

ISOLATION AND IDENTIFICATION OF A GROWTH INHIBITORY SUBSTANCE FROM *HELIOTROPIMUM INDICUM* L.

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Heliotropium indicum L. belongs to the family Boraginaceae. The plant has been used as a folk medicine because it contains substances of various biological activities. It is also identified as a common weed which grows wildly in crop fields in tropical and subtropical regions of the world. However, there is little information on the allelopathic effect in this plant. Therefore, this study was undertaken to investigate the growth inhibitory effect and to identify the growth inhibitory substances in *H. indicum*. An aqueous methanol extract of *H. indicum* inhibited shoot and root growth of barnyard grass, foxtail fescue, timothy, cress, lettuce and rapeseed at concentrations higher than 10 mg dry weight equivalent extract/mL. The concentrations required for 50% growth inhibition (I_{50}) of those test plants ranged from 3–282 mg dry weight equivalent extract/mL. The extract was then separated using a sequence of chromatographic fractionations and a growth inhibitory substance was isolated and identified by spectral analysis as methyl caffeate. Methyl caffeate inhibited the growth of lettuce and foxtail fescue at concentrations higher than 1.0 mM. The results suggest that methyl caffeate may contribute to the growth inhibitory effect of *H. indicum* and may play an important role in the allelopathic effect of *H. indicum*.

Keywords: Growth inhibitory effect – *Heliotropium indicum* – aqueous methanol extract – methyl caffeate – growth inhibitory substance

INTRODUCTION

Heliotropium belongs to the family Boraginaceae that consists of about 250 species of annual and perennial plants. *Heliotropium indicum* has been previously reported to have been used in folklore medicine for wound healing, fever, insect bites, abdominal pain and malaria treatments because it possesses anti-inflammatory, antimicrobial, antitumor- and cardiogenic activities [1, 14, 18, 25]. However, this plant is also classified as a weed in rice and corn fields and roadsides [12, 30]. Since the plant is distributed throughout tropical and subtropical regions in the world [10], it could adapt itself to survive in drought and wet areas and efficiently compete with crops for

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moisture and nutrients [12]. Thus, we were interested in studying allelopathic effects of this plant.

The allelopathic effect of several weed species have been studied and reported to contain allelopathic substances. For example, the methanol extracts of twenty-two weed species inhibited the germination of *Abutilon theophrastii*, *Amaranthus retroflexus*, *Avena sterilis*, *Salvia officinalis*, *Laurus nobilis* and *Artemisia vulgaris* [17]. Ali et al. [4] found ten allelochemicals in the methanol extract of shoots, roots and seeds of *Cephalaria syriaca*, which is an endemic weed. Plants in the genus *Heliotropium* have also been reported to have allelopathic effects. Abdulghader et al. [2] stated that the aqueous extract of leaves of *H. europaeum* inhibited the germination of dodder seeds and radish seeds. In addition, ethanol extract of the aerial part of *H. indicum* has also been reported to decrease the germination and growth of lettuce and black gram [9]. However, there have been no reports about the isolation and identification of the growth inhibitory substances in *H. indicum*. Therefore, the present study was conducted to evaluate for the allelopathic activity and to isolate the growth inhibitory substances in *H. indicum*.

MATERIALS AND METHODS

Plant materials

Whole plants (roots, stems, leaves and flowers) of *Heliotropium indicum* (L.) were collected from Chiang Rai, Thailand. The plants were washed several times with tap water and dried in the shade. The dried plants were ground into powder by a grinding machine. The plant powder was then packed in vacuum-sealed plastic bags and kept at 4 °C. Monocotyledonous plants, barnyard grass (*Echinochloa crus-galli* (L.) P. Beauv.), foxtail fescue (*Vulpia myuros* (L.) C.C.Gmel.) and timothy (*Phleum pratense* L.); and dicotyledonous plants, cress (*Lepidium sativum* L.), lettuce (*Lactuca sativa* L.) and rapeseed (*Brassica napus* L.) were selected to determine the growth inhibitory effect of the extracts.

Extraction and bioassay

The powder (500 g) of *H. indicum* was extracted with 2.5 L of 70% (v/v) aqueous methanol for 48 h and filtrated through a filter paper (No. 2; Toyo, Tokyo, Japan). The residue was extracted again with methanol for 24 h and filtrated. Both filtrates were combined and concentrated with a rotary evaporator at 40 °C. The crude extract was dissolved in methanol to obtain the final concentrations 1, 3, 10, 30, 100 and 300 mg dry weight equivalent extract/mL. Each concentration of the extract was added to a sheet of filter paper (No. 2) in each a 28 mm Petri dish and the solvent was then evaporated until it dried in a draft chamber. Then, the filter paper was moistened with 0.6 mL of 0.05% (v/v) aqueous solution of Tween 20 (Polyoxy-

ethylene sorbitan monolaurate, Nakalai Tesque, Inc, Kyoto, Japan). Ten germinated seeds of barnyard grass, foxtail fescue and timothy were placed separately on the filter paper in Petri dishes after pre-germination for 72 h, 48 h and 60 h at 25 °C in darkness, respectively. Ten seeds of cress, lettuce and rapeseed were also placed separately. The germinated seeds and seeds with only the aqueous solution of Tween 20 were used as control treatments. Shoot and root length of the test plants were measured at 48 h after incubation at 25 °C in darkness. The inhibition percentage was calculated using the following formula: Inhibition (%) = $[1 - (\text{length of treatment} / \text{length of control})] \times 100$. The experiment was performed on thirty seedlings for each treatment. Concentrations required for 50% growth inhibition (I_{50} values) on test plants were calculated by the regression equation of the concentration-response curves.

Isolation and purification of active substances

The aqueous methanol extract of *H. indicum* was concentrated using a rotary evaporator at 40 °C to produce an aqueous residue. The aqueous residue was adjusted to pH 7.0 with 1 N NaOH and partitioned 5 times against 100 mL of ethyl acetate. The ethyl acetate fraction was concentrated to dryness and separated using a column of silica gel (60 g, silica gel 60, spherical, 70–230 mesh: Nacalai Tesque, Kyoto, Japan), eluted with 20, 30, 40, 50, 60, 70, 80, 90% of ethyl acetate in *n*-hexane (v/v; 150 mL per step) and methanol (300 mL). The active fractions were found in fractions obtained by elution with 60 (F5) and 70% (F6) of ethyl acetate in *n*-hexane. The two active fractions were combined and evaporated to dryness. The residue was dissolved in 20% aqueous methanol and loaded onto a Sephadex LH-20 column (100 g, GE Healthcare Bio-Science AB, Sweden), eluted with 20, 40, 60, 80% of aqueous methanol (v/v; 150 mL per step) and methanol (300 mL). The active fraction was found in fraction obtained by elution with 80% of aqueous methanol and concentrated until dry. The residue was dissolved in 20% (v/v) aqueous methanol and loaded into reverse-phase C₁₈ cartridge (1000 mg/6 mL, 6 nm, YMC Dispo SPE, YMC Ltd., Kyoto, Japan), eluted with 20, 30, 40, 50, 60, 70, 80, 90% of aqueous methanol (v/v; 15 mL per step) and methanol (30 mL). The active fraction was found in fraction obtained by elution with 30% aqueous methanol and concentrated to dryness. The residue of active fraction was purified by reverse-phase HPLC (500 × 10 mm I.D, ODS AQ-325; YMC Ltd, Kyoto, Japan), eluted with 35% (v/v) aqueous methanol at a flow rate of 1.5 mL/min, and detected at 220 nm. The growth inhibitory effect in each isolation and purification step was determined using a cress bioassay. An active substance was identified by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), ¹H NMR (400 MHz, CD₃OD) and ¹³C NMR (100 MHz, CD₃OD).

Bioassay of methyl caffeate

The growth inhibitory effect of methyl caffeate was determined using lettuce and foxtail fescue bioassay. The compound was dissolved in 1 mL of methanol to prepare final concentrations of 0.1, 0.3, 1, 3 and 10 mM. Each concentration was dropped into a sheet of filter paper (No. 2) in each a 28 mm Petri dish, as described above. Shoot and root length of test plants were measured at 48 h after incubation at 25 °C in darkness. The inhibition percentage was calculated using formula as described above. The I_{50} values on two test plants were calculated by the regression equation of the concentration-response curves.

Statistical analysis

The bioassay experiment was completed with thirty seedlings for each treatment and repeated twice. Treatments were prepared in a completely randomized design. Data were analyzed by SPSS version 22.0 using one-way ANOVA.

RESULTS

The inhibitory effect of H. indicum extract on shoot growth

The extract of *H. indicum* inhibited shoot growth of test plants at concentrations greater than 10 mg dry weight equivalent extract/mL (Table 1). The extracts at 30 mg dry weight equivalent extract/mL inhibited shoot growth of cress, lettuce and rapeseed seedlings by 95.5, 93.0 and 78.3%, respectively. At 300 mg dry weight equivalent extract/mL, the extract completely inhibited cress, lettuce and rapeseed seedlings, and inhibited foxtail fescue and timothy seedlings by 99.0% and 91.0%, respectively. The I_{50} values of shoot growth of all test plants were in the range of 7–282 mg dry weight equivalent extract/mL. On the basis of I_{50} values, cress shoots were the most sensitive to the extract followed by lettuce and rapeseed shoots. While, barnyard grass shoots were less sensitive than foxtail fescue, timothy, rapeseed, lettuce and cress shoot.

The inhibitory effect of H. indicum extract on root growth

The extract of *H. indicum* inhibited root growth of test plant at concentrations greater than 10 mg dry weight equivalent extract/mL. The extracts at 30 mg dry weight equivalent extract/mL completely inhibited the root growth of timothy seedlings and inhibited lettuce, cress, foxtail fescue, rapeseed and barnyard grass seedlings by 95.6, 93.5, 92.0, 88.1 and 58.6%, respectively. At 300 mg dry weight equivalent extract/mL, the extract completely inhibited root growth of all test plants (Table 2). The I_{50}

Table 1
The inhibition percentage of *H. indicum* extracts on shoot growth of six test plant species

Concentration (mg dry weight equivalent extract/mL)	Inhibition (%)					
	Test plants species					
	Cress	Lettuce	Rapeseed	Banyard grass	Foxtail fescue	Timothy
1	4.55±14.1 A	2.95±17.3 AB	-8.69±33.7 A	-5.24±10.4 A	-15.5±23.2 A	-11.5±29.9 A
3	20.5±15.5 B	14.7±14.5 B	-8.69±45.1 A	-12.1±15.1 A	-13.6±19.9 A	-1.28±40.7 A
10	60.2±14.9 C	47.1±16.1 C	8.70±42.8 A	-5.64±19.0 A	-4.9±27.1 A	29.5±26.5 B
30	95.5±3.9 D	93.0±12.3 D	78.3±17.6 B	-6.45±16.9 A	27.2±53.6 B	62.8±22.6 C
100	100±0.6 D	100±0.0 D	100±2.3 B	19.4±15.2 B	92.2±11.5 C	69.2±18.3 CD
300	100±0.0 D	100±0.0 D	100±0.0 B	64.1±10.1 C	99.0±1.3 C	91.0±6.6 D

Note: Mean ±SD, mean with the same letters in a column is not significantly different at $P < 0.05$.

Table 2
The inhibition percentage of *H. indicum* extracts on root growth of six test plant species

Concentration (mg dry weight equivalent extract/mL)	Inhibition (%)					
	Test plants species					
	Cress	Lettuce	Rapeseed	Banyard grass	Foxtail fescue	Timothy
1	12.9±13.0 A	23.7±11.5 A	-5.43±24.3 A	-0.59±10.3 A	0.00±13.5 A	12.2±26.1 A
3	20.2±18.6 A	34.2±7.9 B	-9.78±28.7 A	-0.59±13.9 A	18.4±12.8 B	50.0±13.2 B
10	54.1±14.8 B	57.0±12.0 C	52.2±25.6 B	29.6±8.6 B	65.7±6.4 C	82.9±6.3 C
30	93.5±2.9 C	95.6±6.3 D	88.1±5.9 C	58.6±9.7 C	92.0±6.3 D	100±0.6 D
100	99.5±0.4 C	100±0.0 D	96.8±1.8 C	86.4±7.8 D	98.8±0.3 DE	100±0.0 D
300	100±0.0 C	100±0.0 D	100±0.0 C	99.4±0.5 E	100±0.0 E	100±0.0 D

Note: Mean ±SD, mean with the same letters in a column is not significantly different at $P < 0.05$.

Table 3

*I*₅₀ values of the *H. indicum* extracts on shoot and root growth of six test plant species

Test plant species	<i>I</i> ₅₀ (mg dry weight equivalent extract/mL)	
	Shoot	Root
Cress	7	7
Lettuce	9	5
Rapeseed	20	12
Barnyard grass	282	23
Foxtail fescue	48	7
Timothy	27	3

Note: The values were determined by a logistic regression analysis after bioassays.

values of root growth of all test plants were in the range of 3–23 mg dry weight equivalent extract/mL. Timothy roots were the most sensitive to the extract followed by lettuce and foxtail fescue roots (Table 3).

Isolation and identification of a growth inhibitory substance

The residue of ethyl acetate fraction was loaded into silica gel column and the fractions with high inhibitory activities were found in F5 and F6 (Fig. 1). At the concentration 0.6 g dry weight equivalent extract/mL, F5 and F6 completely inhibited (100%) shoot growth of cress and inhibited root growth by 94.5 and 96.2%, respectively.

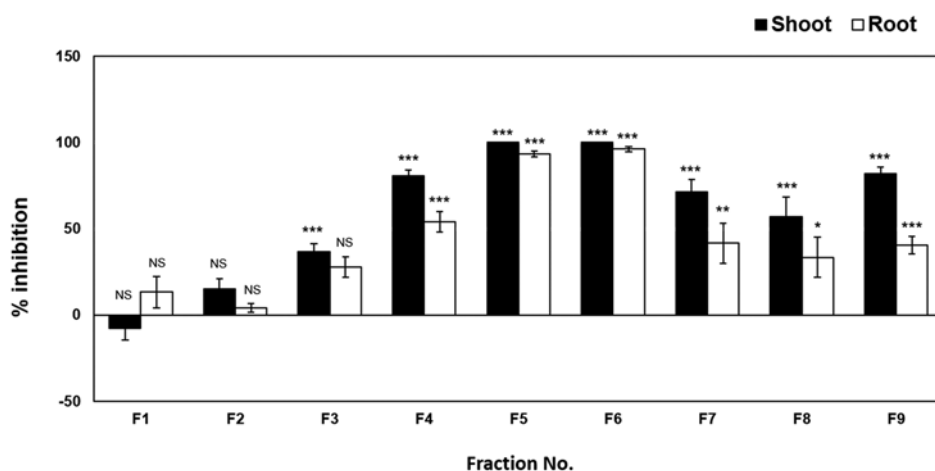


Fig. 1. Inhibitory effects of fractions obtained by the column of silica gel on shoot and root growth of cress seedlings at the concentration 0.6 g dry weight equivalent extract/mL. Values represent means \pm SE from two independent experiments with 10 seedlings for each treatment. Asterisks indicate significant difference between control and treatments: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

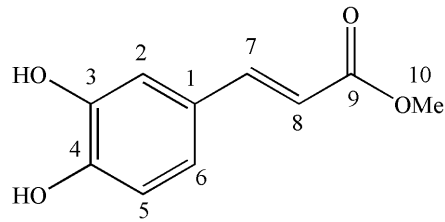


Fig. 2. The chemical structure of methyl caffeate

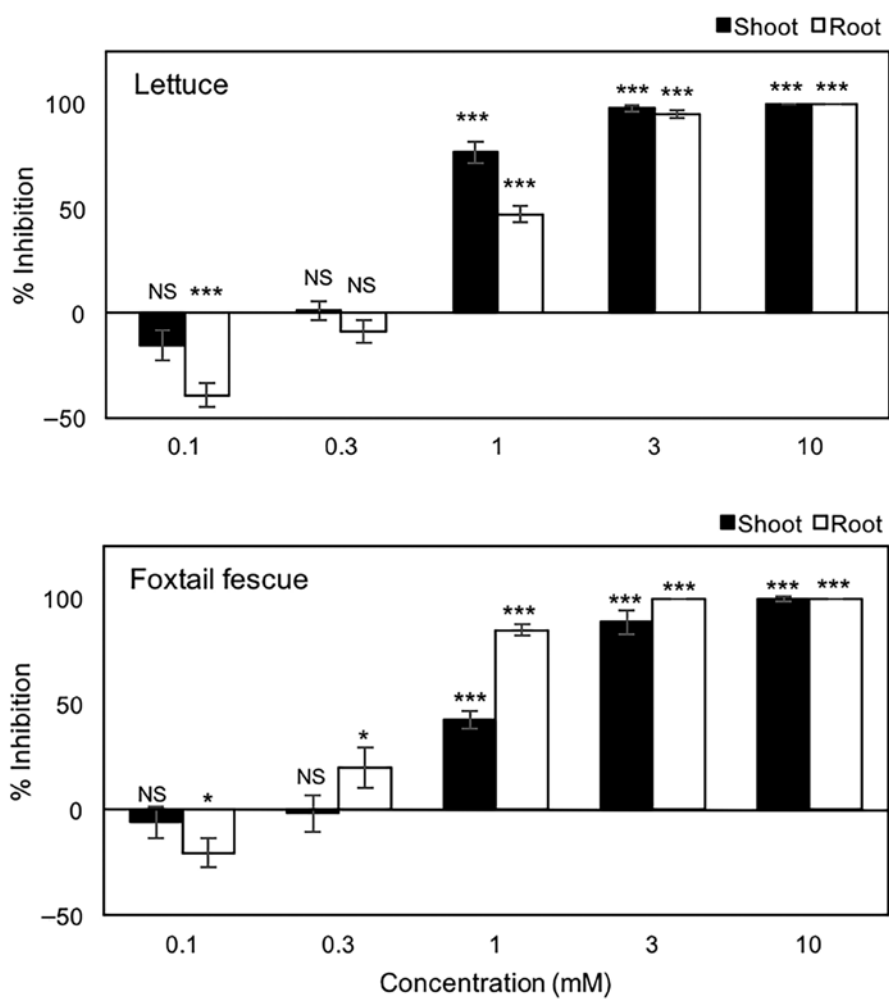


Fig. 3. Effects of methyl caffeate on shoot and root growth of lettuce and foxtail fescue. Values represent means \pm SE from two independent experiments with 10 seedlings for each treatment. Asterisks indicate significant difference between control and treatments: * $P < 0.05$; *** $P < 0.001$; NS: non-significant

tively. The two active fractions were combined and further separated by chromatographic fractionations. An active substance was isolated and identified by spectral analysis. The molecular formula of an active substance was determined as $C_{10}H_{10}O_4$ by HR-ESI-MS m/z 192.9761 ($[M-H]^-$) (calcd for $C_{10}H_9O_4$ 193.0501, $\Delta = -383$ ppm); 1H NMR (400 MHz, CD_3OD) δ_H 7.54 (d, $J = 16.2$ Hz, 1 H, H-7), 7.03 (d, $J = 2.1$ Hz, 1 H, H-2), 6.94 (dd, $J = 8.4, 2.1$ Hz, 1 H, H-6), 6.77 (d, $J = 8.4$ Hz, 1 H, H-5), 6.26 (d, $J = 16.2$ Hz, 1 H, H-8), 3.76 (s, 3 H, H-10); ^{13}C NMR (100 MHz, CD_3OD) δ_C 169.8 (C-9), 149.6 (C-4), 147.0 (C-3), 146.8 (C-7), 127.6 (C-1), 122.9 (C-6), 116.5 (C-5), 115.1 (C-8), 114.8 (C-2), 52.0 (C-10). The substance was characterized as methyl caffeate (Fig. 2) by comparison of the spectroscopic data with previously reported in the literature [23, 32].

Growth inhibitory effect of methyl caffeate

Methyl caffeate inhibited shoot and root growth of lettuce and foxtail fescue at concentrations greater than 1 mM (Fig. 3). Methyl caffeate at 10 mM completely inhibited (100%) shoot growth of the test plants. The inhibitory effects depended on test plant species and methyl caffeate concentration. The I_{50} values of methyl caffeate on lettuce shoots and roots were 0.7 and 1.0 mM, respectively, and those on foxtail fescue shoots and roots were 1.3 and 0.5 mM, respectively.

DISCUSSION

The aqueous methanol extracts of *H. indicum* exerted inhibitory effects on seedling growth of six test plant species. The variation of growth inhibition depended on the test plant species and the extract concentrations. The results are in agreement with previous studies demonstrations also that the inhibitory effects of the extract on seedling growth depended on the plant species and the concentrations applied [16, 27]. The plant extracts showed higher inhibition on roots than shoots of all test plant species. The reduction of root growth might be due to the direct contact between roots and the putative allelochemicals which affect the morphological and physiological process such as cell division, cell elongation, membrane permeability and ion uptake [26]. Afef et al. [3] reported that allelopathic effect of aqueous and methanol extract of *Capparis spinose* leaves and *Cleome arabica siligues* showed a cytotoxic effect on the root tip of *Lactuca sativa* including cell membrane damage and reduction of mitotic index. Moreover, Lara-Nuñez et al. [19] and Ma et al. [22] reported that the inhibition of root growth and root cell damage were found in a state of allelopathic stress. Regarding I_{50} values, our results show that dicotyledonous plants are more sensitive to the extracts than monocotyledonous plants. Similar results were also found by Ma et al. [21], who reported that the germination and growth of rapeseed and sesame (dicotyledonous plants) were more sensitive to an aqueous extract of *Stellala chamaejasma* than wheat and maize (monocotyledonous plants). These result

suggests that *H. indicum* may possess an allelopathic effect and contain growth inhibitory substances.

The investigation of the inhibitory effect of *H. indicum* extract led to the isolation and identification of an active substance. A growth inhibitory substance was characterized as methyl caffeate, which is the derivative of caffeic acid, and is oxidized to quinone [5, 20]. In the present study, methyl caffeate had a growth inhibitory effect on lettuce and foxtail fescue seedlings. The growth of the two test plant seedlings was reduced by increasing methyl caffeate concentrations. Considering the quinones, some compounds that have allelopathic property have been reported to possess a quinoidal structure [11, 28]. Moreover, Inbaraj and Chignell [15] reported that the mechanism of quinones (juglone and plumbagin) affected the redox cycling, increasing H₂O₂ generation and the oxidation of glutathione (GSH), which is a reason for its toxicity. The growth inhibitory effect of methyl caffeate may be a result of its quinone derivatives. Methyl caffeate has been isolated from several plants, such as *Magnolia obavata* [24], *Polygonum amplexicaule* [31] and *Solanum torvum* [7]. This compound has been reported to possess antihyperglycemic [13], antimicrobial [6], anticancer [8] and antioxidant activities [29]. Although methyl caffeate has been isolated from several plants, its presence has never been reported in *H. indicum*. Therefore, this may be the first report on methyl caffeate as a growth inhibitory substance in *H. indicum*.

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