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Table: 1, Figs : 4

Potential of *trans-p*-coumaric acid released from *Rottboellia cochinchinensis* for weed control in vegetable fields

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ABSTRACT

We characterized the phytotoxic allelochemicals in itchgrass (*Rottboellia cochinchinensis*) and assessed their phytotoxic effects on early seedling growth of *Ageratum conyzoides* L., *Bidens pilosa* L. var. *radiata* Sch. Biq., *Echinochloa crus-galli* (L.) P. Beauv. and *Lactuca sativa* L. The aqueous methanol extract of itchgrass inhibited the growth of *A. conyzoides*, *B. pilosa*, *E. crus-galli* and *L. sativa*. Aqueous methanol extract at 1.50 mg/mL inhibited shoot growth of *L. sativa* by 65.8 % than control. These results indicated that itchgrass contains growth inhibitory substances (allelochemicals), which were detrimental to target plant species. Based on electrospray ionization-mass spectrometry (ESI-MS) data and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra, the allelochemical isolated from the aqueous methanol extract was identified as *trans-p*-coumaric acid. Both isolated and the commercial *trans-p*-coumaric acids inhibited the growth of *A. conyzoides*, *B. pilosa*, *E. crus-galli* and *L. sativa* at > 0.08 mg/mL concentration than control, except the root growth of *L. sativa*. Considering the endogenous level and the inhibitory effects the *trans-p*-coumaric acid may be responsible for the growth inhibitory effects, of itchgrass and may play an active role in the allelopathy of itchgrass for weed control.

Key words: Allelochemical, allelopathy, itchgrass, phytotoxic, *trans-p*-coumaric acid.

INTRODUCTION

Allelopathy plays significant role in agricultural systems. Some plant species produce and release allelochemicals into the environment, which directly or indirectly inhibits the germination and growth of others plants (1,3,20,21,25,27). The development of naturally-derived products as new, effective and environmental friendly alternatives for chemical weed management is an important area of research in weed science (8,9). The use of allelopathy for weed control may lead to the reduction in use of synthetic herbicides input in sustainable crop production systems and consequent environmental pollution.

Itchgrass [*Rottboellia cochinchinensis* (Lour.) W.D. Clayton] is an annual grass widely distributed in tropical and subtropical regions (2,19). In some areas of Thailand, it has become dominant weed in maize (*Zea mays* L.) and sugarcane (*Saccharum*

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officinarum L.) fields or in orchards (18). Farmers in Chaehom-Lampang (CH-LP) area, Northern Thailand, cultivate itchgrass for use as mulch to control weeds in vegetable crops as an alternative to synthetic herbicides (4). The allelochemicals (phytotoxic substances) released from itchgrass into the soil suppresses the germination and growth of *Ageratum conyzoides*, *Bidens pilosa*, *Echinochloa crus-galli*, *Lactuca sativa*, *Mimosa pudica*, *Oryza sativa* and *Raphanus sativus* (4,12,18).

Although itchgrass may be helpful in controlling the weeds, but the responsible allelochemicals for weed control have not yet been isolated and characterized. This study aimed to isolate and characterize the potent allelochemicals from itchgrass and assess their phytotoxic effects on the early seedling growth of *A. conyzoides*, *B. pilosa*, *E. crus-galli* and *L. sativa*.

MATERIALS AND METHODS

Itchgrass plants were harvested at the mature stage (97-116 days after planting) in October, 2014 from a farmer's field in Chaehom city, Lampang province of Northern Thailand (18°42′46″ N, 99°33′27″ E). The aerial parts were cut into 1-2 cms pieces and dried in oven at 40 °C for one week. The aerial parts were ground into powder using an electrical grinder and then sieved through a 0.5 mm screen mesh. The powder was stored in plastic bottles at -30 °C until extraction. *Ageratum conyzoides* L., *Bidens pilosa* L. var. *radiata* Sch. Biq., *Echinochloa crus-galli* (L.) P. Beauv. and *Lactuca sativa* L. were used as test plant species.

Extraction and purification of active compound

One kg itchgrass powder was extracted with 5 L *n*-hexane and kept at room temperature (25 °C) for 3 days. It was sonicated daily for 1 h and filtered at the end of extraction period. The residual plant material was further extracted with 5 L each of dichloromethane and methanol for 3 days (Fig. 1). Each extract was evaporated to dryness *in vacuo* and kept at -30 °C until further use.

The dried methanol extract (RcM) was re-dissolved in 50 % (v/v) aqueous methanol solution and was further partitioned to obtain *n*-hexane, dichloromethane, ethyl acetate, butanol and water layers, respectively. The water layer (RcMW) was then evaporated to dryness *in vacuo*, and 14.98 g of RcMW was dissolved in 200 mL of hot propanol (100 °C) to separate into supernatant (RcMWS) and precipitate (RcMWP) fractions using low temperature (0 °C). The supernatant fraction (RcMWS) was collected by decantation and evaporated to dry *in vacuo*. The RcMWS was then subjected to centrifugal concentrator (MWCO: 3000, Vivaspin 20 ml, Sartorius Stedim Biotech, Goettingen, Germany). It was centrifuged at 3,000 rpm for 20 min. The pass-through fraction of supernatant (RcMWST) was further purified by high-performance liquid chromatography (DP-8020 pump and PD-8020 photodiode array detector, Tosoh Corporation, Tokyo, Japan) on a normal-phase column (TSK-gel Amide-80, 10 µm, ∅7.8×300 mm, Tosoh Corporation, Tokyo, Japan) at flow rate of 1 mL/min with a linear gradient of 95:5 to 5:95 acetonitrile: water within 50 min and detected at 210 nm. The active peak (retention time: 17-18 min) was collected as single peak. It was an amorphous solid (slightly yellow colour) with output of 14.5 mg.

Trans-p-coumaric acid (**I**) was assigned using NMR spectroscopic techniques. The ^{1}H and ^{13}C NMR spectral data were as follows: ^{1}H NMR (CD_3OD), δ 7.56 (1H, *d*, *J* = 15.8, H-7), 7.42 (2H, *d*, *J* = 8.6 Hz, H-2, 6), 6.77 (2H, *d*, *J* = 8.6 Hz, H-3, 5), 6.25 (1H, *d*, *J* = 15.8 Hz, H-8); and ^{13}C NMR (CD_3OD), δ 171.2 (*s*, C-9), 161.1 (*s*, C-4), 146.6 (*d*, C-7), 131.1 (*s*, C-2, 6), 127.3 (*s*, C-1), 116.9 (*d*, C-3, 5), 115.8 (*d*, C-8).

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured and recorded using a spectrometer (Avance 500, Bruker, Billerica, MA, USA) in CD_3OD. The pentet resonance of CHD_2OD at δ_H 3.3 ppm was used as the internal reference for the ^{1}H NMR spectra. Electrospray ionization-mass spectra (ESI-MS) were recorded using a SYNAPT G2 (Waters, Milford, MA, USA) mass spectrometer.

Bioassay-guided fractionation and purification

The bioassay was done as per Meksawat and Pornprom (17) with some modifications. The RcM showed bioactivity on the growth of test plant seedlings and was selected for further isolation. For this purpose, it was partitioned using four organic solvents (*n*-hexane, dichloromethane, ethyl acetate and butanol) and water. The significant inhibitory activity was observed in RcMW. The RcMW layer was dissolved in hot propanol to separate into RcMWS and RcMWP fractions. Both the RcMWS and RcMWP fractions were further separated into two fractions depending on molecular size (RcMWST, RcMWSR, RcMWPT and RcMWPR) using ultrafiltration technique. Only the RcMWST (< 3,000 MWCO) fraction showed inhibitory effect. The RcMWST fraction was further purified by HPLC with a normal-phased column. According to the HPLC chromatogram, the RcMWST fraction was composed of 9-fractions and these fractions were also subjected to bioassays (data not shown). The compound **I**, which was inhibitory to test seedlings, was eluted between 17 and 18 min (Fig. 1).

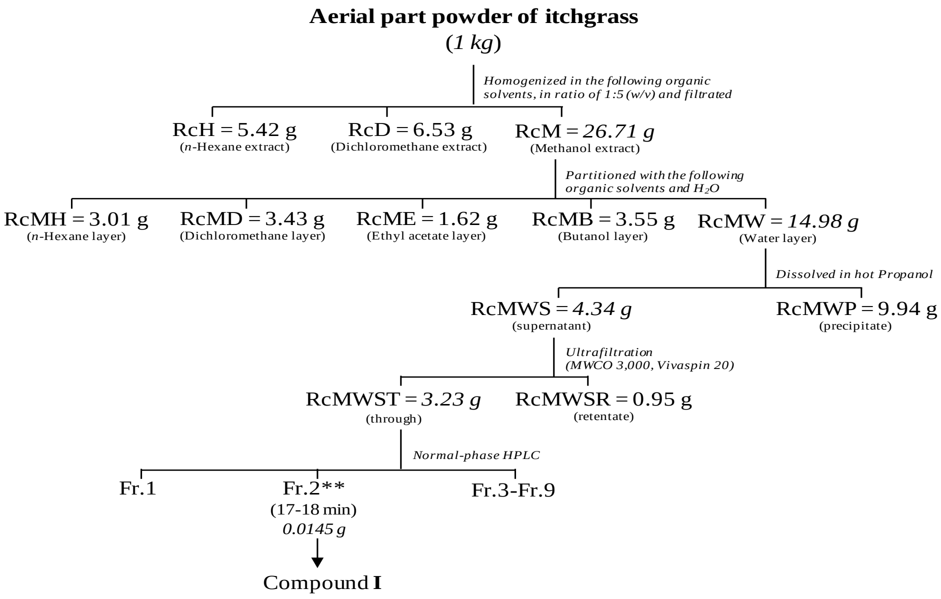

Figure 1. Extraction and purification scheme of biochemicals from the aerial parts of itchgrass.

An aliquot of RcM and *trans-p*-coumaric acid (isolated and commercial compounds) were dissolved in distilled water and diluted into final concentrations of 0.75 and 1.50 mg/mL (RcM) and 0.08 and 0.15 mg/mL (isolated and commercial compounds). A 0.5 mL of test solution was then added to 33 mm Petri dish lined with filter paper. A Petri dish with 0.5 mL distilled water served as control. Five germinated seeds of test plant (radicals of ~ 2 mm) were placed on a filter paper moistened with the test solution. After incubation for 5 days at 25 °C, under continuous light conditions, the length of shoot and root was measured and compared with control.

Statistical analysis

The experiments were set up in completely randomized design with four replications. The % inhibitory/stimulatory effects were calculated using the following equation:

$$\text{Inhibition (+) or stimulation (-) \%} = [1 - (L1/L2)] \times 100,$$

Where, L1: Treatment response and L2: Control response.

Absolute values of percentage varied directly according to allelopathic effects. Data were analyzed by Fisher's protected least significant difference (LSD) test at $P < 0.05$. The statistical analyses were conducted using the R-program version 2.9.1 (22).

RESULTS AND DISCUSSION

Allelopathic effects of crude extract from itchgrass

The three extracts (*n*-hexane extract, dichloromethane extract and RcM [Methanol extract]) of itchgrass and the aqueous methanol extract (RcM) were most phytotoxic to test plant species (Table 1). Therefore, the phytotoxic effects of RcM on the early seedling growth of four test plant species were investigated. The RcM showed significant inhibitory effects on the shoot and root lengths of all test plant spp. (*A. conyzoides*, *B. pilosa*, *E. crus-galli* and *L. sativa*) in dose-dependent manner (Fig. 2). The seedlings shoot and root lengths of all test plant species were inhibited (9-66 %) at 1.50 mg/mL concentration than control. Among the four test plants, the shoot of *L. sativa* was more sensitive (65.8 % inhibition with aqueous methanol extract at 1.50 mg/mL concentration). *A. conyzoides* and *B. pilosa* root elongation was more sensitive than other plant species in response to RcM. Thus, the RcM caused the growth inhibition in test plants in dose-dependent manner, indicating the presence of growth inhibitory substances in itchgrass.

Table 1. Effects of different crude extracts of aerial parts of itchgrass on growth of *Lactuca sativa*

	Lactuca sativa L. var. OP	
Treatment	Shoot length	Root length
RcM (Methanol extract)	+31.57 a	+3.41 a
RcD (Dichloromethane extract)	-16.39 b	-60.61 c
RcH(*n*-Hexane extract)	-22.58 b	-29.83 b
$LSD_{0.05}$	11.23	20.75
Significance	**	**

(+) : % Inhibition and (-): % Stimulation; $^{**}P < 0.01$.

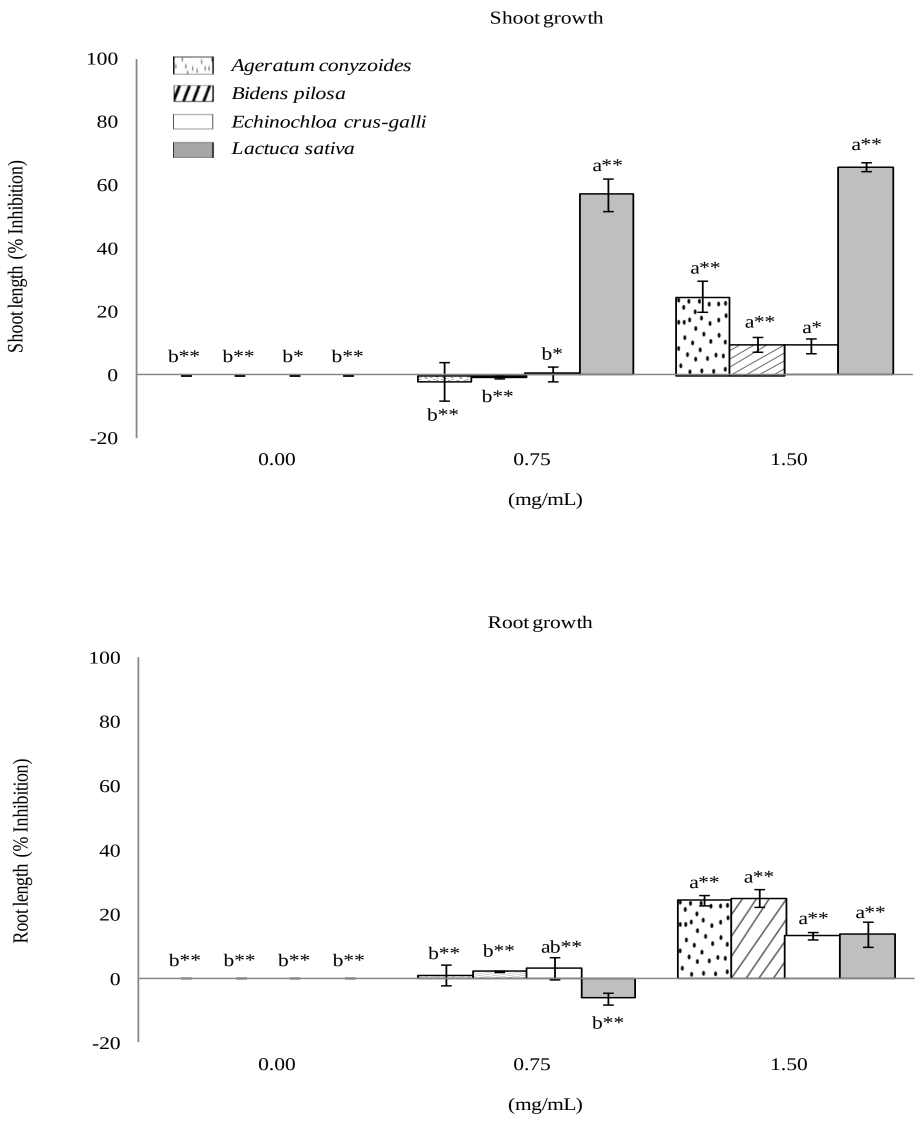

Figure 2. Phytotoxic effects of the RcM extract from itchgrass on shoot growth and root growth of test plant species. Data are means ± standard errors of four replications. The treatment means were compared using least significant difference (LSD) test (*$P < 0.05$; **$P < 0.01$).

The aqueous methanol extract of itchgrass powder was inhibitory to the early seedling growth of tested plant species. This indicated that itchgrass may release growth inhibitory substances (allelochemicals) into soil, when used as mulch. This finding is consistent with the available literature (5,12,18). Therefore, it is important to identify the growth inhibitory substance (allelochemical) in RcM.

Identification of active compound

The molecular ion peak of compound **I** produced a deprotonated molecule at *m/z* 163 $(M\text{-H})^-$ as shown by the data from negative mode ESI-MS, which could confirm the molecular weight as 164. A fragment at *m/z* 119 $(M\text{-H-44})^-$ corresponds to a loss of CO_2 group from the carboxylic acid function, which is consistent with a previous report (24). Therefore, compound **I** was inferred to have the structure of *trans*-*p*-coumaric acid. The NMR data were identical to the data available in literature (28). In addition, the ESI-MS data of compound **I** were identical to those of commercially available authentic chemical agent (from Nacalai Tesque, Inc., Kyoto, Japan). From comparison of these data with those reported in the literature, compound **I** was identified as *trans*-*p*-coumaric acid ($C_9H_8O_3$; FW 164).

The active compound from the aqueous methanol extract was identified as *trans*-*p*-coumaric acid according to its ESI-MS data, ^{1}H and ^{13}C NMR spectra. The signals of both *cis*- and *trans*- forms were detected in ^{1}H NMR spectral analysis. However, the *trans*-form was more abundant in the fraction based on the ^{1}H NMR spectral signals. The *cis*-form might be evident during the extraction process (17) by isomerization through photo-induction (7,13). The *trans*-configuration of *p*-coumaric acid has been identified from the fresh tissues of other plants (17,28) and dried plant material of itchgrass. Thus, *trans*-configuration of *p*-coumaric acid can exist at 40 °C during the drying process. Farmers use the dried material of itchgrass as mulch for 1 to 2 months for weed control in vegetable fields, where temperature sometime reaches up to 40 °C. The *trans*-*p*-coumaric acid is pure isomer that could be released from itchgrass under the field conditions. However, the determination of endogenous levels of *cis*- and *trans*- forms of *p*-coumaric acid in itchgrass and in the rhizosphere soil is necessary for better understanding the allelopathy of itchgrass.

Growth inhibitory effects of *trans*-*p*-coumaric acid on test plants

Phytotoxic effects of compound **I** on the early seedling growth of 4-test plant species was determined. It significantly inhibited the growth of all test plant species in dose-dependent manner. At 0.15 mg/mL concentration, shoot lengths of *A. conyzoides* and *L. sativa* were inhibited by 30.6 and 16.1 %, respectively; however, shoot lengths of *B. pilosa* and *E. crus-galli* were not affected than control (Fig. 3). The 0.15 mg/mL concentration, inhibited the root lengths of *A. conyzoides*, *B. pilosa* and *E. crus-galli* seedlings by 47.4, 35.4 and 26.2 % respectively, except stimulation (9.4 %) in root length of *L. sativa*, than control. The authentic *trans*-*p*-coumaric acid at 0.15 mg/mL concentration, inhibited the shoot lengths of *A. conyzoides*, *B. pilosa*, *E. crus-galli* and *L. sativa* by 31.9, 22.1, 11.5 and 15.8 %, respectively, than seedlings in control (Fig. 4). This concentration reduced the root lengths of *A. conyzoides*, *B. pilosa* and *E. crus-galli* by 25.7, 14.2 and 37.3 % respectively, however, the root length of *L. sativa* was stimulated by 8.9 %. The authentic *trans*-*p*-coumaric acid exhibited similar inhibitory effects like compound **I**.

In higher plants, phenolic compounds are the major groups of allelochemicals (15). Although coumaric acid has been isolated from the plants of Poaceae family (23) [such as *Oryza sativa* (6), *Sasa cernua* (14), *Triticum aestivum* (26) and *Avena fatua* (10)]. This is the first report of the identification of an allelochemical in itchgrass. Considering the inhibitory effects, *trans-p*-coumaric acid may play an active role in the allelopathy of itchgrass and may be responsible for growth suppression of neighbouring weeds. However, the isolation and identification of "major" allelochemical(s) in the RcM fraction is under investigation.

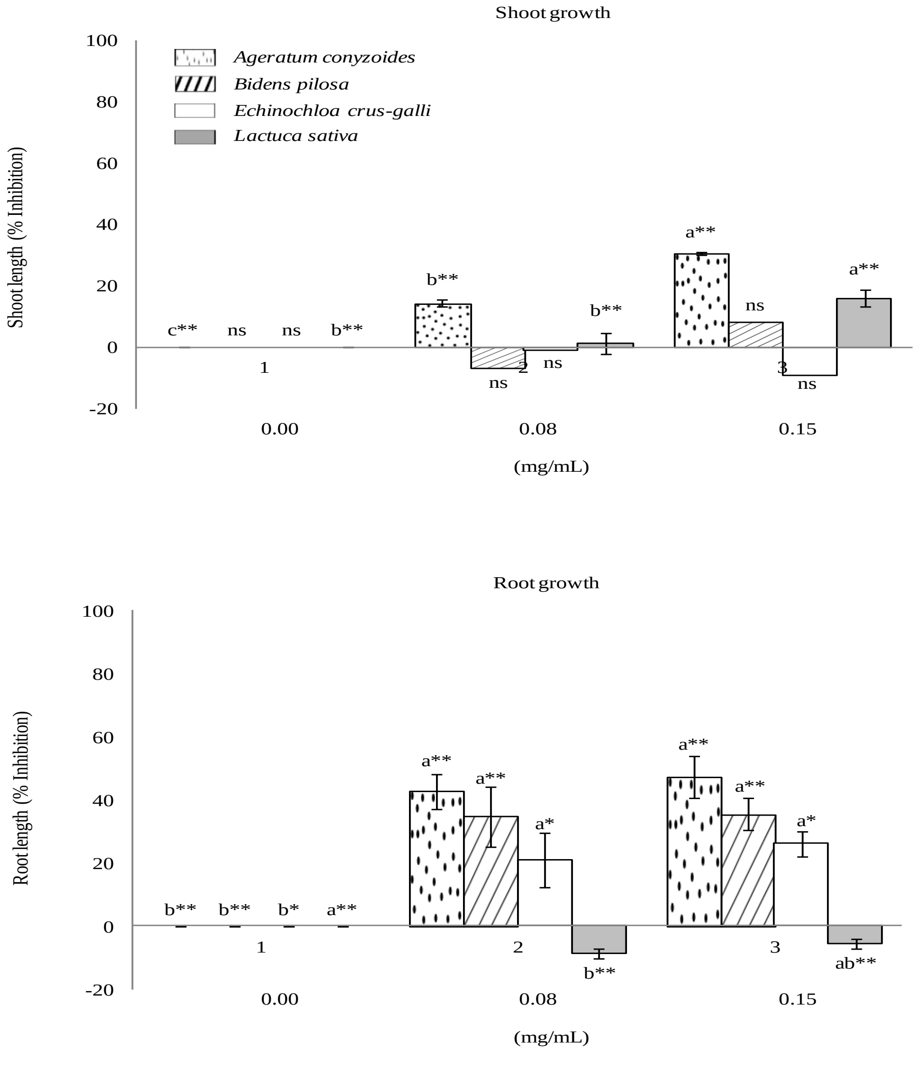

Figure 3. Phytotoxic effects of compound **I** on the shoot growth and root growth of test plant species. Data are means ± standard errors of four replications. The treatment means were compared using least significant difference (LSD) test (ns, not significant; *P < 0.05; **P < 0.01).

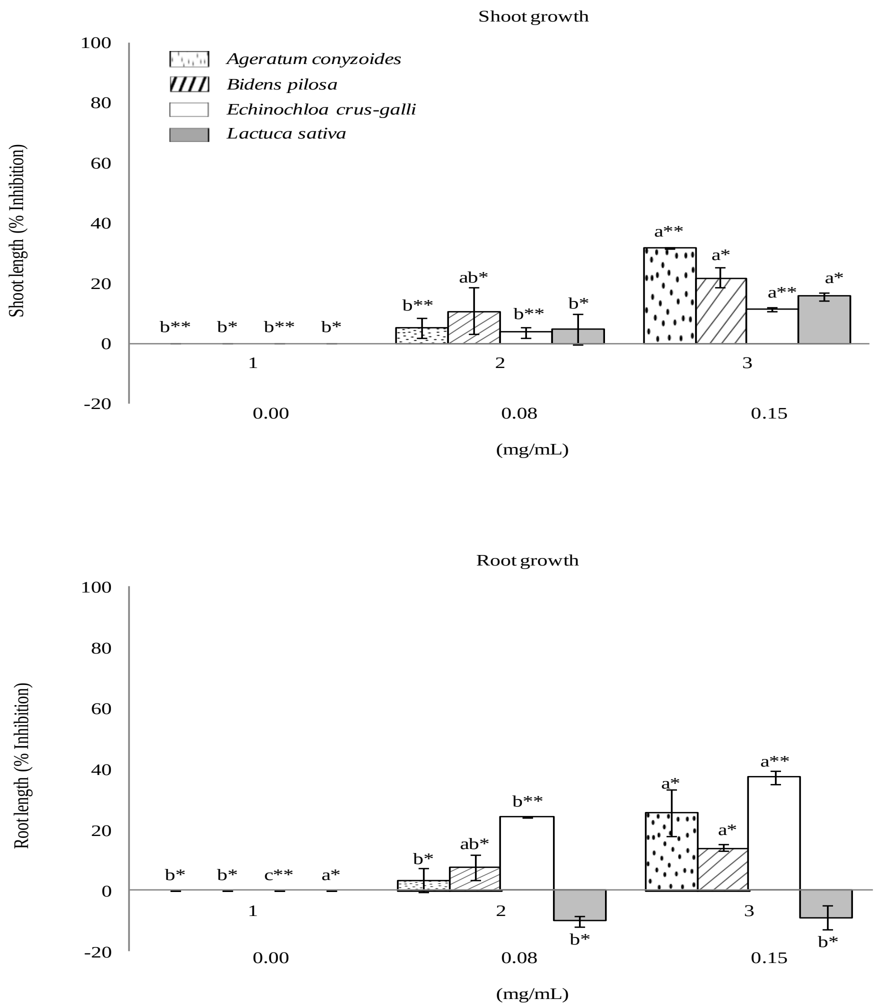

Figure 4. Phytotoxic effects of authentic *trans-p*-coumaric acid on the shoot growth and root growth of test plant species. Data are means ± standard errors of four replications. The treatment means were compared using least significant difference (LSD) test (*$P < 0.05$; **$P < 0.01$).

In recent years, the applied aspects of allelopathy for sustainable agricultural production and environmental protection through ecologically friendly control of weeds and the synthesis of novel agrochemicals based on allelochemicals have attracted an increased attention. Some studies have reported that plants can produce allelochemicals with promising weed suppressing potential, such as flavanones and kavalactones have been identified from *Piper methysticum* roots (29), and some phenolic acids (*p*-hydroxybenzoic, vanillic, syringic, sinapic and benzoic acids) from the extracts of two rice varieties (Koshihikari and Jasmine) seedlings (11) In addition, 9,12,15-octadec-atrienoic acid, 9,12-octadecadienoic acid, hexadecanoic acid and decanoic acid were identified in the ethanolic extracts from *Veronica persica* against the weeds (16). The major challenge faced by weed scientists is to develop ecologically sound and sustainable weed management tool for crop production systems with less reliance on synthetic herbicides. Suitable application of allelopathy in cropping systems could reduce the synthetic herbicide inputs and thereby reduce the environmental pollution and contamination of food and feed. Therefore, the isolation and identification of novel allelochemicals from the plants having allelopathic potential is of great importance to develop an innovative strategy of weed management in sustainable agricultural systems.

CONCLUSIONS

We found that *trans*-*p*-coumaric acid (*trans*-4-hydroxycinnamic acid) was the major allelochemical in itchgrass (confirmed by ESI-MS data, ^{1}H and ^{13}C NMR spectra). The phytotoxic effects of *trans*-*p*-coumaric acids significantly inhibited the early seedling growth of *A. conyzoides*, *B. pilosa*, *E. crus-galli* and *L. sativa*. This is the first report of identification of *trans*-*p*-coumaric acid in itchgrass

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