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Isolation of allelopathic substance from *Piper sarmentosum* Roxb.

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ABSTRACT

Allelopathic activity of *Piper sarmentosum* Roxb. was determined and a potent growth inhibitory substance was isolated. The aqueous methanol extracts of *P. sarmentosum* inhibited the growth of 6-test plant species [cress (*Lepidium sativum* L.), lettuce (*Lactuca sativa* L.), alfalfa (*Medicago sativa* L.), timothy (*Phleum pretense* L.), Italian ryegrass (*Lolium multiflorum* Lam.) and crabgrass (*Digitaria sanguinalis* L.)] to various degrees in a dose-dependent manner. The active substance 3-phenylpropionic acid was purified from the aqueous methanol extract of *P. sarmentosum* and identified by ^{1}H-, ^{13}C-NMR and MS. The threshold of 3-phenylpropionic acid for growth inhibition was 1 µM. The concentrations required for 50% growth inhibition of cress and lettuce were 1.2-9.3 µM and the concentrations required for 50% growth inhibition of timothy and Italian ryegrass were 4.7-51.8 µM. The endogenous concentration of 3-phenylpropionic acid in *P. sarmentosum* was 121 µmol/kg. Considering the endogenous level and the inhibitory activity, 3-phenylpropionic acid may work as allelopathic substance in *P. sarmentosum* through growth inhibition of neighbouring plant species.

Key words: Alfalfa, allelopathic activity, allelopathy, aqueous methanol extract, crabgrass, cress, lettuce, Italian ryegrass, *Piper sarmentosum*, timothy

INTRODUCTION

Widespread use of synthetic herbicides has caused many problems including their persistence in soil, environmental contamination, increase in herbicide-resistant weeds, etc. Hence, effective bio-herbicide chemicals with novel mode of actions, low application rates, low toxicity to humans and animals and more environmental friendly are preferred in ecological agriculture (7). Allelopathy holds promise for the environmentally friendly weed management and offers practical weed management options (34). Numerous plants possess the allelopathic potential and efforts have been made to apply them for weed control. Several studies have been done on the screening for the allelopathic potential of medicinal plants (8,10,13,32,17). Fujii *et al.* (11) evaluated the allelopathic potentials of 239 medicinal species using the Plant Box Method and 223 species of them, suppressed the growth of the tested plant, whereas 17 species enhanced the lettuce radicle growth. Gilani *et al.* (12) also determined the allelopathic potential of 81 Japanese medicinal plants to identify the possible candidates as natural herbicides. Nazir *et al.* (22) evaluated the allelopathic potential of three herbal species (*Rheum emodi, Saussaurea lappa* and *Potentilla fulgens*) against several traditional crops and the germination of all tested crops was reduced significantly by aqueous extracts of *S. lappa* and

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P. fulgens. Medicinal plants are also useful for pest control and soil improvement besides their pharmaceutical properties (18).

Piper sarmentosum Roxb. (*Piperaceae)* is a stoloniferous herb or shrublet widely distributed in Southeast Asia. The extracts of its different plant parts are used to cure many diseases as traditional medicine (29). The plant possesses several pharmacological properties [anti-tuberculosis (16), anti cancer (31), hypoglycaemic (24), antimalarial (21), antioxidant (35), neuromuscular blocker (27) and antiamoebic (30)].

Recently, the phytotoxic substance, sarmentine was isolated from Long pepper (*Piper longum* L.) and exhibited inhibitory activities against several crops and weeds (15). However to date, no information is available on the allelopathic substance of *P. sarmentosum*. In this study, the growth inhibitor causing the allelopathic effect was isolated from the methanol extracts of *P. sarmentosum*. The substance was then characterized, and biological activities and endogenous concentrations of the substances were determined.

MATERIALS AND METHODS

Whole plants (leaves, stem and roots) of *Piper sarmentosum* Roxb. were collected from Chiang Mai province, Thailand, in August 2011. The plants were washed several times by tap water, dried under sunlight and then ground into powder. Cress (*Lepidum sativum* L.), lettuce (*Lactuca sativa* L.), alfalfa (*Medicago sativa* L.) and timothy (*Phleum pratense* L.) were selected as test plants for bioassay because of their known seedling growth behavior. Italian ryegrass (*Lolium multiflorum* Lam.) and crabgrass (*Digitaria sanguinalis* L.) were chosen as test plants for bioassay due to their existence of weeds in crop fields throughout the world.

Extraction and bioassay

Plant powder (500 g) was extracted with 2 L of 80% (v/v) aqueous methanol for two days. The extract was filtered through one layer of filter paper (No. 2; Toyo Ltd., Japan), using a vacuum pump. The residue was extracted again with 2 L cold methanol for one day and filtered. The two filtered were combined and evaporated with a rotary evaporator at 40°C.

I. Bioassay: An aliquot of extract (final assay concentration was 0.01, 0.03, 0.1 and 0.3 g dry weight equivalent extract/mL) was evaporated to dryness at 40°C *in vacuo* by rotary evaporator, dissolved in 3 mL methanol and added to a sheet of filter paper (No. 2) in a 2.8 cm dia Petri dish. The methanol was evaporated in a draft chamber, then the filter paper was moistened with 0.6 mL of 0.05% (v/v) aqueous solution of polyoxyethylenesorbitan monolaurate (Tween 20; Nacalai, Kyoto, Japan), which was used for surfactant and did not cause any toxic effects. Timothy, Italian ryegrass and crabgrass were germinated in dark at 25°C for 48 h, 120 h and 72 h, respectively. Then, 10 seeds of cress, lettuce, alfalfa, timothy, Italian ryegrass, or crabgrass were arranged on the filter paper in Petri dishes. The aqueous solution of Tween 20 without the extract was used as control. The shoot and root lengths of seedlings were measured at 48 h after incubation in dark at 25°C.

The percentage length of seedlings was then determined by reference to the length of control seedlings. The bioassay was repeated thrice with 10 plants for each determination. The inhibition (%) was calculated as under:

$$\text{Inhibition (\%)} = [1- (\text{sample extracts}/ \text{control})] \times 100.$$

II. Purification of active substance in ethyl acetate fraction

Piper sarmentosum Roxb. (500 g dry weight) were extracted as described above and the extract was concentrated at 40°C *in vacuo* to produce aqueous residue. The aqueous residue was adjusted to pH 7.0 with 1 M phosphate buffer, partitioned three times against an equal volume of ethyl acetate (Figure 1). The ethyl acetate fraction was evaporated to dryness and chromatographed on a column of silica gel (100 g, silica gel 60, 70–230 mesh; Merck), eluted stepwise with *n*-hexane containing increasing amounts of ethyl acetate (10% per step, v/v; 100 mL per step). The biological activity of fractions was determined using a cress bioassay as described above and activity in fractions was obtained by elution in 60% ethyl acetate in *n*-hexane and gave 12.4 g residue. After evaporation, the residue was separated by a column of Sephadex LH-20 (100 g, Amersham Pharmacia Biotech, Buckinghamshire, UK), and eluted with 20, 40, 60 and 80% (v/v) aqueous methanol (100 mL per step) and methanol (200 mL). The active fractions were eluted by 20% aqueous methanol and evaporated to dryness. The residue was dissolved in 20% (v/v) aqueous methanol (2 mL) and loaded onto reverse phase C_{18} Sep-Pak cartridges (Waters). The cartridge was eluted with 20, 40, 60, 80% (v/v) aqueous methanol and methanol (15 mL per step). The active fractions was eluted by 40% aqueous methanol and evaporated to dryness. The residue of active fraction was finally purified by reverse-phase HPLC (10 mm i.d. × 50 cm, ODS AQ-325; YMC Ltd., Kyoto, Japan) eluted at a flow rate of 1.5 mL/min with 50% aqueous methanol, detected at 220 nm. The active substance was characterized by ^{1}H- and ^{13}C-NMR and mass spectra.

III. Bioassay of 3-phenylpropionic acid

Growth inhibitory activity was determined by dose-response experiments. Filter paper was placed into Petri dish and test solution of 3-phenylpropionic acid was added on it. Final concentrations of 3-phenylpropionic acid were 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 or 100 mM. After the solvent evaporated, 10 seeds of cress and lettuce or 10 germinated seeds of timothy and Italian ryegrass were arranged on the filter paper in the Petri dishes. For control treatments, seedlings were placed into the filter paper moistened with the aqueous solution of Tween 20 without the solution of 3-phenylpropionic acid.

Shoot and root lengths were measured after 48 h incubation in dark at 25°C. The percentage length of the seedlings was then determined by reference to the length of control seedlings. The concentrations required for 50 % inhibition (defined as I_{50}) of the test plants in the assay was calculated from the regression equation of the concentration-response curves.

Statistical analysis

All experimental treatments were replicated thrice and repeated twice. Treatments were prepared in a completely randomized design. Data were analyzed by SPSS version 16.0 using One-way ANOVA.

RESULTS AND DISCUSSION

Shoot growth

The extracts obtained from 0.1 g dry weight of *P. sarmentosum* plants inhibited the shoot growth of cress, lettuce, alfalfa, timothy, Italian ryegrass and crabgrass by 98.7, 100.0, 97.6, 86.4, 75.2, and 69.5%, respectively (Table-1). Exposure to 0.3 g/mL concentration, the shoot growth of crabgrass was drastically inhibited (97.1%), whereas, shoot growth of cress, lettuce, alfalfa, timothy, Italian ryegrass was completely inhibited.

Root growth

At 0.1 g/mL concentration, the root length of cress, lettuce, alfalfa, timothy, Italian ryegrass and crabgrass was drastically inhibited by 99.5, 100.0, 94.6, 83.7, 84.0 and 78.8%, respectively (Table 1). Exposure to 0.3 g/mL concentration, severely inhibited the root growth of crabgrass (97.4%). Root growth of cress, lettuce, alfalfa, timothy, Italian ryegrass were completely inhibited.

Table 1. Inhibitory effects of aqueous methanol extracts of *P. sarmentosum* on the shoot and root growth of test plant seedlings

Test plant species	Inhibition (%)							
	Shoot				Root			
	0.01	0.03	0.1	0.3	0.01	0.03	0.1	0.3
Cress	50.6^{a}	71.3^{b}	98.7^{c}	100.0^{c}	84.3^{a}	91.3^{b}	99.5^{c}	100.0^{c}
Lettuce	-5.7^{a}	84.3^{b}	100.0^{b}	100.0^{b}	54.1^{a}	92.8^{b}	100.0^{b}	100.0^{b}
Alfalfa	79.0^{a}	87.1^{a}	97.6^{b}	100.0^{b}	84.7^{a}	89.1^{ab}	94.6^{bc}	100.0^{c}
Timothy	45.3^{a}	59.6^{b}	86.4^{c}	100.0^{c}	28.6^{a}	85.5^{b}	83.7^{b}	100.0^{b}
Italian ryegrass	0.0^{a}	34.4^{b}	75.2^{c}	100.0^{c}	42.5^{a}	60.6^{b}	84.0^{c}	100.0^{d}
Crabgrass	18.2^{a}	40.5^{b}	69.5^{c}	97.0^{d}	28.6^{a}	57.0^{b}	78.8^{c}	97.3^{c}

Mean with same letters in row is not significantly different at $P < 0.05$

The aqueous methanol extract of *P. sarmentosum* inhibited the roots and shoots growth of all test plant species at concentrations > 0.03 g dry weight equivalent extract/mL and increasing the extract concentration increased the magnitude of inhibition. Such inhibition in the growth of test plant species might be due to the presence of allelochemicals in *P. sarmentosum*. Similar results were reported by Randhawa *et al.* (26), who found that the germination of *Trianthema portulacastrum* was suppressed by higher concentration of sorghum water extract. The results are in agreement with earlier studies reporting that degree of inhibition increase with increased extract concentration (1-6,14,20,26).

The extract of *P. sarmentosum* were more inhibitory to growth of roots than shoots of all test plant species. Salam and Kato-Noguchi (28) reported that the extracts of allelopathic plants had more inhibitory effect on root growth than on hypocotyl growth, because root is the first organ to absorb allelochemical from the environment. Furthermore, the permeability of root tissues to allelochemicals was greater than shoot tissue (23). Results of this study also showed the inhibitory effects of *P. sarmentosum* were variable on test plant species. This unequal susceptibility to the extracts could be due to inherent differences in various biochemicals involved in the process (17). The aqueous methanol extract of *P. sarmentosum* plants showed allelopathic effects on all test plant species (Table 1). This study has successfully isolated allelopathic substance from *P. sarmentosum* plant using biological assay guided isolation approach. It could therefore be concluded that the aqueous methanol extract of *P. sarmentosum* may possess allelopathic potential and may contain growth inhibitory substances.

Purification of allelopathic substance and biological activity

The active component in the aqueous methanol extract of *P. sarmentosum* was purified by bioassay-guided fractionation (Figure 1). Two fractions of aqueous and ethyl acetate were separated from the aqueous methanol extract and their biological activities were determined. Both fractions suppressed the root and shoot growth of cress seedlings. The ethyl acetate fraction proved more inhibitory (Fig. 2). Thus, allelopathic active substances were isolated using the ethyl acetate fraction.

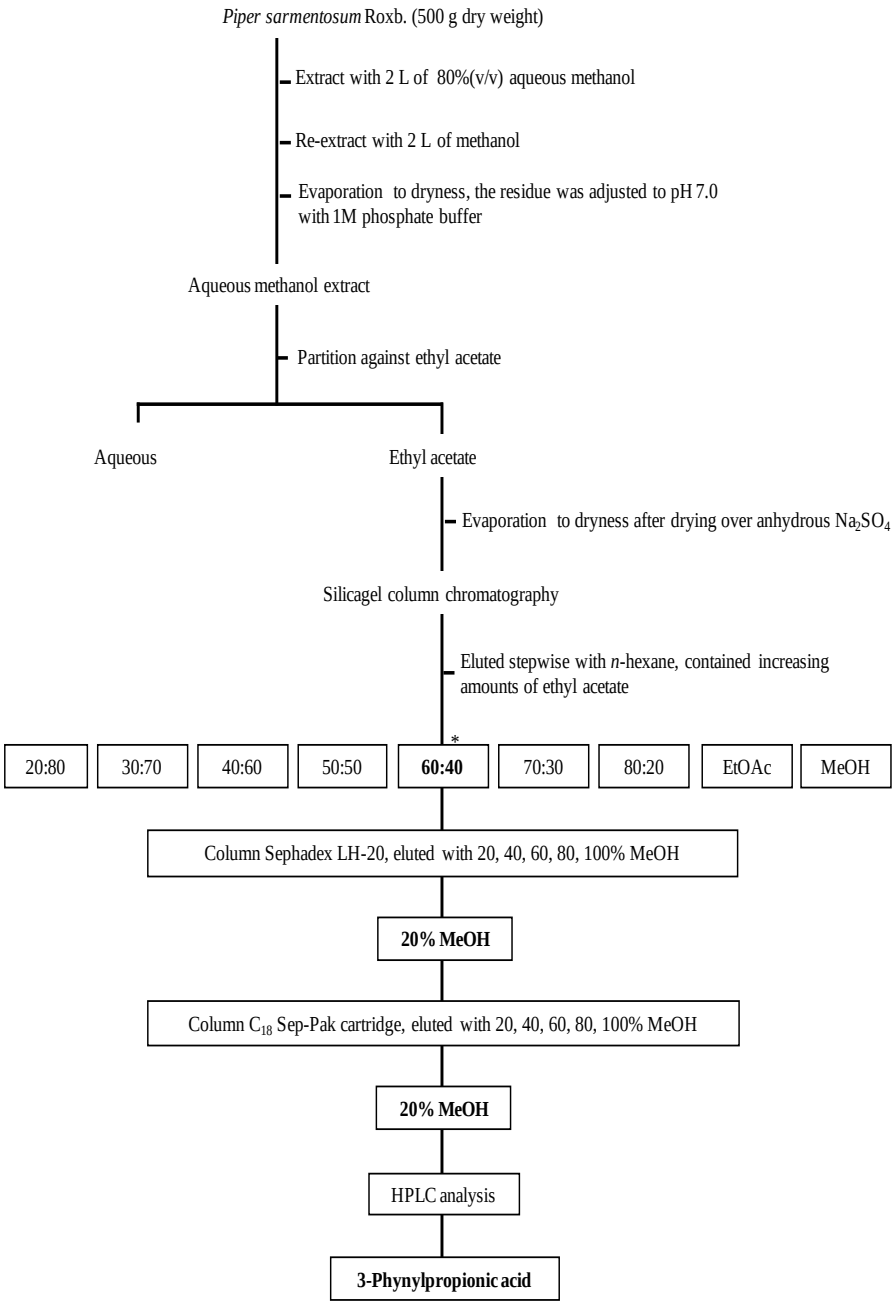

Figure 1. Procedure for isolation of active compound from *P. sarmentosum*, * Active fractions

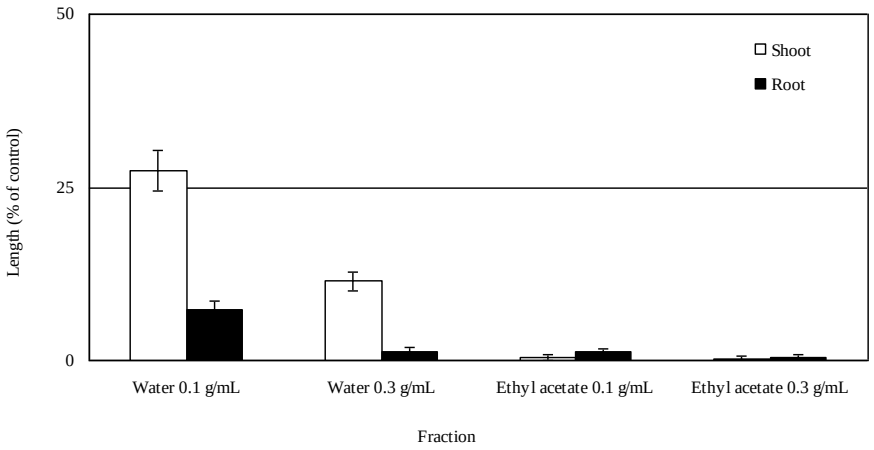

Figure 2. Effects of water and ethyl acetate fractions obtained from *P. sarmentosum* on the shoot and root growth of cress seedlings. Means ± SE from three independent experiments with 10 seedlings for each determination are shown. Asterisk indicates significant difference between control and treatment: *$P<0.05$, **$P<0.01$, ***$P<0.001$ (Student's t-test).

The ethyl acetate fraction was separated by columns of silica gel and the biological activity was found in the fractions eluted with 60% ethyl acetate in n-hexane. The active residues were purified by columns of Sephadex LH-20, C_{18} Sep-Pak cartridges and the active substance was isolated by reverse phase HPLC at the retention time of 5.3 to 8.0 min, yielding an active compound (9.1 mg) as white residue.

Characterization of compound

The ^{1}H NMR (400 MHz, CD_3OD) spectrum of compound showed; HRESIMS *m/z* 149.0597 $[M-H]^-$, Δ= -2.8 mmu, (calcd for $C_9H_9O_2$, 149.0625); δ_H 7.22 (m, 2 H), 7.20 (m, 2 H), 7.19 (m, 1 H), 2.90 (t, J = 8.0 Hz, 2 H), 2.59 (t, J = 8.0 Hz, 2 H); The ^{13}C NMR (100 MHz, CD_3OD) spectrum of the compound showed; δ_C 176.1, 142.0, 128.2, 127.0, 36.2, 32.0. From the comparison of data with the literature (25), the spectrum indicated that this compound was 3-phenylpropionic acid (Fig. 3).

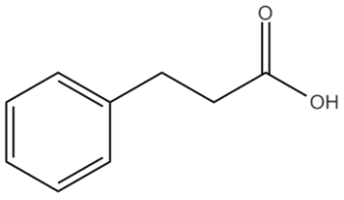

Figure 3. Chemical structure of 3-phenylpropionic acid

3-Phenylpropionic acid isolated from the *P. sarmentosum*, inhibited the roots and shoots growth of cress, lettuce, timothy, and Italian ryegrass at concentrations > 1 µM (Fig. 4). The I_{50} values were calculated by a logistic regression analysis after the bioassays (Table 2). The I_{50} in the roots and shoots of cress in the assay were 1.2 and 8.22 µM, respectively, and on those of lettuce

were 5.52 and 9.34 µM, respectively. The I_{50} in the roots and shoots of timothy were 51.76 and 18.15 µM, respectively and on those of Italian ryegrass were 6.51 and 4.65 µM, respectively. Comparing I_{50} values, effectiveness of 3-phenylpropionic acid on cress and lettuce (dicotyledonous plants) was much greater than on timothy and Italian ryegrass (monocotyledonous plants).

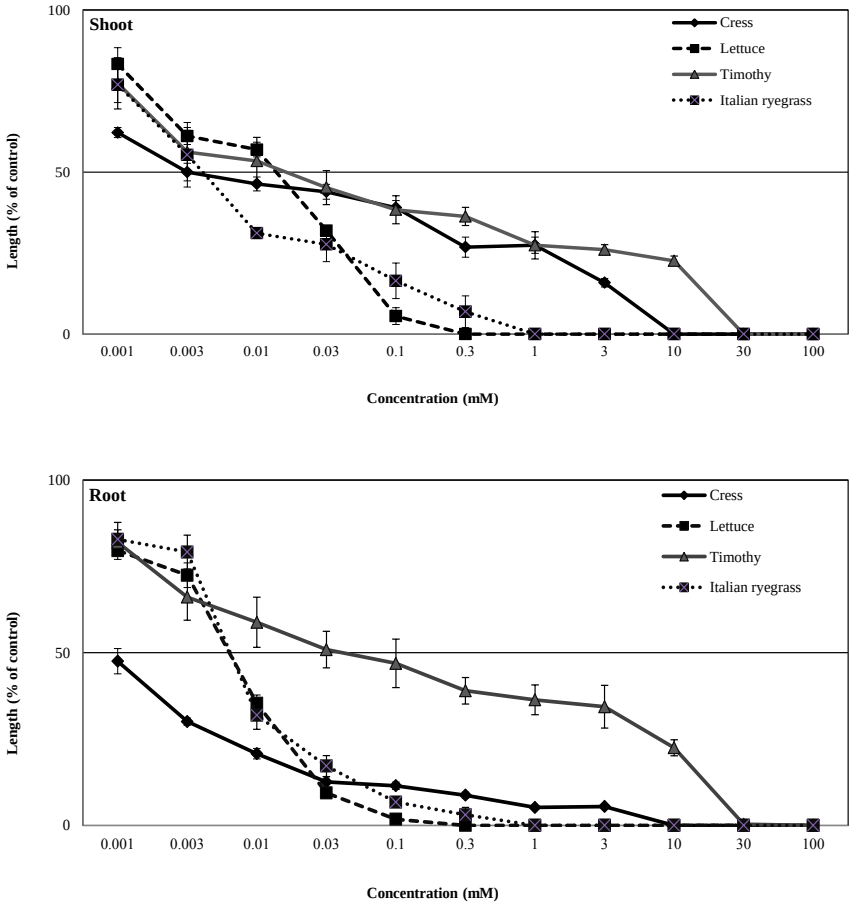

Figure 4. Effects of 3-phenylpropionic acid on the shoot and root growth of cress (●), lettuce (■), timothy (▲), and Italian ryegrass (▼) seedlings as affected by different concentrations. Means ± SE from three independent experiments with 10 seedlings for each determination are shown.

An allelopathic substance was isolated from an aqueous methanol extract and determined by spectral data as 3-phenylpropionic acid. The inhibitory activity of this allelopathic 3-phenylpropionic acid and its occurrence in *P. sarmentosum* suggest that 3-phenylpropionic acid may play an important role in the allelopathic properties of *P. sarmentosum*. The 3-Phenylpropionic acid is present in the root residues, root exudates and rhizosphere of certain plants like cucumber (*Cucumis*

Table 2. I_{50} values of 3-phenylpropionic acid for shoots and roots of test plants.

Test plant species	3-phenylpropionic acid I_{50} (µM)	
	Shoot	Root
Cress	8.22	1.2
Lettuce	9.34	5.52
Timothy	18.15	51.76
Italian ryegrass	4.65	6.51

I_{50} values were determined by a logistic regression analysis after bioassays ($P < 0.05$).

sativus L.) (38). This substance also inhibits the germination and growth of Florida's rosemary scrub (*Ceratiola ericoides*) and the grass *Schizachyrium scoparium* and has been proposed as the allelochemical (9,33,36). Furthermore, Li *et al.* (19) isolated the 3-phenylpropionic acid from the aqueous leachates of decaying Italian ryegrass residues. This substance inhibited the elongation of rice seedling roots at 0.1 mM, whereas restrained the shoots only at 1 mM.

The endogenous concentration of 3-phenylpropionic acid was at least 121 µmol/kg, because of 9.1 mg of the substance (MW 150.18) was isolated from 500 g dry weight of *P. sarmentosum*. The threshold of 3-phenylpropionic acid for growth inhibition was 1 µM. Considering the endogenous level and the inhibitory activity, 3-phenylpropionic acid may provide the competitive advantage to *P. sarmentosum* as an allelopathic substance through the growth inhibition of neighboring plant species. The results may have value in enabling weed control based on natural plant extracts and hence this plant could be used for the development of bioherbicide in weed management. In addition, further evaluation of allelopathic substance under field conditions and studied on the mode of action of this allelopathic substance are required to assist in the identification of novel target sites of action for weed control.

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