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Research article

ANTIBACTERIAL ACTIVITY OF THE CRUDE EXTRACT OF *PIPER SARMENTOSUM* AGAINST *PSEUDOMONAS FUSCOVAGINAE*

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ABSTRACT: Kadok or *P. Sarmentosum* Roxb. is a terrestrial herb of the Piperaceae family widely distributed throughout South East Asian in the sub-tropical and tropical region of the world. All part of the plant is used as vegetables, commercially and medicinally valuable in many regions for treating many ailments. The possible benefits of *P. sarmentosum* are enormous which supported by a number of investigations which suggest the existence of antimicrobial compounds in the all plant parts. A study was conducted to evaluate the antibacterial activity of the methanolic fruit extract of *P. sarmentosum* against *P. fuscovaginae* by agar well diffusion assay and macro broth dilution method. The results were measured by diameter of inhibition zones produced, determination of Minimum Inhibition Concentration (MIC) and Minimum Inhibition Concentration (MBC) and the Inhibition Concentration (IC). The fruit extract which showed positive inhibition against the tested bacteria with diameter ranging from 9.33±0.58mm to 19.33±1.15 mm. At lowest concentration, 25 mg/mL of fruit extract recorded inhibition zone value of 9.33±0.58 mm for *P. fuscovaginae*. The highest concentrations of 200 mg/mL of fruit extract showed higher inhibition zone for 18.33±0.58 mm for *P. fuscovaginae* compared to positive control, Streptomycin sulphate 15.67±5.13 mm. The MIC and MBC value obtained was 12.5 mg/mL and 25 mg/mL, respectively. The IC_{50} and IC_{90} values were also determined. The fruit extract exhibited IC_{50} values of 28.08 mg/mL and IC_{90} values of 353.77 mg/mL against *P. fuscovaginae*. The antibacterial activity of streptomycin was clearly higher (50% growth inhibition at 0.072 mg/mL and 90% growth inhibition at 1.049 mg/mL against *P. fuscovaginae*. The results obtained from this study suggest that the fruit extract of *P.sarmentosum* has a potential to be developed as a novel bactericide.

Key words: Antibacterial, Inhibition concentration, Medicinal plant, Plant extracts, Rice plant

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INTRODUCTION

Kadok or *P. sarmentosum* is a terrestrial herb of the Piperaceae family that is widely distributed throughout South East Asia and the sub-tropical and tropical regions of the world (Taweechaisupapong *et al*., 2010). All parts of the plant are used as vegetables, and it is both commercially and medicinally valuable in many regions for treating many ailments (Diastutia & Delsy, 2012, Jalil *et al*., 2012, Scott *et al*., 2008). The possible benefits of *P. sarmentosum* are enormous, as supported by a number of investigations, which suggest the existence of antimicrobial compounds in all plant parts (Ruangsang, 2006). Several pharmacological and scientific studies have been done on different parts of *P. sarmentosum.* The water extract of *P. sarmentosum* showed hypoglycemic activity on normal and streptozocin-induced rats (Peungvicha *et al*., 1998).

The methanolic extract of the leaves of *P. sarmentosum* showed a neuromuscular blocking activity at the neuromuscular junction (Ridtitid *et al*., 1998). Najib Nik A Rahman *et al*. (1999) reported the chloroform extract of *P. sarmentosum* was the most effective extract for anti-malarial activity. The methanol extract of the leaves of *P. sarmentosum* also demonstrated potential antibacterial activity against gram negative *Pseudomonas aeruginosa* and both gram positive *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Zaidan *et al*., 2005). A recent study demonstrated the potent antibacterial activity of both the stem and leaf extracts of *P. sarmentosum* on *Pseudomonas aeruginosa* (Puzi *et al*., 2011). The antimicrobial properties of *P. sarmentosum* could be due to the presence of flavonoids and alkaloids, as indicated in the TLC analysis in which the crude methanolic extract exhibits bactericidal effects against MRSA (Fernandez et al., 2012). Therefore, the objective of this study was to screen for the antibacterial activities of the crude methanolic fruit extract of *P. sarmentosum* against *P. fuscovaginae.*

MATERIALS AND METHODS

Preparation of the extracts

The fruit part of the plant was collected and air-dried for three days and then extracted using methanol three times, followed by filtration through No. 1 Whatman filter paper prior to evaporation using a rotary vacuum evaporator (Zaidan et al 2005 and Singh et al 2010). The extracts were kept in a chiller (Chill-300 Protech) at 4°C until further use.The extraction efficiency was quantified by determining the weight of each of the extracts and the percentage yield was calculated (Mushore & Matuvhunye, 2013).

In vitro assay antibacterial activity

The assay for antibacterial activity of fruit extract was tested by agar well diffusion method as described by Patel et al (2012) with slight modification. Bacterial suspensions were cultured in Mueller Hinton Broth for 24hours and 0.2 ml of this culture was spread on Mueller Hinton Agar in petri dishes. Wells with 8mm diameter were cut in agar plates and filled with 50 µL of different extracts concentrations of 25, 50, 100 and 200 mg/mL. Two other wells were filled with 50 µL of streptomycin sulfate (30 µg/mL) and served as positive control, and 50 µL of aqueous methanol 80% (v/v) served as negative control and were left to dry for 3 hours. The plates were inverted and incubated for 24 hrs at 28°C. The test was done in three replicates. Microbial growth was determined by measuring the diameter of the zone of inhibition of the extract using a transparent ruler in millimeters (mm).

Determination of minimum inhibitory concentration (MIC)

Minimum Inhibitory Concentration (MIC) was determined by the macro broth dilution method after incubation for 24 hours at 28 ºC where plants extract concentration ranged from 25-200 mg/ml. 2, 3, 5-triphenyltetrazolium chloride (TTC, 2 mg/mL) (Sigma) aqueous solution used as dye to indicate the bacterial growth. The MIC value was taken as the lowest extract concentration, which showed no colour changes (Basri *et al*., 2011).

Determination of minimum bactericidal concentration (MBC)

The MBC value was determined by sub culturing the bacteria from the test tubes from MIC test which showed no colour changes into the sterile MHA plates. The least concentration that showed no visible growth on the agar plates was considered as the MBC value (Taweechaisupapong *et al*., 2010).

Statistical analysis

The results were analysed using statistical software JMP (version 9.0). Data were subjected to one-way analysis of variance (ANOVA), and significant difference between means, where the significance ($P<0.05$) is determined by Tukey's standardized range test (HSD) (Mushore & Matuvhunye, 2013, Basri et al., 2011). The relationship between the diameter zone of inhibition and extract concentration was determined by regression analysis of the data obtained for the fruit and leaf extracts. To determine the inhibition concentrations at IC_{50} and IC_{90} levels, the means of inhibition zonediameter data were subjected to log-probit analysis using Polo Plus V2 (Finney, 1972). To compare the antimicrobial activity, streptomycin sulfate at the concentrations of 0.01, 0.05, 0.1 and 0.5 mg/mL was used as standard. For *P. sarmentosum* fruit extract, four concentrations with three replicates were used to obtain concentration-mortality data. All the parameters including IC_{50} and IC_{90} and their 95% confidence interval were obtained from the regression line between the logarithmic dose and probit inhibition.

RESULTS AND DISCUSSION

In vitro assay antibacterial activity

The antibacterial activity of the fruit extract against the pathogenic bacteria *P. fuscovaginae* was measured by the diameter of inhibition zones produced and the MIC and MBC values. The fruit extract showed antibacterial activity against the tested pathogen (Table 1 and Figure 1) showing a diameter of inhibition zone ranging from 9.33±0.58 mm to 18.33±0.58mm. The positive control served by streptomycin sulfate showed inhibition towards all the tested bacterial isolates with the range of 15.67±5.13mm to 21.00±3.46 mm. Negative control (well containing aqueous methanol 80% (v/v)) showed no zone against any microorganisms in both the leaf and fruit extracts.

There was no significant difference between the fruit extract of *P. sarmentosum* at 100 and 200 mg/ml and streptomycin sulfate against *P. fuscovaginae*. The effect was negligible at low concentration, 25 mg/mL. Bactericides often act bacteriostatically at low concentrations, and are only bactericidal at higher concentrations. For bactericides to be effective, they must attain a sufficiently high concentration at the target site in order to exert their antibacterial action (Cloete, 2003). At the higher concentrations, the extracts inhibited the growth of the tested bacteria with a good potency. Generally, antimicrobial substances target a range of cellular loci, from the cytoplasmic membrane to respiratory functions, enzymes and the genetic material. However, different bacteria react differently to bactericides, either due to inherent differences, such as unique cell envelope composition and non-susceptible proteins, or to the development of resistance, either by adaptation or by genetic exchange (Cloete, 2003). This could be related to the beneficial effects of phytochemicals through the additive or synergistic action of several bioactive compounds that act at single or multiple target sites (Wink, 1999). The antibacterial activity, or so called antibiosis relationship between extracts and the bacteria, showed that the diameter of inhibition zones increased when the concentration level increased.

Determination of minimum inhibitory concentration (MIC)

The MIC and MBC of the methanolic extract of fruit of *P. sarmentosum* against *P. fuscovaginae* using the broth micro-dilution method are presented in Table 2. The *P. sarmentosum* leaf extract possessed killing effects against the bacterium. The lowest concentration of methanol extract that was able to inhibit the rice pathogenic bacteria was recorded at a concentration of 12.5 mg/mL.

Table 1: Antibacterial activity of *P. sarmentosum* methanol fruit extract by the agar well diffusion method

Extract	Concentration (mg/mL)	Microorganism / Inhibition zone (mm)(Mean ±SD)
		Pseudomonas fuscovaginae
Fruit	Positive control	15.67 ± 5.13^{ab}
	Negative control	0^{d}
	25	9.33 ± 0.58^{c}
	50	10.67 ± 0.58^{bc}
	100	12.00 ± 1.00^{bc}
	200	18.33 ± 0.58^{a}

Positive control: Streptomycin sulfate (30 µg/mL); Negative control: Methanol 80% (v/v). Within rows, values with different letters differ significantly (Tukey Kramer HSD, p = 0.05). All the tests were performed in triplicate.

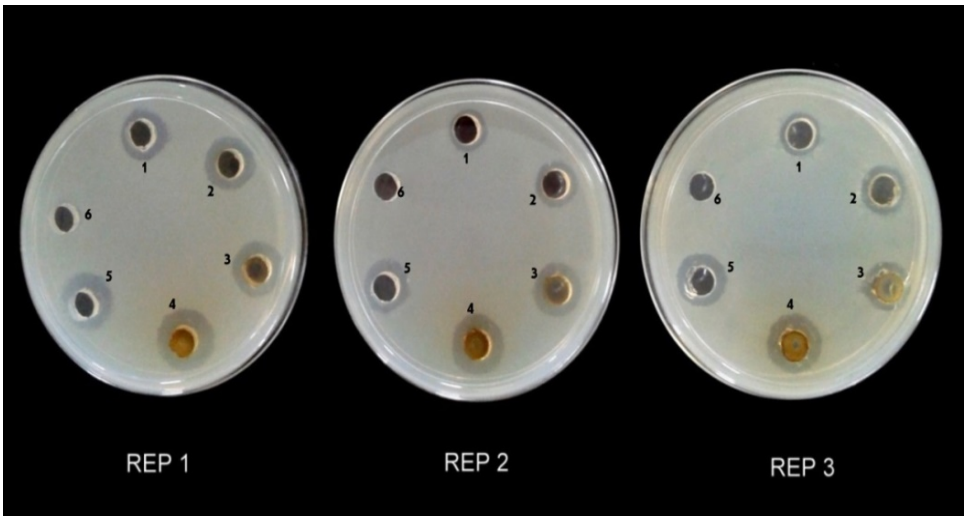

Figure 1: Inhibitory effects of the methanolic fruit extracts against *P. fuscovaginae*. The plates were inverted and incubated at 28°C for 24hrs. The concentrations of the extracts were indicated as (1) 25 mg/mL, (2) 50 mg/mL, (3) 100 mg/mL, (4) 200 mg/mL, (5) Positive control (Streptomycin), (6) Negative control (Methanol 80% (v/v))

Table 2: Probit regression line parameters and Inhibition Concentration (IC) of *P. sarmentosum* methanol fruit extract against *P. fuscovaginae*

Extract	Bacteria	Regression equation	Chi-square (df)	IC50 with fiducial limits	IC90 with fiducial limits
Fruit	*P. fuscovaginae*	Y=68.73+0.122 x	0.811 (1)	28.08 (16.52-38.82)	353.77 (202.11-1095.61)
PC	Streptomycin sulfate	Y=0.33+1.041x	3.517 (2)	0.072 (0.039-0.132)	1.049 (0.416-8.410)

All values of IC_{50} and IC_{90} along with their fiducial limits are in mg/mL
PC: Positive control, df: degree of freedom

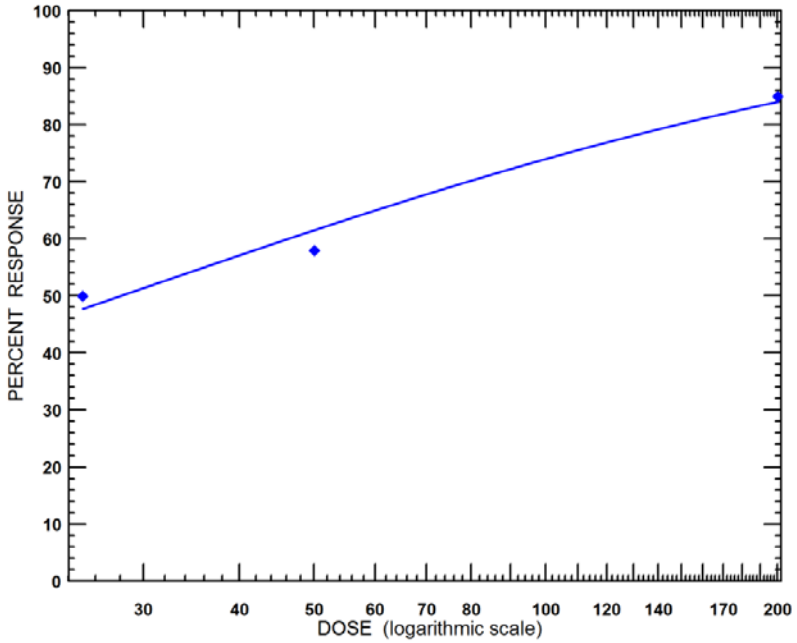

Figure 2: Typical relationship observed between concentrations of fruit extract and the diameter of the inhibition zone against the bacterial strains.

Table 3: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of *P. sarmentosum* methanol fruit extracts by the macro-broth dilution method

Tested bacteria	MIC (mg/mL)	MBC (mg/mL)	MIC index
Pseudomonas fuscovaginae	12.5	25	2

Determination of minimum bactericidal concentration (MBC)

For MBC, the lowest concentration that exhibited the bactericidal effect was at the concentration of 25 mg/mL of fruit extract recorded for*P. fuscovaginae*. The *P. sarmentosum* methanolic fruit extract exhibited broad-spectrum antibacterial activity, and had a bacteriostatic effect against all the tested rice pathogenic bacteria. The ability of methanolic fruit extract to inhibit pathogenic bacteria at the lowest concentration was also supported by the previous studies conducted by Zaidan et al. (2005) who reported that *P. sarmentosum* methanolic leaf extract had a lower MIC against most of the tested microbes, and the study conducted by Fernandez et al. (2012) who showed that *P. sarmentosum* inhibited the growth of MRSA with a MIC value of 50 mg/mL, and confirmed the presence of flavonoids and alkaloids in the extract. In a study performed by Masuda et al. (1991), they found the presence of the antimicrobial compounds, which stop the growth of *Escherichia coli* and *Bacillus subtillis*.

Determination of inhibition concentration (IC) of *P. sarmentosum* methanolic fruits extracts against *P. fuscovaginae.*

Table 2 summarizes the IC_{50} and IC_{90} values. Fruit extract exhibited IC_{50} values of 28.08 mg/mL and 8.4 mg/mL against *P. fuscovaginae*. The antibacterial activity of streptomycin was clearly higher (50% growth inhibition at 0.072 mg/mL and 90% growth inhibition at 1.049 mg/mL against *P. fuscovaginae*. In our study, the IC_{50} and IC_{90} values for the plant extracts were clearly higher than those of streptomycin, which is not surprising. Higher activities could also be expected if isolated compounds with antibacterial activity from the extracts had been employed in the assay (Syed Ab Rahman et al., 2014). The chi-square test values revealed that none of the tested bacteria has significant heterogeneity in the test population.

CONCLUSION

The methanolic fruit extract of *P. sarmentosum* was found to be effective as antibacterial agents against the causal agent of sheath brown rot, *P. fuscovaginae*. From the foregoing date, we can conclude that the fruit extract from this plant can be introduced further as the alternatives of the current control options with low cost, non-toxic and better effectiveness at a lower concentration and developed as a plant-based formulation.

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