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Antimicrobial activity of the crude extract of *Piper sarmentosum* against methicilin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Vibrio cholera* and *Streptococcus pneumoniae*

L. FERNANDEZ, K. DARULIZA, S. SUDHAKARAN, R. JEGATHAMBIGAI

Institute for Research in Molecular Medicine (INFORMM) University Sains Malaysia, 11800 Penang (Malaysia) and Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Jalan Semeling-Bedong 3 1/2, Bukit Air Nasi, Bedong 08100, Kedah (Malaysia)

Abstract. – BACKGROUND AND AIMS, The emergence of novel diseases caused by microbial pathogens and the undesirable side effects of certain antibiotics has been a recent dilemma in the medical arena. Consequently, it has stirred the discovery of many naturally occurring agents which could possibly provide important ramifications against various pharmacological targets and to combat various ailments. The main aim of the present study was to determine the antimicrobial activity of the crude methanolic extract of *Piper (P.) sarmentosum* against methicillin resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Vibrio cholera* and *Streptococcus pneumoniae*.

MATERIALS AND METHODS, The plant materials were extracted by percolation in 70% methanol. Only the leaves were used in this study. Initial antimicrobial screening using disc diffusion assay was conducted and further screening of the antimicrobial properties in the plant was performed using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The phytochemical constituents in the plant were evaluated via phytochemical screening and thin layer chromatography (TLC).

RESULTS, *P. sarmentosum* inhibited the growth of MRSA with an inhibition zone of 10.0 mm. There was no inhibition zone observed for the other microbes tested. MIC test showed a value of 50mg/ml and MBC results showed no Colony Forming Unit (CFU) at 100 mg/ml against MRSA. Phytochemical screening of the crude extract indicated the presence of tannins, flavonoids, alkaloids glycosides and anthraquinone. Thin-layer chromatography (TLC) further confirmed the presence of flavonoids and alkaloids in the extract.

CONCLUSION, *P. sarmentosum* has shown to have some antimicrobial properties against MRSA. Based on the MIC index (MBC/MIC), the extract exhibits bactericidal effects against MRSA. TLC analysis indicated the presence of flavonoids and alkaloids which could have contributed to the antimicrobial activity of the plant extract.

Key Words:

Piper sarmentosum, Antimicrobial screening, Disc diffusion, MIC.

Introduction

Piper (P.) sarmentosum which *belongs* to the Piperaceae family is a fast-growing vine which can be found in many parts of Asia. The leaves are used traditionally to treat toothaches, fungal dermatitis on the feet, asthmatic coughing and pleurisy[1]. Chloroform extracts from *Piper sarmentosum* have also exhibited antimalarial activity against *Plasmodium falciparum* and *Plasmodium berghei*[2].

Previous investigations on the medicinal properties of this species make it commendable for further studies. Since the species does exhibit some antimicrobial properties, it is worth enquiring in-depth properties possessed by this plant by challenging it with more lethal bacteria such as *Vibrio (V.) cholera* and *Streptococcus (S.) pneumoniae* which may perhaps provide positive ramifications for harmful bacterial infections. Hence, in the quest to fight various infectious maladies and ailments using naturally-occurring entities, methanolic leave extract of *P. sarmentosum* was screened for its antimicrobial activities against MRSA, *E. coli*, *V. cholera, and S. pneumoniae* strains.

Corresponding Author: R. Jegathambigai, MD; e-mail: jegathas@yahoo.com

Objectives of Study

The main objective of this study was to determine the antimicrobial activity of the crude methanolic of *P. sarmetosum* against MRSA, *Escherichia (E.) coli*, *V. cholera, S. pneumoniae* strains using disc diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Besides this, this study was also conducted to analyze the phytochemical constituents and to identify the presence of active compounds in the plant methanolic extract by thin-layer chromatography (TLC) screening.

Materials and Method

Materials

Plant Material

The plant material was collected from Kedah. The plant was identified and classified by a botanist in the Biotechnology Department of AIMST University.

Chemicals

Methanol, ethyl acetate, hydrochloric acid, sulphuric acid, chloroform, ammonia, and ethanol from R&M Chemicals (Essex, UK) Benedict reagent and Ferric chloride from (Bendosen Laboratory Chemicals, Bendosen, Norway) silica gel pre-coated aluminium backed TLC plates (Kieselgel 60 F254; 20 × 20 cm) from Merck (Darmstadt, Germany) and sodium hydroxide, Meyer's reagent, distilled water, Luria-Bertani (LB) agar, Luria-Bertani broth.

Equipments

Micropipete (P20, P1000, P5000), conical flasks, test tubes, Eppendorf tubes, L-rod, universal bottles, disposable Petri dishes, beakers, filter paper (6 mm), gloves, sterile swab sticks, parafilm, magnetic stirrer, TLC chamber (Cole-Parmer, Vernon Hills, IL, USA) 24-well Microtitre plates, Weighing machine, Vortex machine, Laminar flow, Incubator, Bunsen burner, Autoclave machine, Spectrophotometer.

Methods

Preparation of Plant Materials

The leaves were separated from the rest of the plants. The leaves were then washed thoroughly under running tap water in order to reduce the microbial contamination. The plant materials were then spread on trays and allowed to air dry for about a week. Direct sunlight was avoided as the UV rays may destroy the active compounds present in the plants. The leaves were then grounded into fine powder. This was done to increase the surface area of the plant materials when exposed to the extraction solvent. The ground plant materials were then placed in jar bottles and stored at room temperature.

Extraction of Plant Materials

The plant material was extracted through percolation with 70% methanol. 100 g of plant material were soaked in 500 ml of methanol[3]. The mixtures were soaked for four days at room temperature (30°C) and they were stirred every day with a sterile glass rod. The extract was then clarified by filtration through Whatman No. 1 filter papers and then was concentrated using an oven at 37°C for 24 hours to obtain the crude extract. The extract was then stored in vials at -20°C to prevent the loss of the bioactive compounds until further use.

Preparation of Different Concentration of the Plant Extract

A working solution of 100 mg/ml of the plant extract was prepared by re-dissolving appropriate amounts of distilled water to the crude methanolic extract by using the standard formula ($M_1V_1=M_2V_2$).

Bacterial Strains Used for Antimicrobial Activity

The bacterial strains used in this study were *V. cholera* and *E. coli* gram (–ve) and MRSA and S. *pneumoniae* gram (+ve). The strains were obtained from the Culture Collection Centre, Department of Biotechnology, AIMST University. The bacterial cultures were streaked on Luria Bertani (LB) agar plates and single colonies were selected and cultured in Luria Bertani broth to be used for antimicrobial testing. In addition, glycerol stocking was also done for long term storage of the strains.

Antimicrobial Screening of the Crude Methanolic Extract of *P. sarmentosum*

Disc Diffusion Assay

The disc diffusion assay was employed to determine the antimicrobial activity of the plant ex-

tract against the four selected bacterial strains. The method used was according to a modified version of NCCLS 2001[4]. A concentration of 100 mg/ml of each plant extracts were prepared. Sterilized paper discs were also prepared and soaked in the extracts and allowed to dry for 1 hour. Overnight log phase cultures were used for the screening. The bacterial inoculums were standardized to 0.5 Mc Farland. A cotton swab was used to streak the bacteria on LB agar. The impregnated discs were placed on the agar using a sterile forceps. The plates were placed in 4°C for 1 hour to allow prediffusion of the extracts onto the agar[5]. The plates were then incubated for 24 hours at 37°C. Chloramphenicol (30 ug/ml) was used as the positive control where as distilled water was used as the negative control. The zone of inhibition was measured in millimeters and recorded.

Minimum Inhibitory Concentration (MIC)

MIC is the lowest concentration of drug or in this case, the plant extract that inhibits the growth of bacteria. It is the concentration that gives the least inhibitory mode of action and below which there is no further inhibition. A method by Andrews, 2001 was used to determine the MIC[6].

For this test, a 24-well microtitre plate was used. Precisely, 1 ml of LB broth was placed in all the wells. 1 ml of 200 mg/ml plant extract was placed in the well labeled 'A'. A two-fold serial dilution was performed. The mixtures were re-suspended with a pipette each time before they were transferred to the next well. A 100 ul of bacteria was inoculated in all the wells. Chloramphenicol was used as the reference drug where as distilled water was used as the negative control. The plates were incubated at 37°C for 24 hrs. The wells were observed for turbidity & non-turbidity.

Minimum Bactericidal Concentration (MBC)

Following defining MIC, the dilution with no growth was used as inoculum for MBC determination. A 100μl of contents in the well which did not show visible growth was platted on fresh LB agar using an L-rod. The plates were incubated for 24 hours at 37°C. The MBC endpoint was the lowest concentration of extract at which there was no visible growth.

MIC Index

The MIC index (MBC/MIC) was calculated to determine if the plant extract had bactericidal or bacteriostatic properties. When the (MBC/MIC is ≤4), the extract is bactericidal and when (MBC/MIC >4), the extract is bacteriostatic[6,7].

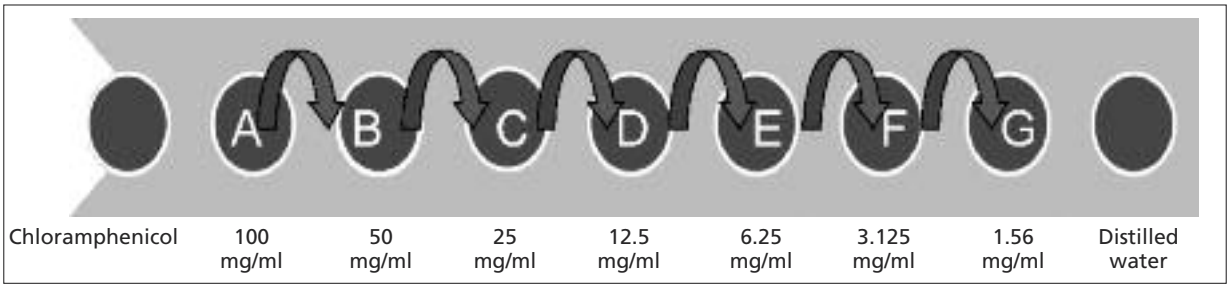
Qualitative Phytochemical Screening of the Plant Crude Methanolic Extract

The phytochemical screening was done according to a modified method by Onwukaeme et al[8]. 1 g of the crude extract was used for the phytochemical qualitative tests to identify the presence of reducing sugars, saponins, anthraquinone, flavonoids, tannins and alkaloids. The tests were:

Benedict's test for reducing sugar (glycosides) 2 ml of plant methanolic extract was placed in a test tube. 1 ml of Benedict's solution A and B was added. The mixture was shaken and heated in water bath for 10 minutes. The colour change was recorded. A brick red precipitate indicates presence of reducing sugar.

Frothing test for saponins 2 ml of plant methanolic extract was placed in a test tube. The extract was shaken vigorously and allowed to stand for 10 minutes. A thick persistent froth indicates the presence of saponins.

Borntrager's test for anthraquinone derivatives 2 ml methanolic extract was placed in a test tube. 1 ml of 10% ammonia was added and the mixture was shaken vigorously. The colour change was recorded. Pink-red colour in the ammoniacal layer shows the presence of anthracene derivatives.



Test for flavonoids 2 ml of plant methanolic extract was placed in a test tube. Diluted NaOH was added followed by diluted HCl. The solubility and colour change was noted. A yellow solution with NaOH which turns colourless with diluted HCl confirms the presence of flavonoids.

Ferric chloride solution test for tannins 2 ml of plant methanolic extract was placed in a test tube. A 15% ferric chloride test solution was added. The colour change was recorded. A blue or green colour indicates the presence of tannins.

Colour test for alkaloids 2 ml of methanolic extract was added to a test tube. 10 ml of ammonical chloroform was added followed by 10 drops of 10% H_2SO_4 and finally 0.5 ml of Meyer's reagent. Precipitate indicates presence of alkaloids.

Qualitative Thin Layer Chromatography of the Plant Crude Methanolic Extract

TLC screening of the crude extract was done. Silica gel pre-coated aluminum backed TLC plates (Kieselgel 60 F254; 20 × 20 cm) were dried in the oven at 90°C for 10 minutes. The extract was spotted at the bottom of the plates and transferred into a separating chamber. The mobile phase was composed of ethyl acetate: methanol (50:50) ratio, (40:60) ratio, (30:70) ratio and (20:80) ratio. The chamber was equilibrated for 15 minutes with the mobile solvents. The plates were developed and observed under UV light to determine the spots.

Statistical Analysis

The result of this study was analyzed using Student's *t*-test (SPSS version 17, Chicago, IL, USA).

Results and Discussion

Antimicrobial Activity of the Crude Methanolic Extract of *P. sarmentosum*

Disc Diffusion Test

Table I shows the zone of inhibition of the methanolic extract of *P. sarmentosum* against the four selected bacterial strains. The results showed an inhibition zone of 10.0 ± 0.0 mm at a concentration of 100 mg/ml when tested against MRSA where as the zone of inhibition of the commercial antibiotic, chloramphenicol (30 ug/ml) was 14.0 ± 0.0 mm. The zone of inhibition between the crude extract and commercial antibiotic is considered to be statistically significant ($p = 0.0018$). However, the extract may show better activity if tested at higher doses. The plant extract did not show any activity against *E. coli*, *V. cholera* and *S. pneumoniae*. There was no zone of inhibition observed in the disc impregnated with distilled water.

The agar disc diffusion method is ideal for screening antimicrobial activity in the plants crude methanolic extracts. However, it does not give any qualitative information about the active compounds present in the extracts.

Determination of Minimum Inhibitory Concentration (MIC) of the Crude Methanolic Extract of *P. sarmentosum*

The MIC values for *C. asiatica* and *P. sarmentosum* against MRSA and *A. tuberosum* against *V. cholerae* are given in Table II. MIC is defined as the lowest concentration that is able to inhibit any visible microbial growth and below that concentration which there is no further inhibition. *P. sarmentosum* inhibited MRSA at 50 mg/ml. The wells with concentrations below the mentioned MIC values for the respective plants showed turbidity. The turbidity in the wells was due to the growth of the microbes.

The advantages of MIC over disc diffusion technique include increased sensitivity for small quantities of extracts which is important if the antimicrobial is scarce as in the case of many natural products; the ability to distinguish between bacteriostatic and bactericidal effects; and quantitative determination of the MIC[9]. This method can also be used for a wide variety of microorganism, it is not expensive and it presents reproducible results.

Determination of Minimum Bactericidal Concentration (MBC) of the Plant Crude Methanolic Extract

As depicted in Table III, the MBC screening indicated no colony forming unit (Figure 1) for *P. sarmentosum* tested against *MRSA* at 100 mg/ml; hence deducing that the MBC value for this extract was 100 mg/ml. The plant extracts may have inhibited the bacterial growth either through the disruption of DNA replication[10] or by impairing cellular function and membrane integrity through cellular leakage of nucleotides and proteinacious materials[11].

Table I. The table above shows the zone of inhibition of the crude methanolic extract of *P. sarmentosum*.

Disc diffusion assay				
	Zone of inhibition (mm)			
Sample	*E. coli*	*MRSA*	*S. pneumonia*	*V. cholerae*
P. sarmentosum (100 mg/ disc)	–	10.0 ± 0.0 mm	–	–
Chloramphenicol (30 ug/disc) (positive control)	22 mm	14 mm	16 mm	18 mm
Distilled water (negative control)	6 mm	6 mm	6 mm	6 mm

Table II. The table above shows the MIC values of *P. sarmentosum*. The test was done in triplicates (n=3).

Minimum inhibitory concentration		
Plants	MIC values (mg/ml)	Microbial strain tested
P. sarmentosum	50	MRSA

Table III. The table above shows the MBC value for *P. sarmentosum*. The test was done in triplicates (n=3).

Minimum bactericidal concentration	
MBC (mg/ml) for *P. sarmentosum*	Colony forming unit (CFU)
100 mg/ml	0

Figure 1. Shows the plate where MBC for *P. sarmentosum* was determined. At 100 mg/ml, no colony forming unit was observed, deducing that 100 mg/ml was the MBC value for the plant extract.

MIC Index

MIC index = MBC/MIC
= 100/50
= 2

Based on the MIC index calculation, the index value was 2. As the value is less than 4 and there was an absence of bacterial growth observed on the plate at 100 mg/ml, it can be inferred that the plant extract possesses bactericidal effect against MRSA.

Qualitative Phytochemical Screening of the Crude Methanolic Extract of P. sarmentosum

Phytochemical constituents of the crude methanolic extract of the *P. sarmentosum* were identified based on the phytochemical tests. Table IV shows the results obtained from the phytochemical screening. Based on the results, all the tested phytochemicals were present in the extract except for saponins. It is believed that plants which are rich in a wide variety of secondary metabolites belonging to chemical classes such as flavonoids and alkaloids are generally superior in their antimicrobial activities and these compounds are generally soluble in polar solvents such as methanol[12]. However, tannins, saponins and glycosides have also been associated with antimicrobial activities[13].

Table IV. Phytochemical screening of the crude extract of *P. sarmentosum*.

Phytochemical screening	
Phytochemical tests	*P. sarmentosum*
Flavonoid test	Flavonoids present
Ferric chloride test	Tannins present
Colour test for alkaloids	Alkaloids present
Benedict's test	Glycosides present
Frothing test	Absent
Bontrager's test	Anthraquinone present

TLC Screening of the Crude Methanolic Extract of the P. sarmentosum

TLC screening was done to separate and identify the chemical constituents that were present in the methanolic extracts of *P. sarmentosum*. This was done by using ethyl acetate and methanol as the mobile phase at four different concentrations (50:50, 40:60, 30:70 and 20:80 of ethyl acetate to methanol ratio). Two different solvents and different concentrations of the solvents were used to facilitate the partitioning of the extracts on the silica gel column. Based on the observations, the concentration of 30:70 of ethyl acetate to methanol ratio showed the best separation. The separated fragments were observed under UV light. For *P. sarmentosum,* a reddish brown spot and a purple spot was observed on the TLC plate (Figure 2). The reddish brown colour indicated the presence of flavonoids and the purple colour indicated the presence of alkaloids[14,15]. These two compounds may be responsible for the antimicrobial activity of the plant extracts. Nevertheless, further studies on the isolation and characterization of the compounds should be done as to provide new leads towards discovering novel antimicrobial agents. High Performance Liquid Chromatography (HPLC) can be used to analyse the plant extracts by using a flavonoid and alkaloid standards. A comparison can be made based on retention time, peak area or peak height ratio. Then again, it is not necessarily the contribution of only a single active compound but the contribution of all the active compounds may provide more powerful healing and remedial effects.

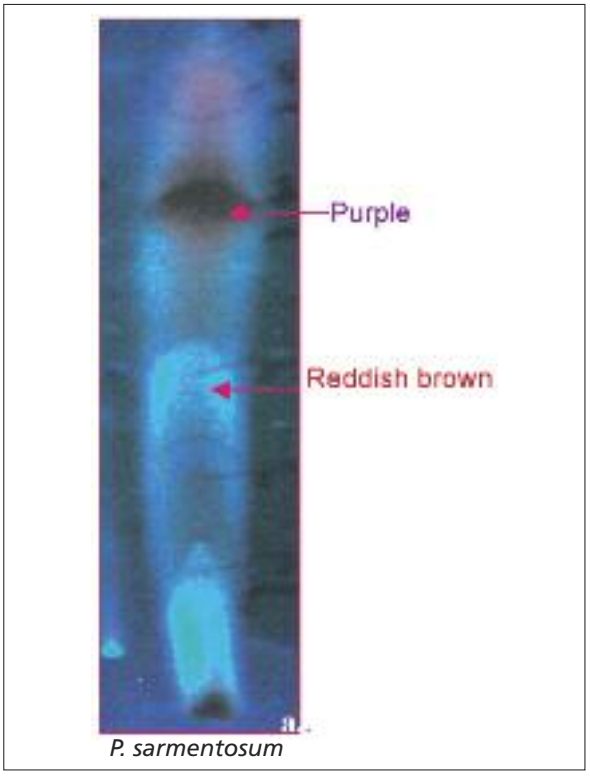


Figure 2. Shows the TLC plates observed under UV light. In TLC screening, the solvent mixture is drawn up the plate via capillary action. Since different plant compounds ascend the TLC plate at different rates, separation is achieved. The mobile phase used in this separation was ethyl acetate and methanol (30:70 ratios).

Conclusions

This investigation confirms that *P. sarmentosum* does have a scientific merit in its ability as an antimicrobial agent. The results obtained from the diffusion method showed that the extract exhibits a favourable antimicrobial activity against MRSA. The zone of inhibition obtained was significant (p=0.0018) between crude extract and commercial antibiotic for MRSA. Although it showed relatively lower antimicrobial activity when compared to standard chloramphenicol, it possesses bactericidal activity which could contribute towards the unending quest for novel antimicrobial drugs.

This study can be taken a step further in future by isolating and identifying the bioactive compounds present in the plant extracts by using either HPLC or NMR. The concentration of the plant extracts can also be increased to observe its effects.

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