper sarmentosum terhadap patogen terpilih telah dijalankan. Dalam kajian ini, ekstrak daun Piper sarmentosum menggunakan larutan metanol telah dijalankan untuk mengenal pasti kehadiran bahan fitokimia. Aktiviti antimikrobial secara in vitro telah diuji ke atas ekstrak mentah melalui kaedah 'disc diffusion' dan 'microdilution broth'. Kajian ini menunjukkan aktiviti antimikrobial ekstrak daun terhadap Staphylococcus aureus (7 mm) dan Escherichia coli (6.5 mm). Walau bagaimanapun, tiada zon perencatan ditunjukkan oleh Pseudomonas aeruginosa. Sementara itu, nilai MIC bagi Staphylococcus aureus ialah 6.25 mg/mL manakala Escherichia coli mencatatkan 12.5 mg/mL. Di samping itu, hasil pemeriksaan fitokimia mendedahkan bahawa ekstrak mengandungi glikosida, flavonoid, terpenoid, alkaloid dan fenolik. Kesimpulannya, ekstrak metanol daun Piper sarmentosum mempunyai potensi sebagai alternatif yang berkesan berbanding ubatan yang sedia ada untuk jangkitan berkaitan dengan kulit. Penemuan daripada kajian ini adalah penting dalam memperolehi maklumat terkini tentang khasiat tumbuhan ini yang boleh digunakan sebagai asas untuk kajian semasa dan pada masa akan datang serta menyiasat tumbuh-tumbuhan lain yang bermanfaat untuk kesihatan sejagat, terutamanya untuk memerangi jangkitan berkaitan dengan kulit.

Kata kunci: Piper sarmentosum; antimikrobial; patogenik; fitokimia; ekstrak metanol

INTRODUCTION

Wound infections on the skin are common throughout the world and can occur almost every day in our lives. The infection due to break in the skin integrity usually caused by bacteria, fungi or viruses (Mousa 2016). Normally, one can easily obtain the over-the-counter drug or medication to treat the wound. However, the usage of antiseptic for treatment had become a huge issue over the years due to its decreasing effectiveness and possible cytotoxicity that can be harmful to humans (Hirsch et al. 2010). The ever-increasing numbers of antimicrobial resistance towards

modern medicine are of public concern and rather alarming. The development of new, alternative herbal remedies is essential in assisting treatment of diseases. Hence, numerous studies had been carried out to determine other possible approaches including herbal remedies.

Piper sarmentosum (P. sarmentosum) is one of the plants that are beneficial to human health and mostly grown in tropical and subtropical regions (Ugusman et al. 2012), especially in shady and moist areas. Although it looks similar to *Piper betle (P. betle)*, the anatomy of *P. sarmentosum* is slightly different (Lakshmi & Naidu 2010; Vasuki et al. 2011; Raman et al. 2012). Several studies have documented that the genus *Piper* has many constituents that include lignans and alkaloids, to name a few (Ghosh et al. 2014). The plant has been widely used over the generations to treat various diseases (Ugusman et al. 2012), namely diabetic and inflammatory diseases (Peungvichaa et al. 1998; Stohr et al. 2001; Rahman et al. 2016). Besides, it also possessed high antioxidant efficacy (Hafizah et al. 2010). Therefore, *P. sarmentosum* has a great potential to be developed as an antimicrobial agent. Hence, the aim of this research is to investigate the antimicrobial activity and phytochemical compounds of *P. sarmentosum* leaves extract against selected pathogens.

EXPERIMENTAL METHODS

PLANT MATERIAL AND SAMPLE COLLECTION

Fresh leaves were collected from Kampong Ketemba Luar, Kuala Pegang, Baling, Kedah, Malaysia. The leaves were washed for several times to remove other foreign substances. The leaves were then cut into smaller pieces and dried under a shade before grounded into powder form.

EXTRACTION METHOD

The extraction was performed according to Vijayakumar (2012) with slight modification. 200 g of *P. sarmentosum* powder was weighed and added in 800 mL of the methanol solution, using the ratio of 1:4. Once the crude extract was obtained, it was then stored in a sealed petri dish at 4°C until further use.

SELECTED MICROORGANISMS AND ANTIBIOTICS

Twenty µL of 10% Dimethyl sulfoxide (DMSO) was used as negative control while 30 µg of Amikacin, 10 µg of Gentamicin and 10 µg of Streptomycin were chosen as positive control for each tested microorganisms of *Pseudomonas aeruginosa (P. aeruginosa)* (ATCC 27853), *Staphylococcus aureus (S. aureus)* (ATCC 25923)and *Escherichia coli (E. coli)* (ATCC 25922), respectively. Both antibiotics and bacterial strains used for this study were obtained from Microbiology Laboratory, Centre of Medical Laboratory Technology, Faculty of Health Sciences, UiTM Puncak Alam Campus, Selangor. Similarly based on Sahalan et al. (2018), the bacteria confirmation tests were also included, such as Gram staining and cultivation on several media (i.e. Nutrient, Eosin Methylene Blue (EMB), MacConkey and Mannitol salt agars) as well as biochemical tests (i.e. Catalase, Oxidase, Coagulase, Phenylalanine deaminase, Motility, Citrate, Triple sugar iron (TSI), Indole, Methyl red and Voges Proskauer tests).

DISC SUSCEPTIBILITY TEST

Three to five colonies were inoculated into 3 mL of Tryptic Soy Broth (TSB) prior to incubation at 37°C for 2 h. Meanwhile, the turbidity was adjusted according to 0.5% MacFarland standard. A sterile cotton swab was dipped into the bacterial-contained broth and streaked onto Muller Hilton Agar by rotating the agar plate at 60°. The impregnated disc with *P. sarmentosum* extract was placed onto the surface agar together with negative and positive control before overnight incubation at 37°C. After incubation, sensitivity or resistance of the inhibition zones were determined by the measurement of diameter in millimeter (mm).

DETERMINATION OF MIC VALUES VIA MICRODILUTION BROTH METHOD

The inoculum suspension of bacteria used to determine Minimum inhibitory concentration (MIC) values via two-fold broth microdilution method in 96-well microtiter plate were prepared, only after the bacteria had shown sensitivity against the impregnated-extract disc of the leaves during disc susceptibility test. The procedure was based on Mazumder et al. (2014) with minor modifications. Three to five colonies were inoculated into 5 mL of TSB. The suspension was then incubated at 37ºC for 3 to 6 h with turbidity equivalent to 0.5 MacFarland standards. The plate was labelled from number 1 to 13 with well 11 as positive control followed by well 12 as negative control and well 13 as antibiotic control. The plate was then sealed with aluminium foil and incubated at 37°C for 18 to 24 h. This procedure was performed in triplicates.

PHYTOCHEMICAL ANALYSIS

The qualitative test of preliminary phytochemical screening was done according to the method performed by Banu and Cathrine (2015) with slight modification. The methanol extract of *P. sarmentosum* leaves was screened for the presence of glycosides (using Glycosides test method), flavonoids (using Alkaline Reagent test method), terpenoids (using Salkowski test method), alkaloids (using Wagner's test method) and phenolics (using Ferric Chloride test method). The results were based on the reaction and colour changes.

RESULTS

From 200 g of the dried-powder form of *P. sarmentosum*, 18.93 g of crude extract was obtained with 9.2% of weight extraction. Each tested organisms were observed on several media with *S. aureus* streaked onto Mannitol salt and Nutrient agars followed by *P. aeruginosa* on Nutrient, MacConkey and EMB agars, then MacConkey and EMB agars were used to test for *E. coli*. Subsequently, for biochemical test, catalase and coagulase tests showed positive for *S. aureus* with the former exhibited bubble formation that indicates the presence of catalase enzyme while the latter formed clot that revealed the presence of coagulase enzyme. For *P. aeruginosa*, oxidase, motility, citrate and methyl red tests showed positive results whilst phenylalanine deaminase, indole and Voges Proskauer tests were negative. Furthermore, for triple sugar iron test revealed alkaline slant and alkaline butt that indicates glucose, lactose and sucrose while absence of bubble or crack indicates no gas, followed by none black precipitate showed absence of hydrogen sulphide (H_2S). Then, for *E. coli*, motility, indole and methyl red tests showed positive results whilst phenylalanine deaminase, citrate and Voges Proskauer tests revealed negative results. Moreover, for triple sugar iron test performed on *E. coli* revealed acid slant and acid butt that indicates glucose, lactose and sucrose fermenter with bubble that showed the presence of gas, while no black precipitate was observed had revealed the absence of H_2S.

In addition, the findings from disc diffusion method revealed that the mean zone of inhibition for 25 mg/ml and 50 mg/ml of *P. sarmentosum* extract against *S. aureus* was measured 0 mm and 7 mm, respectively. The positive control was revealed 22.33 mm while negative control showed 0 mm. For *P. aeruginosa*, both 25 mg/ml and 50 mg/ml were measured 0 mm. However, the positive control revealed 23 mm while negative control showed 0 mm. Then, the findings of 25 mg/mL and 50 mg/ml extract against *E. coli* were measured 0 mm and 6.5 mm, respectively, with positive control showed 15.33 mm whilst negative control revealed no inhibition zone. The results are further tabulated in Table 1. Furthermore, the MIC test values for *S. aureus* and *E. coli* were inhibited at the concentration of 6.25 mg/mL and 12.5 mg/mL, respectively, as shown in Table 2. However, MIC value for *P. aeruginosa* was not performed as no zone of inhibition was observed during the earlier test. In addition, the phytochemical screening tests showed the presence of glycosides, flavonoids, terpenoids, alkaloids and phenolics as described in Table 3.

TABLE 1. Disk diffusion tests for *P. sarmentosum* methanolic leaves extract against selected bacterial strains

Bacterial Strains	Mean Zone of Inhibition/mm			
	Positive control	Negative control	25 mg/mL	50 mg/mL
S. aureus (ATCC 25923)	22.33	0	0	7
P. aeruginosa (ATCC 27853)	23.00	0	0	0
E. coli (ATCC 25922)	15.33	0	0	6.5

ATCC = American Committee of Clinical Laboratory Standards; mm = millimeter; mg/mL = milligram per milliliter

TABLE 2. MIC values for *P. sarmentosum* methanolic leaves extract against selected bacterial strains

Bacterial Strains	Concentration of leaves extract/mgmL^{-1}	Result	MIC Value/ mgmL^{-1}
S. aureus (ATCC 25923)	100	-	6.25
	50	-	
	25	-	
	12.5	-	
	6.25	-	
	3.125	+	
	1.563	+	
	0.782	+	
	0.391	+	
	0.2	+	
	Positive Control	+	
	Negative Control	-	
	Antibiotic Control	-	
E. coli (ATCC 25922)	100	-	12.5
	50	-	
	25	-	
	12.5	-	
	6.25	+	
	3.125	+	
	1.563	+	
	0.782	+	
	0.391	+	
	0.2	+	
	Positive Control	+	
	Negative Control	-	
	Antibiotic Control	-	

MIC = Minimum Inhibitory Concentration; ATCC = American Committee of Clinical Laboratory Standards; mm = millimeter; mg/mL = milligram per milliliter; (-) = clear, absence of growth; (+) = turbid, presence of growth

DISCUSSION

There are numerous studies from the Southeast Asian region that indicate the beneficial values of *P. sarmentosum* (i.e. fruit, leave, stem) to cure various illnesses (Seyyedan et al. 2013). In Malaysia, *P. sarmentosum* has long been used to treat diabetes mellitus, mouth-related problems such as gum infection and curing other ailments as well as reducing white discharge during the menstrual cycle (Subramaniam et al. 2003). In this study, dried leaves of *P. sarmentosum* were used. The leaves were dried under the shady area to prevent the degradation of bioactive

TABLE 3. Phytochemical compound tests of *P. sarmentosum* leaves extract

Phytochemical compound	Observation	Result
Glycosides	Brick-red precipitate formed	+
Flavonoids	Yellow colour formed	+
Terpenoids	Yellow colour formed	+
Alkaloids	Reddish-brown precipitate formed	+
Phenolics	Dark-green colour formed	+

(+) = Presence of compound

compounds (Hossain et al. 2014). The dried leaves were further grounded into a fine, powder to increase the surface area, hence, escalating the extraction rate (Tiwari et al. 2011). Meanwhile, methanol solvent was used in this study as it showed a high degree of inhibition as stated by Sen et al. (2012) and Bhat and Al-Daihan (2013). Other studies had also recorded highest extraction yield by using methanol as compared to other solvents (Cowan 1999; Anokwuru et al. 2011; Gami & Parabia 2011; Basri et al. 2014).

The disc diffusion method was performed in determining the antimicrobial activity of *P. sarmentosum* leaves extract against selected pathogens and was chosen due to it's cost-effective and easy interpretation of findings. From this study, antimicrobial activity was observed against *S. aureus* (7 mm) while the extract showed a slight effect against *E. coli* with 6.5 mm. A similar study carried out on *Piper betle* also revealed that the leaves have the ability to inhibit *S. aureu*s and *E. coli* (Hoque et al. 2011; Jayalakshmi et al. 2015). Nonetheless, the methanolic extract of *P. sarmentosum* leaves were found to be more effective against gram-positive bacteria in comparison to gram-negative bacteria. This may be due to the presence of lipopolysaccharide that acts as an effective permeability barrier to the gram-negative bacteria (Zhang et al. 2013). However, *P. aeruginosa* showed no inhibition zone around the disc. This situation revealed that the extract was unable to inhibit the bacteria. A previous study by Giraud and de Bentzmann (2011) further proof that the presence of intrinsic resistance of AmpC β-lactamase and efflux pump cause *P. aeruginosa* being able to acquire further resistance mechanism to multiple group of anti-microbial agents. Another report carried out by Selim et al. (2013) stated that the bacteria might need a higher concentration of extract or different types of solvent such as aqueous or chloroform to further enhance its ability to inhibit.

Meanwhile, the determination of MIC values were performed as it is considered a goal standard in identifying the sensitivity of microorganism against antimicrobial agent (Yilmaz 2012). In this study, two-fold broth microdilution method was carried out as it only used a small amount of crude extract. The MIC value revealed that the minimum concentration of the plant extract to inhibit *S. aureus* was 6.25 mg/mL while *E. coli* showed 12.5 mg/mL. Contradict to our findings, Basri and Mohd Nor (2014) had exhibited MIC values with 0.391 mg/mL for both acetone and methanol extracts for *Canarium odontophyllum* leaves against *S. aureus* via similar method. In addition, two previous studies by Eloff (2004) and Kuete (2010) had purported that the best MIC value is lower than 0.1 mg/mL. However, our results are considerably higher in comparison to other studies. This may be due to the light exposure during experiment, resulting in degradation of bioactive compounds and decreasing of antimicrobial activity. Besides, prolonged storage of crude extract can also influence the results.

In addition, the qualitative phytochemical screening was also conducted based on the reactions of the plant's extract with respective reagents. A study by Trivedi et al. (2011) had documented that two plants that belong to the same family as *P. sarmentosum* contained similar bioactive properties that are useful for therapeutic purposes. Each of the compounds has its own function. Nonetheless, several previous studies strongly support that phenolics and flavonoids content in plants display a good anti-microbial activity (Daglia 2011; Baba & Malik 2015) with the former playing an important role in plant's defense against pathogen, hence, usually used to control human pathogenic infection. In addition, terpenoids also act as anti-inflammatory, anti-malarial and anti-bacterial as stated by Wang et al. (2005) while the presence of alkaloids are commonly used to reduce fever and headache as it contained analgesic properties (Petruczynik 2012). Recent report by Aslam et al. (2017) reviewed that *P. sarmentosum* leaves contained phenyl propanoids, followed by alkaloid amide and piperamide while the presence of benzene and amide alkaloids had further showed that *P. sarmentosum* possess anti-microbial and anti-fungal properties (Masuda et al. 1991; Tuntiwachwuttikul et al. 2006).

CONCLUSION

Methanolic leaves extract of *P. sarmentosum* showed antimicrobial activity against *S. aureus* and *E. coli*. All of the phytochemical compounds that include glycosides, flavonoids, terpenoids, alkaloids and phenolics were present in the extract. However, further test by using High Performance Liquid Chromatography (HPLC) or Gas Chromatography Mass Spectroscopy (GC-MS) can be carried out to further confirm our results. In conclusion, the leaves extract can be a possible source to obtain a new, effective herbal remedy in treating skin-related infections, hence, further proven the potential of natural-based approach in replacing current drugs that are highly toxic and harmful for humans.

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