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Two Allelopathic Substances from *Plumbago rosea* Stem Extracts and Their Allelopathic Effects

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Abstract: The plant *Plumbago rosea* Linn., belonging to the Plumbaginaceae family, is an important medicinal herb distributed in part of Southeast Asia, and there are many reports of its pharmacological properties. However, the allelopathic activities of *P. rosea* have not been examined. Thus, the present study was conducted to assess the allelopathic activity of *P. rosea* and to identify its allelopathic substances. The aqueous methanol stem extract of *P. rosea* significantly suppressed the seedling growth of barnyard grass (*Echinochloa crus-galli* L. P. Beauv.), Italian ryegrass (*Lolium multiflorum* Lam.), timothy (*Phleum pretense* L.), cress (*Lepidium sativum* L.), lettuce (*Lactuca sativa* L.), and alfalfa (*Medicago sativa* L.). The extract of *P. rosea* was then purified through chromatographic steps, and two active substances were isolated and determined as 7,4′,5′-tri-*O*-methyl dihydroquercetin and 7,4′,5′-tri-*O*-methylampelopsin. The two compounds significantly inhibited the seedling growth of cress, with 7,4′,5′-tri-*O*-methylampelopsin showing a greater inhibitory effect than 7,4′,5′-tri-*O*-methyl dihydroquercetin. This result may be due to the 3′-OH group in 7,4′,5′-tri-*O*-methylampelopsin. The effective concentrations of both compounds required for 50% growth inhibition (EC_{50} values) of cress seedlings were 0.24 mM and 0.59 mM for root and shoot, and 0.07 mM and 0.21 mM, respectively. These findings suggest that the two compounds may contribute to the allelopathic effect of *P. rosea* and could be used as a natural source of allelopathic substances.

Keywords: *Plumbago rosea*; allelopathic substances; 7,4′,5′-tri-*O*-methyl dihydroquercetin; 7,4′,5′-tri-*O*-methylampelopsin

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1. Introduction

Weeds are one of the important factors in agriculture that influence crop production for competing natural resources. Presently, almost all farmers are dependent on using synthetic herbicides to control weeds effectively [1]. However, the overuse of synthetic herbicides severely pollutes the environment [2], harms human health [3], and destroys biodiversity [4]. Therefore, many researchers are focused on alternative, plant-based natural sources, such as secondary metabolites, to control weeds because they can degrade easily, are not toxic to humans, and are safe for environmental pollution [5–8]. Allelopathy is a biochemical interaction among living organisms that provides both inhibitory and stimulatory roles in plant processes [9]. Allelochemicals are released into the environment through volatilization, leaching, root exudation, and the decomposition of plant residues in soil [10]. These allelochemicals can be found in all plant parts, including leaves, roots, rhizomes, stems, flowers, pollens, fruits, and seeds [11]. Upon release from the source plants, these substances affect the germination, growth, or establishment of other plant species in their vicinity, even the source plants themselves (autotoxicity) [12–14]. As a natural approach to weed control, allelopathic plants have been used in various ways [15,16], such as mulching, intercropping, and applying plant extracts as herbicides,

or isolating and identifying their active compounds and using them as a tool for natural herbicide development.

Plumbago rosea Linn. (syn. *Plumbago indica* Linn.) from the family of Plumbaginaceae is commonly known as rose leadwort. This species originates from India and is now mostly cultivated in regions of Southeast Asia, Africa, China, the Arabian Peninsula, and Europe for its roots [17,18]. *Plumbago rosea* is a perennial plant and a spreading evergreen shrub with oval leaves and racemes of deep pink or scarlet flowers in winter. This plant has a sharp, hot taste and is considered good for digestion, slowing aging, and supporting longevity and strength. It is also traditionally used to treat inflammatory disorders, skin diseases [19], gastric acidity [20], constipation [20], abdominal pain [20], and as an abortifacient [21,22]. Moreover, the roots of *P. rosea* have been reported to possess antitumor [23] and antiatherogenic [24] activities. It also has many pharmacological activities, such as abortifacient [25], antiarthritic [26], anticancer [27–29], anticoagulant [30], antifeedant [31], and antifungal [32]. Several researchers described that *P. rosea* constitutes many active biochemicals, such as plumbagin [33], hydroxy-1,4-napthaquinone, sitosterol glycoside, fatty alcohol, and tannins [34]. Because of its different bioactivities, it is believed that it may also possess allelopathic activity. However, there has been no research, report, or information regarding its allelopathic properties yet. Hence, the present study has the following objectives: (1) To assess the allelopathic potential of *P. rosea* on the seedlings' growth of six tested plants; (2) To isolate and identify its allelopathic substances; (3) To determine the effect of identified compounds on the test plants under laboratory conditions.

2. Materials and Methods

2.1. Collection of Plant Materials

Stems of *P. rosea* were collected from different regions of Mandalay Division, Myanmar from July to August 2020 (Figure 1). After collection, the stems were washed under tap water and air-dried in the shade. The dried samples were then ground into powder and stored at 2 °C in a vacuum-sealed plastic package until extraction. The seeds of three dicotyledons (cress (*Lepidium sativum* L.), lettuce (*Lactuca sativa* L.), and alfalfa (*Medicago sativa* L.)) and three monocotyledons (barnyard grass (*Echinochloa crusgalli* (L.) P. Beauv.), Italian ryegrass (*Lolium multiflorum* Lam.), and timothy (*Phleum pratense* L.)) were used for testing the allelopathic potential of the extracts.

(**a**) (**b**)

Figure 1. *Plumbago rosea*: (**a**) whole plant, (**b**) stems.

Lepidium sativum L. (cress) was used for testing the inhibitory effects of allelochemical substances on germination because of its wide range, well-known growth behaviors, and stable germination rate.

2.2. Extraction and Growth Assay

Plumbago rosea stem powder (100 g) was extracted with 500 mL of 70% (*v*/*v*) aqueous methanol for 48 h. The extracts were filtrated using a layer of filter paper (No. 2, 125 mm; Advantec, Toyo Roshi Kaisha Ltd., Tokyo, Japan), and the residues were re-extracted with an additional 500 mL of 100% methanol for 24 h and then filtrated again. The two filtrates were mixed and evaporated with a rotary evaporator at 40 °C (Yamato Scientific Co., Ltd., Tokyo, Japan). The extracts were dissolved in methanol (100 mL) for the germination test. The germination test was conducted using six extract concentrations (1, 3, 10, 30, 100, and 300 mg dry weight (DW)-equivalent extract/mL) and controls (0.6 mL of a 0.05% (*v*/*v*) aqueous solution of polyoxyethylene sorbitan monolaurate (Tween 20)). An exact amount of each concentration was added to filter papers (No. 2, 28 mm; Toyo Roshi Ltd., Tokyo, Japan) in Petri dishes (diameter 28 mm). The Petri dishes were dried in a draft chamber. After drying, 0.6 mL of a 0.05% (*v*/*v*) aqueous solution of polyoxyethylene sorbitan monolaurate (Tween 20; Nacalai Tesque Inc., Kyoto, Japan) was added to each Petri dish, and only 0.6 mL of a 0.05% (*v*/*v*) aqueous solution of Tween 20 was applied for the control. Ten dicotyledonous seeds and ten pre-germinated (36 h at 25 °C in the dark) monocotyledonous seeds were then placed in each Petri dish. The experiment was conducted with three replications and repeated two times (10 seedlings/replication) using a completely randomized design. The lengths of the shoots and roots were measured with a ruler after 48 h incubation in the dark at 25 °C. The inhibition of root and shoot growth was calculated by comparing them to the control. The percentage of inhibition was estimated using the following equation: *(*%) seedling growth = (1 − the length of the treated seedling/the length of the control seedling) × 100.

2.3. Separation and Isolation of the Allelopathic Substances from the Plumbago Rosea Extracts

An aqueous methanol extract was obtained by soaking 3.2 kg of *Plumbago rosea* stem powder in 20 L of 70% (*v*/*v*) aqueous methanol for 48 h. The extract was filtered through a sheet of filter paper (No. 2; 125 mm; Toyo Ltd., Tokyo, Japan). The residue was extracted again with 20 L of methanol for 24 h and filtered. The two filtrates were combined and removed from the solvent at 40 °C using a vacuum rotary evaporator to obtain an aqueous residue. The aqueous residue was adjusted to pH 7.0 with 1 M phosphate buffer and partitioned four times with an equivalent volume of ethyl acetate (EtOAc). The EtOAc fraction was used for the isolation process. The EtOAc fraction was filtrated and evaporated overnight using anhydrous Na_2SO_4. The extract was subjected to a column of silica gel (60 g, silica gel 60, 70–230 mesh; Nacalai Tesque). The column was eluted with ethyl acetate in *n*-hexane 20%, 30%, 40%, 50%, 60%, 70%, 80% (10% per step *v*/*v*, 150 mL), ethyl acetate (150 mL), and methanol (300 mL). The last fraction (methanol fraction) was separated again using a column of silica gel (60 g, silica gel 60, 70–230 mesh; Nacalai Tesque). The solvent of ethyl acetate: acetone: methanol was used in the ratios of 100:0:0, 75:25:0, 50:50:0, 75:25:0, 0:100:0, 0:75:25, 0:50:50, and 0:25:75 (*v*/*v*/*v*), 150 mL per step and methanol (300 mL). The active fraction eluted with the 100% ethyl acetate fraction was evaporated to dryness and subjected to a column of Sephadex LH-20 (100 g; GE Healthcare, Uppsala, Sweden). The column was eluted with aqueous methanol 20%, 40%, 60%, 80% (20% per step *v*/*v*, 150 mL) and methanol (300 mL). The active fraction was eluted with 80% aqueous methanol. After evaporation of the active fraction, the residue was separated using a reverse-phase C_{18} cartridge (1.2 × 6.5 cm; YMC Co., Ltd., Kyoto, Japan). The cartridge was loaded with aqueous methanol 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% (10% per step *v*/*v*, 15 mL) and methanol (30 mL). The fraction eluted by 50% aqueous methanol was active and was then purified using a reverse-phase High-Performance Liquid Chromatography (HPLC; Shimadzu Corporation, Kyoto, Japan) column (500 × 10 mm I.D., ODS AQ-325; YMC Co., Ltd., Kyoto, Japan), eluted at a flow rate of 1.5 mL/min with 50% (*v*/*v*) aqueous methanol, and detected at 220 nm. The most active fractions were detected at the retention times of 200–210 min and 244–252 min. The active fractions were then purified again using a reverse-phase HPLC column (250 × 4.6 mm I.D., Inertsil ODS-3; GL Science Inc., Tokyo, Japan),

eluted at a flow rate of 0.5 mL/min with 45% (v/v) aqueous methanol, and detected at 220 nm at the retention times of 112–120 min (compound **1**) and 170–180 min (compound **2**). The substances were characterized by atmospheric pressure chemical ionization–mass spectrometry (APCI-MS), electrospray ionization mass spectrometry (ESI-MS), and ^{1}H-nuclear magnetic resonance (NMR) spectra (400 MHz, acetone-d_6).

2.4. Germination Test

The isolated compounds **1** and **2** were dissolved in 0.5 mL methanol to prepare a test concentration and added to a sheet of filter paper (No. 2, 28 mm; Toyo) in 28 mm diameter Petri dishes. The concentrations of 0.03, 0.1, 0.3, 1, and 3 mM for compound **1** and 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 mM for compound **2** were added to each Petri dish. After that, 0.6 mL of a 0.05% (v/v) aqueous solution of Tween 20 was added to each dish, and only 0.6 mL of a 0.05% (v/v) aqueous solution of Tween 20 was used as the control. Ten seeds of cress were then placed in each Petri dish. The germination test was conducted with three replications (10 seedlings/replication) using a completely randomized design. The lengths of the shoots and roots were measured with a ruler after 48 h incubation in the dark at 25 °C. The inhibition of root and shoot growth was calculated by comparing them to the control.

2.5. Statistical Analysis

All collected data were analyzed using the Statistical Package for the Social Sciences (SPSS version 16.0). One-way analysis of variance (ANOVA) was used to analyze the data. Significant differences among the treatments were identified using a post hoc Tukey's test at $p \leq 0.05$. The effective concentration required for 50% inhibition (EC_{50} values) was determined by GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Allelopathic Property of Plumbago rosea Extract

The aqueous methanol extracts of *P. rosea* significantly suppressed the seedling growth of all tested plants (Figure 2). The extracts of *P. rosea* obtained from 30 mg DW-equivalent extract mL^{-1} inhibited shoot growth of the monocot species of timothy, barnyard grass, and Italian ryegrass by 41.2%, 52.3%, and 13.5% of control shoot growth and inhibited root growth by 16.5%, 13.3% and 11.1% of control root growth, respectively (Figure 2). In dicot species, the extract obtained from 3 mg DW-equivalent extract mL^{-1} inhibited the shoot growth of cress, alfalfa, and lettuce by 60.2%, 37.1%, and 27.7% and inhibited root growth by 73.4%, 26.5%, and 29.4% of control shoot and root growth, respectively (Figure 2). On the contrary, the shoot growth of timothy showed growth stimulatory activity at 1 mg equivalent extract mL^{-1} (114%) and 3 mg equivalent extract mL^{-1} (107%), and barnyard grass showed at 3 mg equivalent extract mL^{-1} (101%). This indicated that the interaction between crude extracts and tested plants includes not only stimulatory effects but also inhibitory effects. The EC_{50} values of the extracts against the shoot growth of all tested plants ranged from 0.87 to 33.5 mg equivalent extract mL^{-1} and root growth from 1.13 to 11.68 mg equivalent extract mL^{-1} in the root (Table 1). The EC_{50} values showed that the roots of timothy, barnyard grass, and alfalfa were more sensitive to the crude extracts of *P. rosea* than the shoots; in contrast, the shoots of Italian ryegrass, cress, and lettuce were more sensitive to the crude extracts of *P. rosea* than the roots.

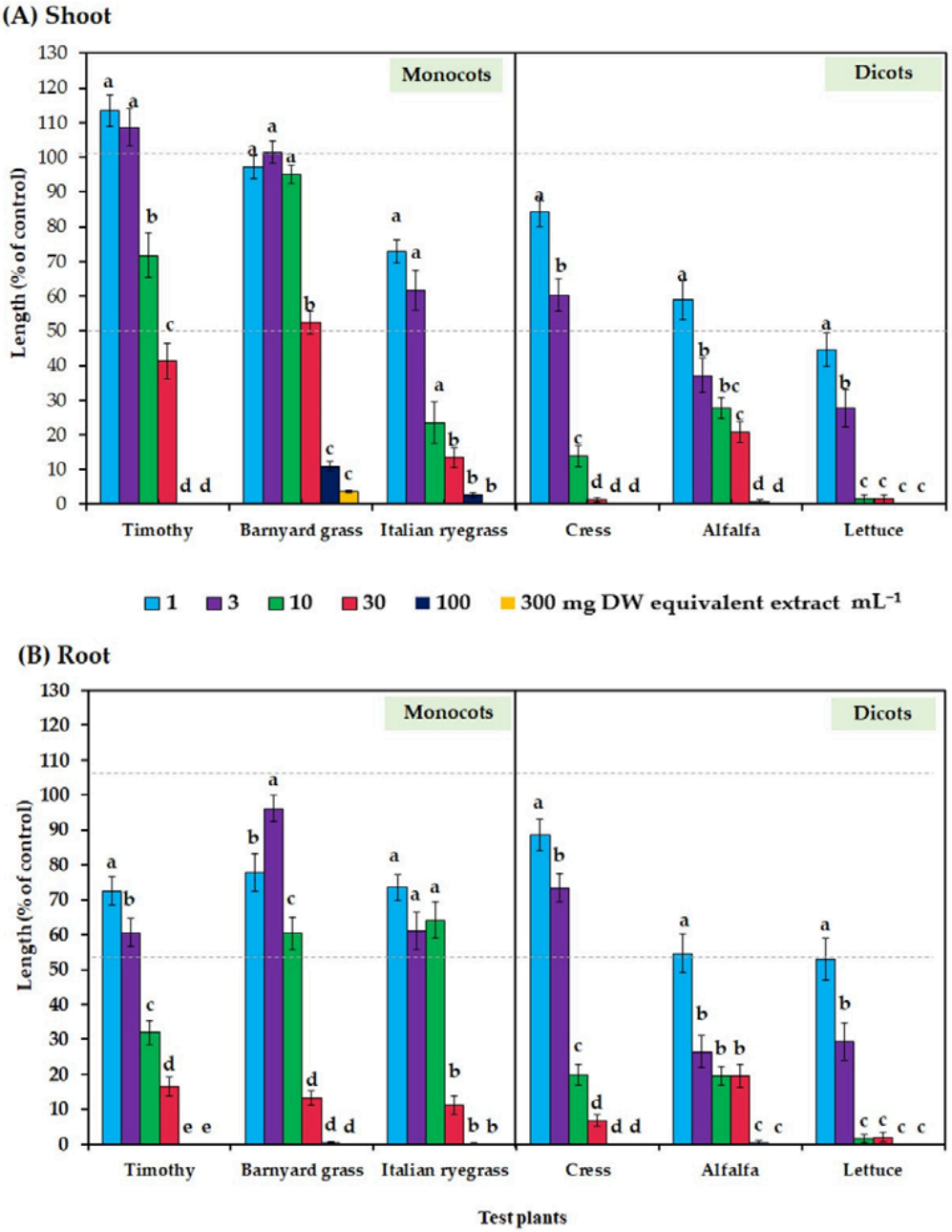

Figure 2. Growth inhibitory effects of the *P. rosea* extract on the (**A**) shoot and (**B**) root growth of all the tested plants. Error bars are standard errors of the mean from two independent experiments repeated three times with 10 seeds for each treatment ($n = 60$). Different letters above the bars indicate significant differences between treatments (Tukey's HSD, $p \leq 0.05$).

Table 1. EC_{50} values of the *P. rosea* extracts for all the tested plants.

Test Plant	Monocot			Dicot		
	Timothy	**Barnyard Grass**	**Italian Ryegrass**	**Cress**	**Alfalfa**	**Lettuce**
Shoot	21.87	33.50	3.71	3.47	1.83	0.87
Root	4.16	11.68	7.02	4.83	1.18	1.13

3.2. Isolation and Identification of the Growth Inhibitory Substances

The aqueous methanol extracts of *P. rosea* were purified through chromatographic steps (silica gel column, Sephadex LH-20, reverse-phase C_{18} cartridge, reverse-phase HPLC), and two growth inhibitory substances, compounds **1** and **2**, were then isolated.

The molecular formula of compound **1** was determined to be $C_{18}H_{18}O_7$ through ESIMS at m/z 347.1130 $[M + H]^+$ (calcd for $C_{18}H_{19}O_7$, 347.1131). The ^1H NMR (400 MHz, acetone-d_6) spectrum of the compound showed δ 11.83 (s, 1H, H-11), 7.23 (d, J = 2.0 Hz, 1H, H-7), 7.12 (dd, J = 8.4, 2.0 Hz, 1H, H-5), 6.96 (d, J = 8.4 Hz, 1H, H-6), 6.13 (d, J = 2.4 Hz, 1H, H-2), 6.07 (d, J = 2.2 Hz, 1H, H-1), 5.54 (d, J = 2.4, 1H, H-4), 5.28 (d, J = 5.9 Hz, 1H, H-12), 4.31 (dd, J = 5.9, 2.2 Hz, 1H, H-3), 3.87 (s, 3H, MeO), 3.82 (s, 3H, MeO), and 3.81 (s, 3H, MeO). The optical rotation of the compound was $[\alpha]_D^{26} = -44$ (c = 0.05, CH_3CN). The compound was identified as 7,4′,5′-tri-*O*-methyl dihydroquercetin (compound **1**, Figure 3) by comparing with previously reported data [22].

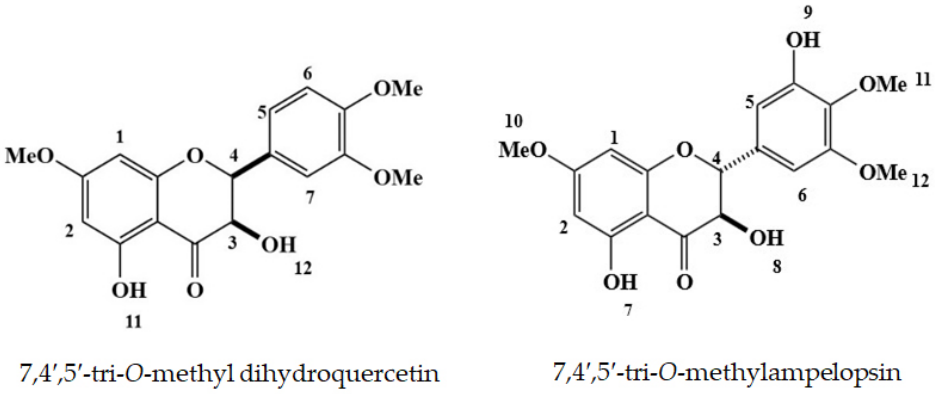

Figure 3. Chemical structures of compounds.

The molecular formula of compound **2** was determined to be $C_{18}H_{18}O_8$ through ESIMS at m/z 363.1097 $[M + H]^+$ (calcd for $C_{18}H_{19}O_8$, 363.1080). The ^1H NMR (400 MHz, acetone-d_6) spectrum of the compound showed δ 11.69 (s, 1H, H-7), 8.01 (s, 1H, H-9), 6.80 (d, J = 1.8 Hz, 1H, H-5), 6.76 (d, J = 1.8 Hz, 1H, H-6), 6.09 (d, J = 2.3 Hz, 1H, H-2), 6.07 (d, J = 2.2 Hz, 1H, H-1), 5.08 (d, J = 11.3, 1H, H-4), 4.82 (brs, 1H, H-8), 4.70 (d, J = 11.3 Hz, 1H, H-3), 3.87 (s, 6H, H-10, 12), and 3.80 (s, 3H, H-11). The optical rotation of the compound was $[\alpha]_D^{23} = +6.6$ (c = 0.12, CH_3OH). The compound was identified as 7,4′,5′-tri-*O*-methylampelopsin (compound **2**, Figure 3) by comparing the spectrum data with published data [23,24].

3.3. Biological Activities of the Two Compounds

The inhibitory activity of the two compounds significantly affected the shoot and root growth of cress ($p \leq 0.05$, Figures 4 and 5). The cress shoots were significantly suppressed at a concentration of 0.3 mM by 7,4′,5′-tri-*O*-methyl dihydroquercetin (58.46%, Figure 4) and 7,4′,5′-tri-*O*-methylampelopsin (38.35%, Figure 5). The roots of cress were significantly suppressed at a concentration of 0.3 mM by 7,4′,5′-tri-*O*-methyl dihydroquercetin (44.40%) and at 0.03 mM by 7,4′,5′-tri-*O*-methylampelopsin (60.88%). The EC_{50} values for 7,4′,5′-tri-*O*-methyl dihydroquercetin against the seedling growth of cress were 0.24 and 0.59 mM for root and shoot, and for 7,4′,5′-tri-*O*-methylampelopsin, were 0.07 and 0.21 mM, respectively. From the EC_{50} values shown in Table 2, the cress roots were more sensitive to both compounds than the shoots.

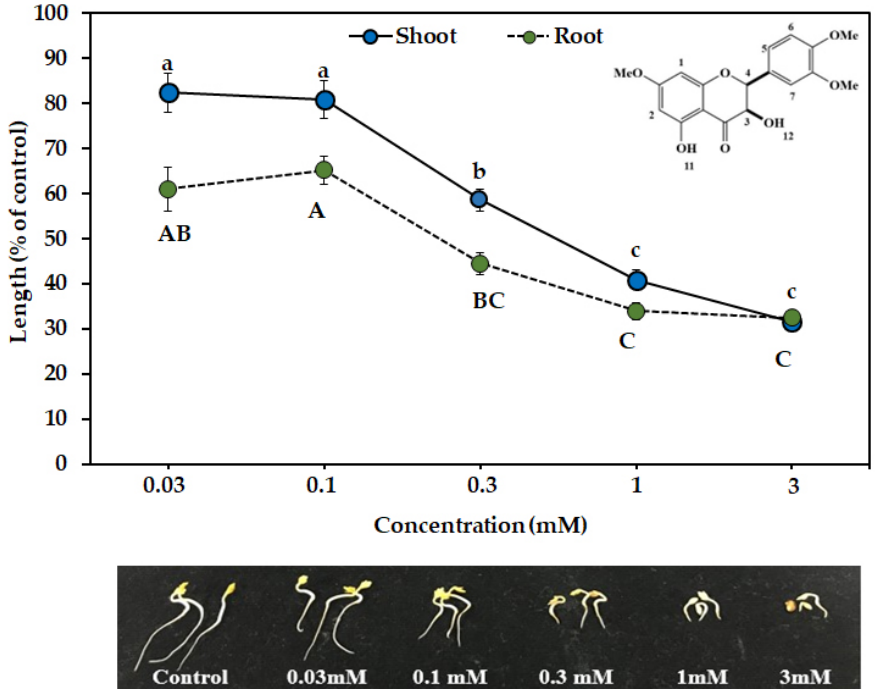

Figure 4. The effect of compound **1** (7,4′,5′-tri-*O*-methyl dihydroquercetin) on the shoot and root growth of the cress seedling. Data are shown as means ± standard errors. Different letters indicate significant differences between treatments for shoots (small letters) and roots (capital letters) (Tukey's HSD, $p \leq 0.05$).

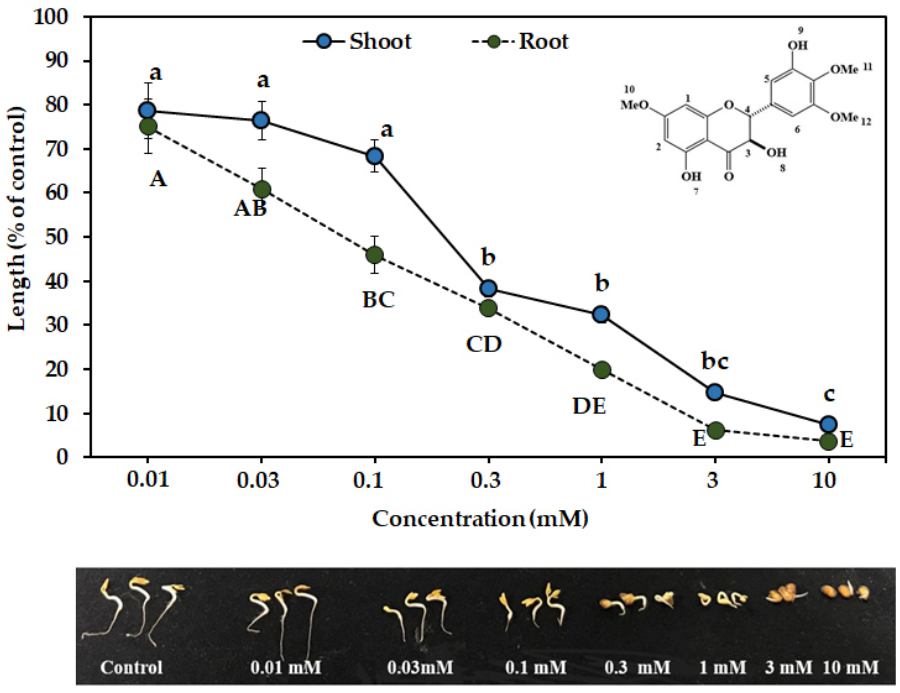

Figure 5. The effect of compound **2** (7,4′,5′-tri-*O*-methylampelopsin) on the shoot and root growth of the cress seedling. Data are shown as means ± standard errors. Different letters indicate significant differences between treatments for shoots (small letters) and roots (capital letters) (Tukey's HSD, $p \leq 0.05$).

Table 2. EC_{50} values of 7,4′,5′-tri-*O*-methyl dihydroquercetin and 7,4′,5′-tri-*O*-methylampelopsin for the growth of cress seedlings.

Compound Name	EC_{50} (mM)	
	Shoot	Root
7,4′,5′-tri-*O*-methyl dihydroquercetin	0.59	0.24
7,4′,5′-tri-*O*-methylampelopsin	0.21	0.07

4. Discussion

The aqueous methanol extracts of *P. rosea* inhibited the growth of all the tested plants in a concentration-dependent manner: the higher the concentration, the greater the suppression (Figure 2). Such a concentration-dependent inhibition of plant extracts has also been reported for *Annona muricana* (L.) [35], *Senna garrettiana* [36], *Leucas cephalotes* [37], *Elaeocarpus floribundus* Blume [38], and *Dregea volubilis* (L.f.) [39]. On the other hand, timothy and barnyard grass showed stimulation effects at some low concentrations. Similar findings of growth stimulation by plant extracts were also reported in previous studies [8,40–42]. The concentration-dependent effects of plant extracts that stimulate cell elongation at low concentrations and inhibit cell elongation at high concentrations are so-called hermetic responses [8]. According to the EC_{50} values (Table 1), the shoots of Italian ryegrass, cress, and lettuce are more sensitive to the *P. rosea* stem extracts than their roots. However, the EC_{50} values of the roots of timothy, barnyard grass, and alfalfa are more sensitive to the *P. rosea* stem extracts than their shoots. Dayan et al. [43] described that different compositions and concentrations of allelopathic properties may be responsible for differences in activity, making the mode of action unique. It could also be caused by changes in the structure of plant cells, cell elongation inhibition, antioxidant system imbalances, breakdown of activities and function of various enzymes, the effects on nutrient absorption in plant roots, or an influence on nucleic acid and protein synthesis [44]. These results suggest that *P. rosea* extracts may contain allelopathic substances.

In this study, two compounds were isolated and characterized as 7,4′,5′-tri-*O*-methyl dihydroquercetin and 7,4′,5′-tri-*O*-methylampelopsin (Figure 3). The two compounds are structural derivatives of flavonoids, which are the largest group of naturally occurring phenols [45]. Flavonoids may be divided into various classes according to the oxidation level of the central ring (ring C). Ampelopsin, also known as dihydromyricetin, is a major secondary metabolite of *Ampelopsin grossedentata*, which belongs to the flavonoid category with reportedly strong antibacterial [46] properties. Dihydroflavonols are found in many plants and are intermediates in the biosynthesis of other flavonoid classes. The most common member of this family is dihydroquercetin (dhq), which occurs in nature as free phenol, as a glycoside, and in the form of free and glycosylated phenol ethers or esters. Many flavonoids of similar structures are known to be effective radical scavengers (antioxidants) [47]. Figures 4 and 5 show that the two compounds inhibited the shoot and root growth of cress. The EC_{50} values in Table 2 show that the inhibitory effect of 7,4′,5′-tri-*O*-methylampelopsin was two to three times greater than 7,4′,5′-tri-*O*-methyl dihydroquercetin. The EC_{50} values of the compounds indicate that 7,4′,5′-tri-*O*-methylampelopsin possesses greater allelopathic activity than 7,4′,5′-tri-*O*-methyl dihydroquercetin. This finding may be due to the 3′-OH group in 7,4′,5′-tri-*O*-methylampelopsin. Some researchers have reported that myricetin (3,5,7,3′,4′,5′-hexa-hydroxyflavone) is a stronger antioxidant than quercetin, which has been attributed to the 5′-OH group that allows further stabilization of the myricetin-derived semi-quinone radical [48,49]. However, the allelopathic activities of 7,4′,5′-tri-*O*-methyl dihydroquercetin and 7,4′,5′-tri-*O*-methylampelopsin had not been reported before. Thus, this study is the first to report on the allelopathic activities of 7,4′,5′-tri-*O*-methyl dihydroquercetin and 7,4′,5′-tri-*O*-methylampelopsin. The present results suggest that the allelopathic effect that *P. rosea* stem and its identified compounds exert could potentially be used as a natural source of herbicide for controlling weeds.

5. Conclusions

The aqueous methanol extracts of stems of *P. rosea* inhibited the growth of three monocot species and three dicot species in a concentration-dependent manner. Two active compounds were isolated from the *P. rosea* extract and identified as 7,4′,5′-tri-*O*-methyl dihydroquercetin and 7,4′,5′-tri-*O*-methylampelopsin. The two compounds significantly inhibited the seedling growth of cress. Of these two compounds, 7,4′,5′-tri-*O*-methylampelopsin exhibited greater allelopathic activity than 7,4′,5′-tri-*O*-methyl dihydroquercetin. The findings of this study showed that the two compounds possess allelopathic potential and may be responsible for the allelopathic activity of *P. rosea*. However, further investigation on the performance of these two compounds is needed against more weed species and in the field.

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