JOURNAL OF PURE & APPLIED MICROBIOLOGY, Nov. 2014. Vol. **8**(Spl. Edn. 2), p. 483-490

Identification and Antibacterial Activity of Phenolic Compounds in Crude Extracts of *Piper sarmentosum* (Kadok)

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(Received: 10 September 2014; accepted: 26 October 2014)

***Piper sarmentosum* L., locally known as Kadok, is a tropical plant that grows in the wild and in cultivation in Southeast Asia. The plant is known for its medicinal properties and variety of active chemical constituents. The antibacterial capacity of methanolic leaf extracts (25, 50, 100, and 200 mg/mL) of *P. sarmentosum* and of phenolic standards was screened against gram-negative bacteria *Pseudomonas fuscovaginae* and *Xanthomonas oryzae* pv. *oryzae*, two major pathogens of rice (*Oryza sativa* L.), by agar-well diffusion and disc-diffusion assays, and compared with that of streptomycin sulfate (30 µg/ml). The inhibition zones for the extracts ranged from 9.0 to 19.33 mm; the minimum inhibitory concentration for both *P. fuscovaginae* and *X. oryzae* was 12.5 mg/mL and the minimum bactericidal concentrations were 25.0 and 12.5 mg/mL, respectively. Four phenolic compounds (gallic acid, tannic acid, quercetin, and naringin) were identified and quantified by reversed phase high-performance liquid chromatography diode-array detection (HPLC-DAD). Antibacterial assays indicated that these four compounds were potential natural antimicrobial agents against *P. fuscovaginae* and *X. oryzae*. The results presented here suggest that leaf extract of *P. sarmentosum* has strong potential to serve as a novel bactericide for disease suppression in crop plants.**

Key words: Leaf extract; *P. fuscovaginae*; *X. oryzae* pv. *oryzae*; agar well diffusion assay; phenolic compounds; minimal inhibition concentration.

Research on chemical compounds found in plants has increased rapidly worldwide, with numerous studies showing large potential for their use as antimicrobial agents (Al- Zubaydi *et al*. 2009). The use of and search for drugs and plant-derived antibiotics has accelerated in recent years, with many studies focusing on producing plant-based antibiotics, bactericides, and bio-pesticides (Bouamama *et al*. 2006). Plant extracts can mitigate harmful effects of, and microbial resistance to, chemical pesticides (Bhardwaj *et al*. 2009). Plant extracts, which contain a variety of phytochemicals, have also been shown to have antimicrobial effects; further investigation into their effectiveness in controlling microbial populations is required.

Phytochemicals plant-based substances and secondary metabolites such as phenolic compounds that are ubiquitous in the plant kingdom can be beneficial to human health, and some have been associated with reduced risk of major chronic diseases (Liu 2004). The universal presence of phenolic compounds in vascular plants has long been considered to be related to active and passive plant defense responses (Nicholson *et al*. 1992). The number of distinct phenolic acids, flavonoids, and polymeric flavonoid compounds produced by plants has been estimated to be as high as 8,000 (Dai and Mumper 2010). Many

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phytochemicals display a variety of biological activities and are purported to be beneficial to human health (Dai and Mumper 2010). In many cases, phenolic compounds serve as plant defense mechanisms that protect plants against microbial infections. Phenolic compounds are produced as a response to combination of stages that the plant obtains mostly in *in vitro* conditions. These compounds are synthesized in response to ecological and physiological pressures such as pathogen and insect attack, UV radiation, and wounding (Maddox *et al*. 2010).

Piper sarmentosum (Piperaceae) is a tropical plant that has been used as a food and traditional medicine to treat various types of diseases and ailments. Although the antibacterial activity of *P. sarmentosum* is well established, the mechanism of the inhibitory action has not been clarified. A number of organic compounds with antimicrobial activity have been identified in extracts from various parts of the plant. Previous studies that reported the presence of chemical constituents in *P. sarmentosum* including naringin (Subramaniam *et al*. 2003); phenylpropanoids (Masuda *et al*. 1991); cinnamic acid (Diastutia and Delsy 2012); and amides, pyrones, flavonoids, sterols, and neolignans (Atiax *et al*. 2010). *Piper sarmentosum* is thought to have a wide range of pharmacological effects and is used for wound healing, treatment of osteoporosis, and as an anti-nociceptive, anti-inflammatory, and antioxidant (Zakaria *et al*. 2010).

Given the above findings, it is relevant to investigate the potential antibacterial activity of *P. sarmentosum* leaf extracts against pathogens of rice (*Oryza sativa* L.). *Xanthomonas oryzae* is the causal agent of bacterial leaf blight (BLB), a vascular disorder that is considered to be one of the most significant and devastating diseases of rice in Asia since the 1980s (Shen and Ronald 2002). Sheath brown rot caused by *P. fuscovaginae* is an additional rice disease that is gaining recognition as a yield constraint. Although sheath brown rot is considered new in Asia, it has been widespread worldwide since at least the 1980s and has been reported on all continents except Antarctica, preferring high-humidity, low-temperature, high-elevation areas in the tropics and subtropics (Miyajima 1983; Adorada *et al*. 2012). Both *X. oryzae* and *P. fuscovaginae* are important pathogens of plant communities, and resistance of these pathogens to chemical control agents and antibiotics has become increasingly common (Miyajima 1983; Razak *et al*. 2009).

The aim of this study was to identify phenolic compounds in crude leaf extracts of *P. sarmentosum* and to evaluate the antibacterial effects of these compounds against two of the most destructive rice pathogenic bacteria, *P. fuscovaginae* and *X. oryzae*, which cause high yield losses in rice.

MATERIALS AND METHODS

Plant materials

Fresh leaves of *P. sarmentosum* were collected from the botanical garden of the Institute of Bioscience, Universiti Putra Malaysia. The taxonomic identification of plants was performed by Dr. Shamsul Khamis, a botanist at the IBS Herbarium and a voucher specimen (SK2171/13) was deposited in the Phytomedicinal Herbarium, Institute of Bioscience, UPM, Serdang, Selangor. The leaves were washed, rinsed with distilled water, placed in plastic bags, and immediately frozen at -80 ºC. The plant material was then freeze-dried.

Antimicrobial activity of crude leaf extracts

Microorganisms and culture conditions

The antibacterial activity of *P. sarmentosum* leaf extract was investigated against two gram-negative bacteria: *Pseudomonas fuscovaginae* T1 strain (accession number JX915743.1 at National Center for Biotechnology Information (NCBI)) and *Xanthomonas oryzae* pv. *oryzae* (accession number CP000967.1 at NCBI). Cultures were maintained in King's B medium at 4 °C for continuous viability and were subcultured regularly. The bacteria were inoculated into 10 ml Mueller–Hinton broth (MHB) (Fisher Scentific) in test tubes, vortexed well, and placed on a rotary shaker for 24hrs at 28°C. Optical density (OD) was measured using a spectrophotometer (DR2800, Hach) and adjusted to 0.1 at 660 nm. The inoculum was then plated out.

Test assay for antibacterial activity. Antibacterial activity was evaluated using the agar well diffusion method as recommended by Clark *et al*. (1981) with slight modification, using Mueller–Hinton agar (MHA) (Fisher Scientific) as the culture media. Six wells were made in an MHA plate that

was previously seeded with standardized bacterium, using a sterile cork borer (4 mm diameter). Leaf extracts (25, 50,100, and 200 mg/mL), a streptomycin standard (30 µg/mL), the positive control) and a dilution of aqueous methanol (80:20 v/v, negative control) were added to the wells. After incubation at 28 °C for 24hrs, the zones of inhibition around the wells were measured using a transparent ruler (mm). The assay was performed on triplicate MHA plates.

Identification of compounds with antibacterial activity in the crude leaf extracts (HPLC-DAD analysis)

Extract preparation. Extraction of *P. sarmentosum* was carried out based on Chen *et al*. (2001) with some modifications. The plant tissue starts the secretion of phenolic compounds after it is collected from the plants (Maddox *et al*. 2010). Dried, powdered leaves (10 mg; 20 mesh) were weighed and 25 mL of methanol were added into 100 mL round-bottomed flasks; 10 mL of 0.01 *M* HCL was added slowly to the solution over 5 min. The solution was stirred using a magnetic stirrer under an N_2 atmosphere at 35 °C for 16hrs. After cooling and filtering through Whatman No. 1 filter paper, 15 mL of the filtrate was evaporated to dryness using a rotary evaporator and a water bath at 35 °C. The residue was redissolved in 3 mL of methanol (HPLC grade). Aliquots (10 µl) of this diluted solution were used for analysis by high-performance liquid chromatography (HPLC).

Analysis of phenolic compounds. The following standards were purchased from Fisher Scientific Sdn Bhd (Malaysia): gallic acid (CAS Number 149-91-7), tannic acid (CAS Number 1401-55-4), quercetin (CAS number 117-39-5), and naringin (CAS number 480-41-1). Quantification of phenolic compounds was performed via calibration with these standards (external standard method). All standards were prepared as stock solutions in methanol. All solvents were HPLC analytical grade (Fisher Scientific Sdn Bhd). Distilled and HPLC water (18.2 M&Ω resistivity) were processed using a water purification system (Elga, USA).

HPLC-DAD system for analysis of phenolic compounds. Chromatographic separation was achieved following the method of Singh *et al*. (2010) using an Ascentis C18 analytical column (60Å, 5 µm, 4.6 × 150 mm, Supelco; Sigma-Aldrich, USA). HPLC analysis was conducted on an Agilent-1200 series instrument equipped with a UV-vis photodiode array detector (DAD), binary pump, vacuum degasser, and autosampler. Two solvent systems were used: solvent A contained 1% trifluoroacetic acid; solvent B contained methanol (80:20 v/v). The chromatographic separation was performed by isocratic elution of the mobile phase (solvents A and B, 80:20 v/v filtered under vacuum through 0.45 µm membrane before use) at a flow rate of 1.0 mL/min^{-1} at 25 °C. Phenolic compounds were detected at 280 nm and were identified by comparing their retention times and absorbance with those of the standards. Chromatograms and quantitative measurements of peak area were recorded and performed with a computer connected to the HPLC using ChemStation software (Agilent, USA).

Determination of minimum inhibitory and bactericidal concentrations

The minimum inhibitory concentration (MIC) of the leaf extracts was determined using the broth micro-dilution method by two-fold serial dilution. Leaf methanolic extract (50 µL) was twofold serially diluted with MHB in a microtiter plate (positive control). The bacterial OD was maintained at 0.1 OD_{660} throughout the experiment; 50 µL of bacterial suspension was added to the leaf extract by two-fold dilution (negative control). Then, 40 µL of 2,3,5-triphenyltetrazolium chloride (TTC, 2 mg/mL) was added as an indicator of bacterial growth before incubating for 24hrs at 28 °C. The MIC value was considered the lowest extract concentration that showed no color changes (Motamedi *et al*. 2010). The minimum inhibitory concentration (MBC) value was determined by subculturing the mixture of bacterium and extracts from wells that showed no color changes on the sterile MHA plates. The lowest concentration that showed no visible growth on the agar plates was considered the MBC value.

Statistical analysis

The results were analyzed using statistical software JMP (9.0) (SAS Institute, Cary, NC). Data were expressed as means ± SD of triplicate samples by one-way analysis of variance (ANOVA) using Tukey's honestly significant difference (HSD) test. Differences were considered significant at $P < 0.05$.

Table 1. Antibacterial activity of methanolic extracts of leaves of *P. sarmentosum* against *P. fuscovaginae* (Accession number JX915743.1) and *X. oryzae* (accession number CP000967.1) determined using the agar well diffusion method. Results are mean values ± standard deviation of three independent experiments

Concentration	Inhibition zones (mm) for each pathogen (mean ± SD)	
(mg/mL)	*P. fuscovaginae*	*X. oryzae*
Positive control	18.00 ± 1.73[a]	17.67 ± 0.58[a]
Negative control	0[c]	0[d]
25	12.67 ± 0.58[b]	9.00 ± 0.00[c]
50	15.67 ± 1.15[ab]	13.67 ± 0.58[b]
100	17.00 ± 2.00[a]	15.33 ± 0.58[b]
200	19.33 ± 1.53[a]	18.33 ± 1.53[a]

Within columns, values with different letters differ significantly (Tukey–Kramer HSD, $P < 0.05$)

RESULTS AND DISCUSSION

Antibacterial activity of crude leaf extracts

The inhibitory effects of *P. sarmentosum* leaf extracts on the rice pathogenic bacterial strains *P. fuscovaginae* and *X. oryzae* are indicated in Table 1. There was no significant difference in inhibition of *P. fuscovaginae* by 50, 100, and 200 mg/mL extract and the positive control. The negative control (aqueous methanol) showed no inhibition of *P. fuscovaginae*. A linear relationship ($R^2 > 0.8$) was obtained between the concentration of phenolic leaf extract and diameter of the inhibition zone (Fig. 1).

The antibacterial assays showed that *P. sarmentosum* leaf extracts had strong activity and that *X. oryzae* was significantly ($P < 0.05$) more resistant to the extract than *P. fuscovaginae*. The larger diameter of the inhibition zone at each

Table 2. Antibacterial activity of phenolic standards (1.0 mg/mL) against *P. fuscovaginae* and *X. oryzae*. The activity was assayed by the agar well diffusion method and expressed as the diameter (mm) of the inhibition zone obtained. Results are means ± standard deviation of three replicates

Plate content (µg)	Control		Tannic acid	Gallic acid	Quercetin	Naringin
	S	M				
P. fuscovaginae	22.00 ± 1.00[a]	nil	17.00 ± 1.00[b]	18.00 ± 1.73[a]	12.67 ± 0.58[c]	18.33 ± 0.58[a]
X. oryzae	22.00 ± 1.00[a]	nil	16.67 ± 1.15[b]	18.33 ± 1.53[a]	12.67 ± 0.58[c]	18.67 ± 0.58[a]

S: streptomycin sulfate; M: methanol (80:20 v/v); nil: no inhibition zone. Within rows, values with different letters differ significantly (Tukey–Kramer HSD, $P < 0.05$)

Table 3. Antibacterial activity of different concentrations of phenolic standards against *P. fuscovaginae* and *X. oryzae* using a broth-dilution assay. Growth of bacterial cultures with outer diameter of 0.1 at 660 nm on plates containing various of extract and incubated at 37 °C for 24 h is indicated. Lack of visible turbidity was considered as absence of growth. These results are the summary of three independent experiments

Conc. (mg/mL) Bacteria	Control	0.098	0.195	0.391	0.781	1.562	3.124	6.25	12.5	25.0
P. fuscovaginae	++	++	++	++	++	++	++	+	-	-
X. oryzae	++	++	++	++	++	++	+	+	-	-

++: growth; +: partial growth; –: no growth.

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. sarmentosum* methanol leaf extract against rice pathogenic bacteria assessed using the macro-broth dilution method

Tested bacteria	MIC (mg/mL)	MBC (mg/mL)
P. fuscovaginae	12.5	25.0
X. oryzae pv. *oryzae*	12.5	12.5

concentration for *P. fuscovaginae* compared to that for *X. oryzae* indicated the higher sensitivity of the former species to the leaf extracts.

The antimicrobial activity of the phenolic compounds (at 1.0 mg/mL) against the rice pathogenic bacteria is described in Table 2. No significant differences were observed in the inhibitory effect of gallic acid or naringin on the bacteria. Both strains showed intermediate sensitivity to tannic acid, while quercetin was less effective against *X. oryzae* and *P. fuscovaginae*. Significant ($P < 0.05$) differences were observed for tannic acid and quercetin against the microorganisms compared to gallic acid, naringin, and the positive control (Table 2).

These results were in agreement with previous reports. Pereira *et al*. (2007) described appreciable antimicrobial activity of naringin against *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 11778, and *Candida albicans*. Cushnie and Lamb (2005) reported antimicrobial activity of naringin, quercetin, and rutin against human pathogenic microorganisms via mechanisms that included effects on energy metabolism, cytoplasmic membrane function, and nucleic acid synthesis. Naringin was also observed to have inhibitory effects against both gram-positive and gram-negative bacteria (Celiz *et al*. 2011). However, reported inhibitory effects of quercetin on clinically isolated bacteria are contradictory. Gatto *et al*. (2002) found no antimicrobial effects of up to 100 µg/mL of quercetin and its esters. However, Susan *et al*. (1985) observed antibacterial activity of quercetin against *Pseudomonas maltophilia* and *Enterobacter cloacae*, with inhibition zones less

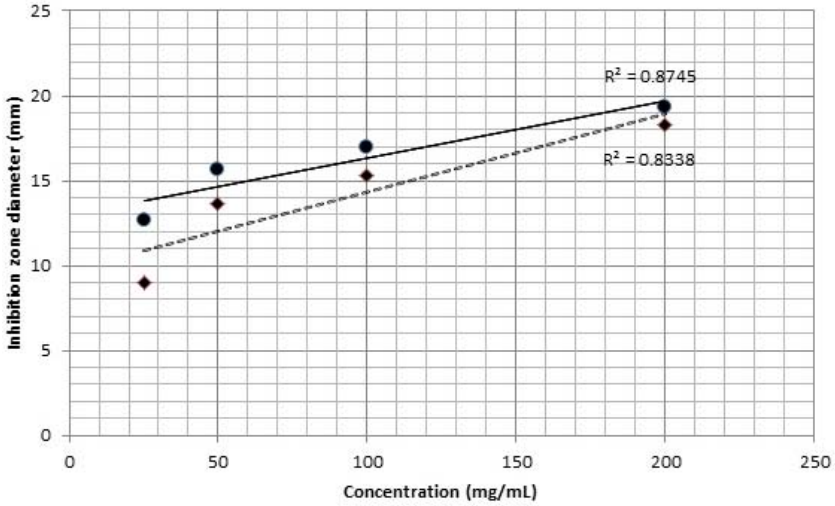

Fig. 1. Typical relationship observed between concentrations of leaf extract and the diameter of the inhibition zone against the bacterial strains. Each point represents the mean value of three replicates. Dashed line shows results for *P. fuscovaginae*; solid line shows results for *X. oryzae*

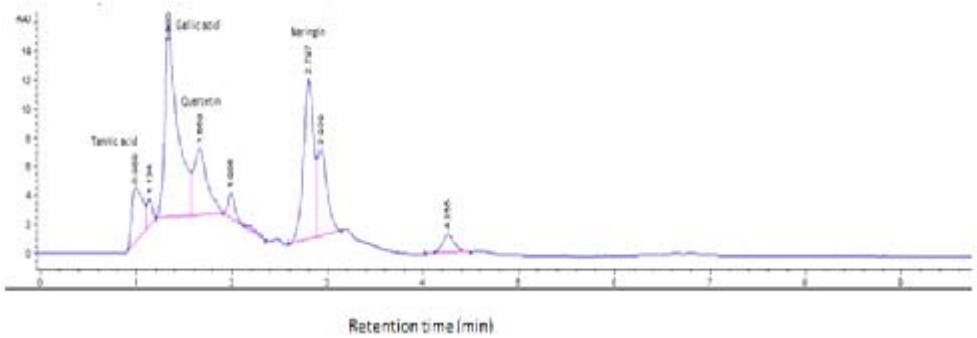

Fig. 2. HPLC chromatogram of phenolic compounds in *P. sarmentosum* leaves

than 7 mm. Gallic acid is known to have pharmacological activity (Inoue *et al*. 1995) and was reported to exhibit strong activity against eight human pathogenic bacteria and six fungal strains (Chanwitheesuk *et al*. 2007). This is supported by Kawada *et al*. (2001), who reported that gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol, can induce apoptosis in four kinds of human lung cancer cell lines *in vitro.* In the present study, the antimicrobial activity of tannic acid, gallic acid, quercetin, and naringin was confirmed.

Identification of compounds with antibacterial activity in the crude leaf extracts (HPLC-DAD) analysis

The HPLC-DAD analysis of *P. sarmentosum* leaf extracts revealed the presence of phenolic acids (tannic acid, gallic acid) and flavonoids (quercetin, and naringin) (Fig. 2). Tannic acid, gallic acid, and quercetin have not been described previously from extracts of this plant (Subramaniam *et al*. 2003). The concentrations of the phenolic compounds ranged from 22.5 to 100.6 µg/g (dry-weight basis, expressed as percentages

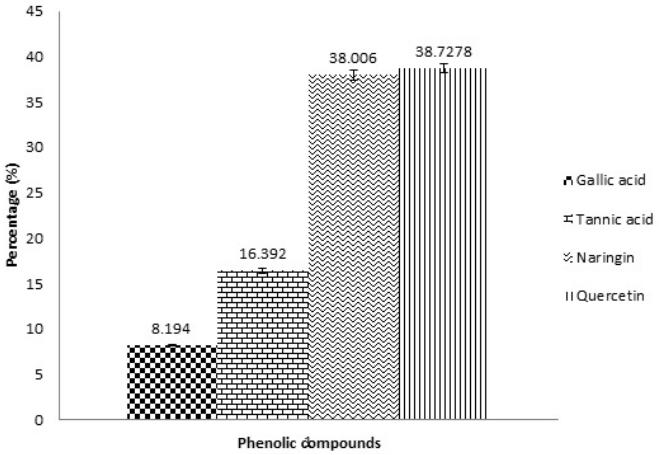

Fig. 3. Phenolic profile of *P. sarmentosum* leaves (percentages of individual compounds relative to the total concentration). Values are means; bars indicate standard error.

in Fig. 3). Flavonoids were always the major compounds, consistent with Pereira *et al*. (2007). All samples showed the same phenolic profile, in which quercetin was the major compound, followed by naringin. Gallic acid was the least prevalent compound (Fig. 3).

Determination of MIC and MBC

Piper sarmentosum leaf extracts had lethal effects on *X. oryzae* and *P. fuscovaginae*; MIC and MBC values are presented in Table 3. The extracts was bacteriostatic at lower concentrations and bactericidal at higher concentrations (i.e., MBC values were higher than MIC values) (Table 4), and showed broad-spectrum antibacterial activity to which both bacteria were highly susceptible. The ability of *P. sarmentosum* methanolic leaf extract to inhibit pathogens at low concentrations was supported by the results of Zaidan *et al*. (2005) who reported lower MIC values against most of the tested microbes. *Piper sarmentosum* inhibited the growth of methicillin-resistant *Staphylococcus aureus* (MRSA), with a MIC value of 50 mg/mL, and the presence of flavonoids and alkaloids in the extract was confirmed (Fernandez *et al*. (2012). In a study performed by Masuda *et al*. (1991), the presence of antimicrobial compounds in *P. sarmentosum* extracts stopped the growth of *E. coli* and *Bacillus subtilis*.

CONCLUSIONS

As antimicrobial properties of plants continue to be discovered, increasing focus is being placed on the use of plant-based extracts against pathogenic bacteria. The antibacterial activity observed in the leaf of *P. sarmentosum* is attributed to the presence of secondary metabolites in the plant extracts, as supported by previous reports, the methanolic leaf extracts of *P. sarmentosum* was found to be effective as an

antibacterial agent against rice pathogenic bacteria. In conclusion, extract from *P. sarmentosum* leaves can provide an alternative to current antibacterial options. This extract may provide a low cost, non-toxic, and more effective agent at lower concentrations and holds promise for development as a bactericide. To our knowledge, this is the first report of the antibacterial activity of phenolic compounds in *P. sarmentosum* extracts against rice pathogenic bacteria.

ACKNOWLEDGEMENTS

Financial support by the Long-term Research Grant Scheme (Rice Research-5525001), Universiti Putra, Malaysia is highly appreciated.

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