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Assessment of Allelopathic Potential of *Senna garrettiana* Leaves and Identification of Potent Phytotoxic Substances

Ramida Krumsri [1,2], Arihiro Iwasaki [3], Kiyotake Suenaga [3] and Hisashi Kato-Noguchi [1,2,*]

[1] Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Miki 761-0795, Japan; ramidakrumsri@gmail.com
[2] The United Graduate School of Agricultural Sciences, Ehime University, Tarumi, Matsuyama 790-8566, Japan
[3] Department of Chemistry, Faculty of Science and Technology, Keio University, Yokohama 223-8522, Japan; a.iwasaki@chem.keio.ac.jp (A.I.); suenaga@chem.keio.ac.jp (K.S.)
* Correspondence: kato.hisashi@kagawa-u.ac.jp; Tel.: +81-87-891-3086

Abstract: *Senna garrettiana* (Craib) Irwin & Barneby (Fabaceae) is a medicinal plant known to be rich in biologically active compounds that could be exploited to produce bioherbicides. The present study was conducted to explore the allelopathic potential and phytotoxic substances of *S. garrettiana*. Extracts of *S. garrettiana* leaves were found to significantly inhibit the growth of *Lepidium sativum* L. and *Echinochloa crus-galli* (L.) P. Beauv. ($p \leq 0.05$). The phytotoxic substances were isolated and identified as vanillic acid and ferulic acid by bioassay-directed fractionation and spectral data analysis. The two compounds were shown to significantly inhibit the seed germination, seedling growth, and dry biomass of *L. sativum*. Based on the concentration required for 50% growth inhibition (defined as IC_{50}), the roots of *L. sativum* were the most sensitive to the compounds, and the inhibitory effect of ferulic acid (IC_{50} = 0.62 mM) was >1.3 times more potent than that of vanillic acid (IC_{50} = 0.82 mM). In addition, a mixture of the two compounds (0.3 mM) resulted in synergistic inhibitory activity against the *L. sativum* roots compared with the individual compounds. These results suggest that the extracts of *S. garrettiana* leaves and their phytotoxic compounds have potential as candidate natural herbicides.

Keywords: phytotoxic activity; phenolic compounds; bioherbicide; sustainable agriculture

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1. Introduction

Allelopathy refers to plants that directly or indirectly release secondary metabolites (called allelochemicals) into the environment [1–3]. These allelochemicals affect the physiological and biochemical processes of neighboring plants and even their plant species to produce an autotoxic effect [4–6]. Allopathic activity has been reported in a large number of *Senna* species. For example, an extract of the aerial parts of *Senna obtusifolia* (L.) Irwin & Barneby inhibited the seed germination and growth of *Lactuca sativa* L. and *Allium cepa* L. [7]. An extract of *S. occidentalis* reduced the growth of *Handroanthus chrysotrichus* (Mart. Ex DC.) Mattos and *Tabebuia roseo-alba* (Ridl.) Sandwith with increased contents of hydrogen peroxide and malondialdehyde in the radicles and leaves. The phytotoxic compounds contained in *Senna occidentalis* (L.) include alkaloids, coumarins, phenols, saponins, free steroids, and tannins [8]. Some phytochemical features that make the extract a potential natural herbicide include its similar mechanisms of action to synthetic herbicides; total or partial solubility for easy application without surfactants; and chemical structures with high oxygen and nitrogen contents that are environmentally friendly, reduce the environmental half-life, and prevent accumulation in the soil [9]. Therefore, researchers have focused on utilizing allelopathic species and their allelopathic compounds as natural herbicides [10–12]. The crude extracts or plant residues can be directly applied as bioherbicides, while the compounds isolated from the allelopathic plants can be used as templates for developing natural herbicides [13–15]. However, differences in the allelopathic compound composition,

concentration, and allopathic properties of the plant species vary significantly with effects unique to the target plant species [16]. Therefore, screening new allelopathic species and identifying active compounds with a high allelopathic activity could augment current weed control approaches. Thus, members of the genus *Senna* are potentially valuable sources of bioactive compounds for alternative weed management strategies.

The genus *Senna* (Fabaceae family) includes approximately 300 species with a tropical distribution, most of which are widely found in the Americas, Africa, Australia, and Asia [17]. This genus contains annuals, subshrubs, and woody and erect plants [7]. The pharmacologically bioactive compounds in various *Senna* spp. have been investigated. Studies have revealed that *Senna* produces more than 120 structurally diverse phytochemicals [18]. The crude extracts, fractions, and compounds obtained from *Senna* spp. have been shown to possess antimalarial, antidiabetic, antimicrobial, antioxidant, anti-inflammatory, analgesic, antitumor, antinociceptive, and anticancer properties [19]. Notably, several members of this genus also have allelopathic potential and contain potent phytotoxic substances [7,20].

Senna garrettiana (Craib) Irwin & Barneby is a Thai medicinal plant in the Fabaceae family, known locally as "Samae-sarn". This plant is widely distributed in all regions of Thailand and is commonly used as an ornamental and food tree [21]. The trunk height is approximately 10 m and has thick dark brown or black bark. The leaves are elongated and oval with a spear-like tip, and its flowers are yellow or golden in color. The skin of the pods is smooth and completely hairless [22] (Figure 1). The heartwood of *S. garrettiana* has been used traditionally to attenuate muscle pain, nourish the blood, and promote menstrual discharge [23]. An extract of *S. garrettiana* has been shown to have anti-hyaluronidase and anti-elastase activities due to the presence of betulinic acid [24]. Many compounds isolated from *S. garrettiana*, such as cassialoin and chrysophanol-9-anthrone, possess anticancer properties [25]. In addition, the bioactive compounds in this species such as piceatannol (trans-3,3′,4,5′-tetrahydroxystilbene), a derivative of phenolic stilbene, exhibit antioxidant, antipyretic, and anti-inflammatory activities [23,26,27]. Researchers have found that pharmacologically bioactive compounds can interact with multiple targets. For example, rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside), found in the *Senna* species [28], has a number of pharmacological activities [29] and possesses the ability to alter the specific expression pattern of the short-root and HD-zip III transcription factor gene family and cause morpho-physiological alterations in *Sorghum bicolor* (L.) Moench roots [30]. Based on its value as a traditional medicine and its secondary substances, it is possible that *S. garrettiana* has significant allelopathic activity with high phytochemical contents. Moreover, it has been observed that the growth and abundance of plant species under the *S. garrettiana* canopy is scant (Figure 1A). One possible reason for this inhibition or suppression could be the allelopathic potential of this species. Although the biological activity of this species has been extensively studied, the allopathic activity of *S. garrettiana* has not been reported. Consequently, the aims of the current research were (i) to assess the allelopathic potential of *S. garrettiana* leaf extracts against the seedling growth of *L. sativum* and *E. crus-galli*, (ii) to isolate and identify the major phytotoxic compounds, and (iii) to identify the biological activity of the candidate phytotoxic compounds against test plant species.

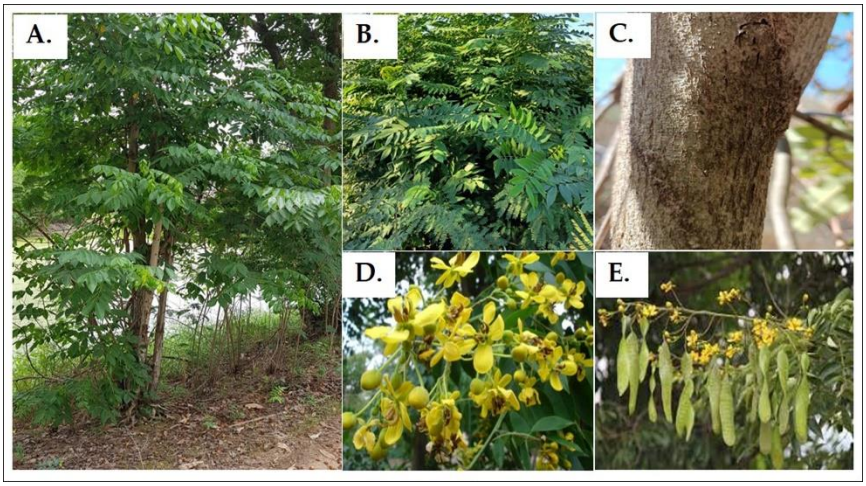

Figure 1. *S. garrettiana* in the natural ecosystem: (**A**) the suppression zone of *S. garrettiana* that reduces the growth of other plant species under the canopy, (**B**) leaves, (**C**) trunks, (**D**) flowers, and (**E**) pods of *S. garrettiana*.

2. Materials and Methods

2.1. Plant Material

Leaves of *S. garrettiana* were obtained from Phitsanulok Province, Thailand (16°49′ N and 100°16′ E). The material was rinsed in tap water to remove impurities and then air-dried in the shade. The material was pulverized, sieved using a 2 mm mesh, and refrigerated at 4 °C until it was extracted. *L. sativum* and *E. crus-galli* were selected as test plant species to evaluate the biological activity of *S. garrettiana* leaf extracts. *E. crus-galli* seeds were used because of their worldwide distribution, whereas *L. sativum* seeds were used because of their well-known growth behavior [31,32].

2.2. Extraction and Bioassay Procedure

Dried leaves of *S. garrettiana* (100 g) were immersed in 500 mL of methanol and distilled water (70:30, *v*/*v*) and kept in the dark for 48 h. The solution was filtered through filter paper (Whatman No. 2, Tokyo, Japan). The residue was re-immersed in an equal volume of methanol for 24 h and the solution was filtered. The two filtrates were mixed and concentrated at 40 °C in a rotary evaporator (Yamato Scientific Co., Ltd., Tokyo, Japan). The dry filtrate was dissolved in methanol and six different concentrations were prepared. Aliquots of the extracts (600 µL) and control (methanol without plant extract) were added to filter paper (Whatman No. 2) in 28 mm Petri dishes. After applying the extracts, the solvent was allowed to dry and then 600 µL of an aqueous solution (0.05% Tween 20 in distilled water) was added. Ten *L. sativum* seeds or ten *E. crus-galli* seedlings (germinated in the dark at 25 °C for 36 h) were arranged on the Petri dishes. The seeds or seedlings were incubated in a growth cabinet in the dark at 25 °C. The shoot and root lengths of the test plant seedlings were measured after 48 h of incubation. The experiments were conducted following a completely randomized design with three replications. The percentage of inhibition was estimated using the following equation: % seedling growth = (the length of the treated seedling/the length of the control seedling) × 100.

2.3. Separation of the Active Fraction of the Aqueous Methanol Extract from the S. garrettiana Leaves

A crude extract of *S. garrettiana* leaves was prepared according to the extraction procedure described in Section 2.2. The crude extract was re-dissolved in distilled water and the pH was adjusted to 7 using 1 M phosphate buffer. The residue solution was partitioned against an equal volume of ethyl acetate (three times) and divided into the ethyl acetate and aqueous fractions. The ethyl acetate fraction was dried and filtered over

anhydrous Na_2SO_4. The solvent from each fraction was dried and re-dissolved in the original solvent. The *L. sativum* and *E. crus-galli* seedlings were used as test plants to assess the biological activity of the *S. garrettiana* leaf extracts at different concentrations. The incubation conditions, experimental design, data collection, and calculations were the same as for the extract bioassay.

2.4. Isolation and Identification of the Phytotoxic Substances in the S. garrettiana Leaves

The ethyl acetate fraction separated from the *S. garrettiana* leaf extracts was chromatographed on a silica gel column, a Sephadex LH-20 column, and a reverse-phase C_{18} SPE cartridge, as described by Krumsri et al. [33], with some modifications. The *L. sativum* bioassay was used to assess the biological activity of all the fractions collected during the isolation steps. The fraction eluted from the silica gel column (silica gel 60, spherical, 70–230 mesh: Nacalai Tesque, Kyoto, Japan) with 60% ethyl acetate in *n*-hexane exhibited the highest inhibitory activity. This fraction was separated using a Sephadex LH-20 column (GE Healthcare Bio-Science AB, Uppsala, Sweden). The fraction eluted with 80% aqueous methanol was active. The active fraction was then subjected to the reverse-phase C_{18} solid phase extraction (SPE) cartridges (YMC Dispo SPE, YMC Ltd., Kyoto, Japan). The active fraction (20% aqueous methanol) was finally purified on high-performance liquid chromatography (HPLC; Shimadzu Corporation, Kyoto, Japan) with a reverse-phase HPLC column (250 × 4.6 mm I.D., 5 µm, Inertsil ® ODS-3; GL Science Inc., Tokyo, Japan). The substances were eluted using an isocratic system of methanol: water (20:80, (*v*/*v*)) at a flow rate of 0.8 mL min^{-1} and detected at 220 nm.

The inhibitory activity was found in peak fractions at retention times of 70–100 min (substance **1**) and 30–50 min (substance **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, CD_3OD).

2.5. Bioassay of the Phytotoxic Substances from the S. garrettiana Leaves

Individual substances were prepared at concentrations of 0.03, 0.1, 0.3, 1, 3, and 10 mM, and a mixture of the two substances was prepared to 0.3 mM (ratio 1:1). Their biological activity *L. sativum* was examined using the above procedure. After a 48 h incubation, the seed germination, shoot length, root length, and dry biomass were measured and calculated in the same way as for the extract bioassay.

2.6. Statistical Analysis

Analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test was used to analyze the effects between treatments by the SPSS version 25.0 software (IBM Corp., Chicago, IL, USA). The significance of the F-values was tested at a p-value ≤ 0.05. The IC_{50} values of the test plant species were determined using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results and Discussion

3.1. Biological Activity of the the Aqueous Methanol Extracts of S. garrettiana Leaf

S. garrettiana leaf extracts had a marked effect on the length of the *L. sativum* and *E. crus-galli* seedlings ($p \leq 0.05$, Table 1) in a dose- and species-dependent manner. The growth of the seedlings of both species was significantly inhibited compared with the control seedlings at extract concentrations ≥3 mg dry weight (DW) equivalent extract mL^{-1}. The extract concentration of 10 mg DW equivalent extract mL^{-1} inhibited the length of the *L. sativum* and *E. crus-galli* shoots by 39.8 and 31.0%, respectively, compared with the control, whereas the root length was inhibited by 36.6 and 39.0%, respectively, compared with the control. Moreover, the results of the ANOVA showed that the extract concentration and test plant species were significant factors in the growth rates of the shoots ($F = 53.6$, $p < 0.001$) and roots ($F = 85.5$, $p < 0.001$).

Table 1. Growth inhibitory effects of the aqueous methanol extracts of *S. garrettiana* leaf against the *L. sativum* and *E. crus-galli* seedlings at different concentrations after 48 h of treatment.

Test Plant Species	Leaf Extract Concentration (mg DW Equivalent Extract mL^{-1})	Shoot Length (mm)	Root Length (mm)
L. sativum	Control	8.47 ± 0.27^{a}	17.50 ± 0.97^{a}
	1	8.12 ± 0.30^{a}	17.27 ± 0.71^{a}
	3	7.24 ± 0.33^{b}	15.87 ± 0.57^{a}
	10	5.10 ± 0.22^{c}	11.10 ± 2.22^{b}
	30	1.08 ± 0.14^{d}	2.22 ± 0.13^{c}
	100	1.25 ± 0.09^{e}	1.07 ± 0.04^{cd}
	300	0.00 ± 0.00^{f}	0.00 ± 0.00^{d}
	F	3.05	24.58
	p-value	<0.001	<0.001
E. crus-galli	Control	19.73 ± 1.02^{a}	15.43 ± 0.99^{a}
	1	18.60 ± 0.52^{a}	15.33 ± 0.46^{a}
	3	15.55 ± 0.66^{b}	12.13 ± 0.55^{b}
	10	13.61 ± 0.58^{b}	9.27 ± 0.57^{c}
	30	10.35 ± 0.69^{c}	4.48 ± 0.52^{d}
	100	3.95 ± 0.18^{d}	1.35 ± 0.12^{e}
	300	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
	F	154.86	1.79
	p-value	<0.001	<0.001
Interaction species × treatment	F	53.85	85.52
	p-value	<0.001	<0.001

Experimental data are the mean (±S.D.) of three replicates (n = 30); different superscript letters in each row-column indicate a statistically significant difference between treatments (Tukey's HSD, at the 0.05 probability level).

Such different degrees of inhibition of the test plants may be related to the bioactive compounds exhibiting increased inhibitory effects on physiological processes as the concentration increased. Our results are consistent with previous research, which reported that *Senna* species have inhibitory activity, such as extracts of *S. obtusifolia* and *S. occidentalis* causing the inhibition of the seed germination and growth of plant species [7,8]. Moreover, the unequal susceptibility of the tested species to the extracts could be due to inherent differences in the biochemicals involved in the process [34]. The species specificity of phytotoxic substances has also been demonstrated for other allelopathic plant species [35–37]. In general, plant extracts comprise a mixture of various bioactive and inactive compounds with different polarities [38]. Therefore, further studies were carried out on leaf extracts to separate the active fractions using different organic solvents.

3.2. Separation of the Active Fraction in the S. garrettiana Leaf Extracts

S. garrettiana leaf extracts were partitioned with different liquid organic solvents. The partitioning results revealed that the aqueous and ethyl acetate fractions had significant inhibitory effects on the *L. sativum* and *E. crus-galli* seedlings compared with the control ($p \leq 0.05$, Figure 2A,B). The IC_{50} values of the test plants showed that the inhibition by the ethyl acetate fraction was greater than that by the aqueous fraction, and *L. sativum* was the most sensitive to both fractions (Table 2).

These results showed that the phytotoxic substances from the *S. garrettiana* leaf extracts had the highest inhibitory activity in the ethyl acetate fraction. Several studies have reported the ability of the growth inhibitory activity of ethyl acetate fractions. Silva et al. [39] showed that the ethyl acetate fraction of *Hydrocotyle bonariensis* Lam. inhibits the growth of *L. sativum*, *Solanum lycopersicum* L., *Allium cepa* L., and *Triticum aestivum* L. seedlings and has high levels of phenolic compounds. Pereira et al. [40] found that the ethyl acetate fraction of *Pancratium maritimum* L. inhibits the tested plants due to the presence of tannins, flavanone, steroids, and saponins. Previous research indicates that active fractions from allelopathic plant extracts contain bioactive compounds. Moreover, Blum [41] and

Mirmostafaee et al. [42] reported that two or more compounds usually act together and have synergistic or antagonistic effects on plant growth, depending on the combination used. Consequently, it is crucial to determine which compounds play a significant role in phytotoxicity and hence have potential use in weed control. The potent phytotoxic substances in the *S. garrettiana* leaf extracts were further isolated using the bioassay-guided fractionation method.

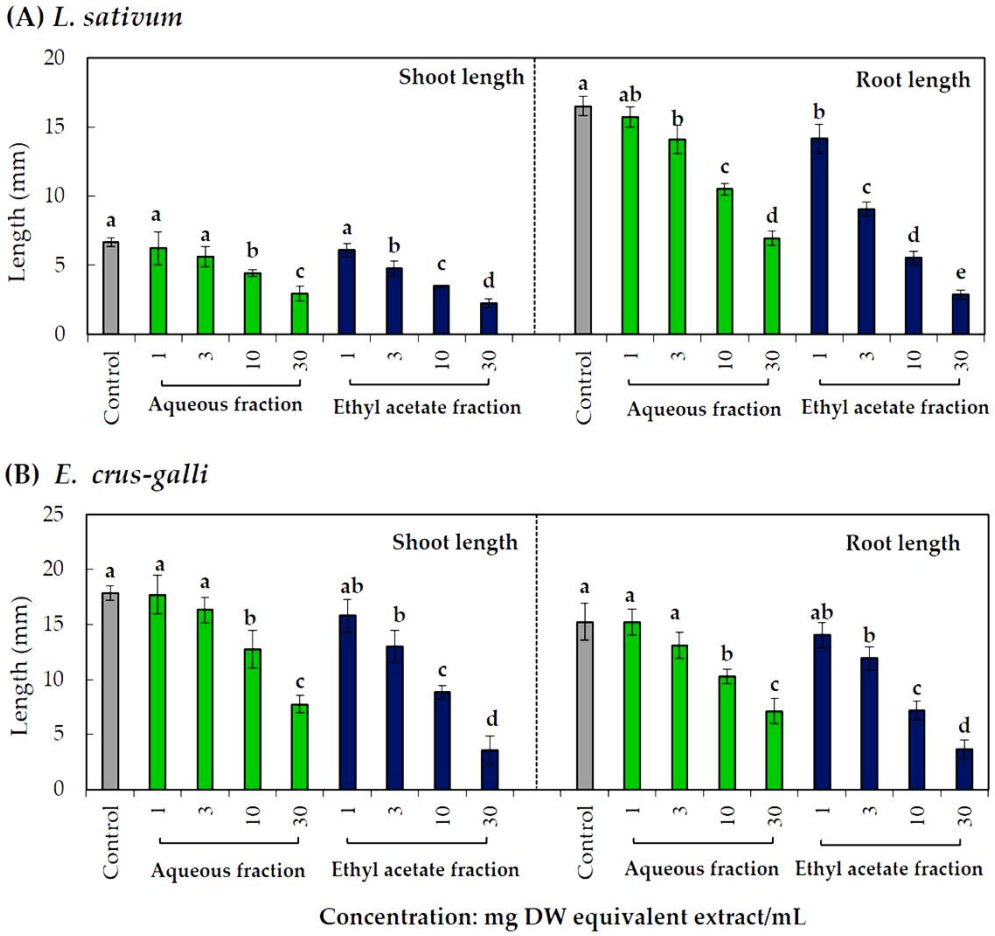

Figure 2. Growth inhibitory effect of the aqueous and ethyl acetate fractions separated from the *S. garrettiana* leaf extracts against the growth of (**A**) *L. sativum* seedlings and (**B**) *E. crus-galli* seedlings. Values are the mean (±S.D.) of three replicates (n = 30); different letters on each bar indicate a statistically significant difference between treatments (Tukey's HSD, at the 0.05 probability level).

Table 2. The concentration required for 50% growth inhibition (IC_{50}) of the shoot and root growth of test plant species by the aqueous and ethyl acetate fractions separated from *S. garrettiana* leaf extracts.

Fraction	IC_{50} Value (mg DW Equivalent Extract mL^{-1})			
	L. sativum		*E. crus-galli*	
	Shoot	Root	Shoot	Root
Aqueous	21.93	18.12	26.23	22.40
Ethyl acetate	16.13	13.32	19.87	18.02

3.3. Isolation and Identification of the Phytotoxic Substances in the S. garrettiana Leaf Extracts

The ethyl acetate fraction from the *S. garrettiana* leaf extracts was fractioned through silica gel, Sephadex LH-20, and reverse-phase C_{18} SPE cartridges. Reverse-phase HPLC was used to purify the active substances and two active substances were detected.

Active substance **1** has the molecular formula $C_8H_8O_4$, as established using APCI-MS at m/z 167.0339 $[M + H]^-$ (calcd. For $C_8H_7O_4$, 167.0344). The spectrum data of ^1H NMR (400 MHz, CD_3OD) presented δ_H: 7.56 (d, J = 1.3 Hz, 1 H, H-3), 7.55 (dd, J = 8.5 Hz, 1 H, H-7), 6.84 (d, J = 8.5 Hz 1 H, H-6), 3.89 (s, 3 H, H-8). These spectroscopic data were compared with previous literature and the substance was identified as 4-hydroxy-3-methoxybenzoic acid [43], which is known as vanillic acid (Figure 3A).

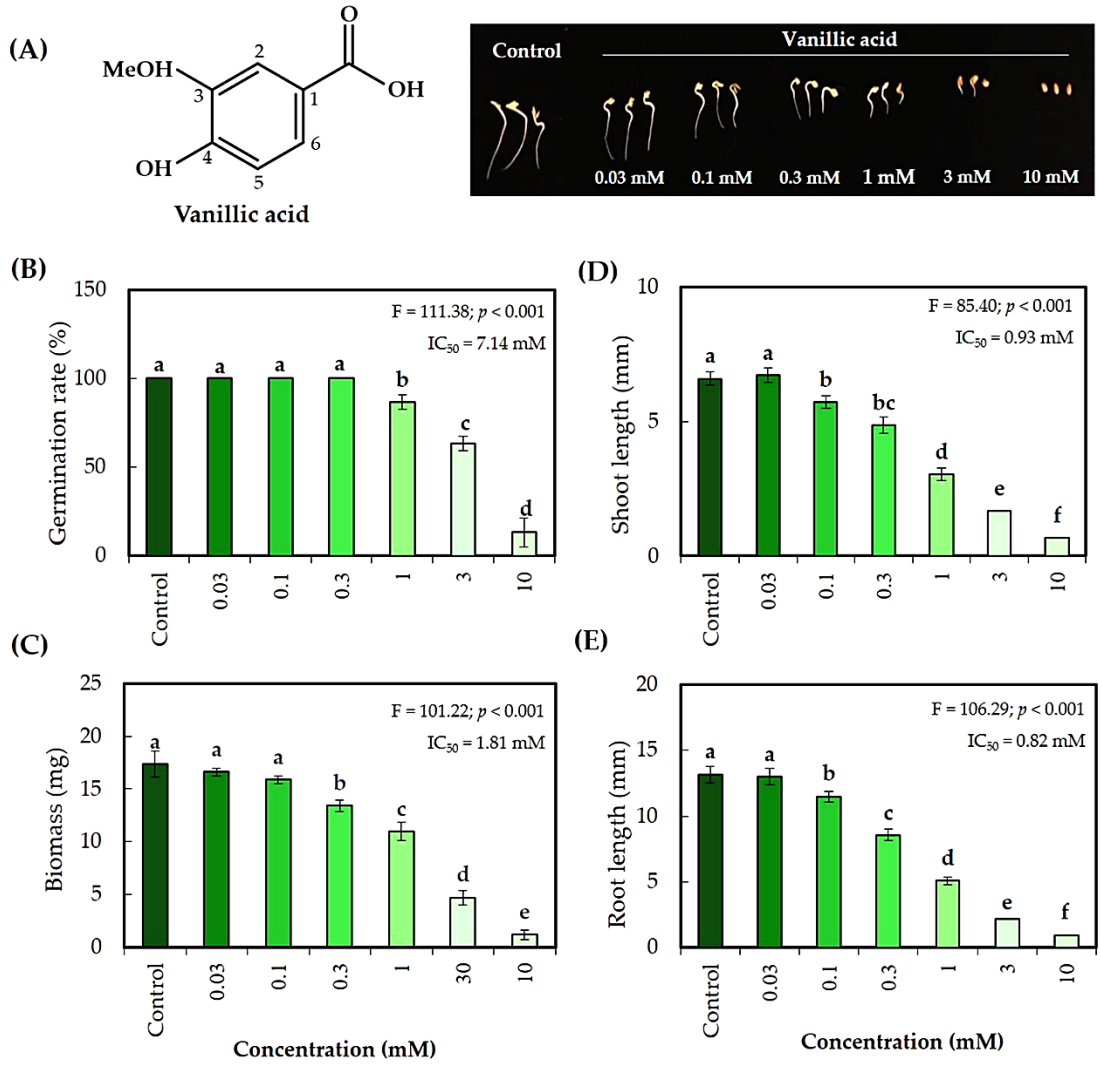

Figure 3. Growth inhibitory effects on *Lepidium sativum* exposed to vanillic acid isolated from the *S. garrettiana* leaf extracts. (**A**) *L. sativum* seedlings after 48 h of treatment, (**B**) seed germination, (**C**) dry biomass, (**D**) shoot length, and (**E**) root length. Values are the mean (±S.D.) of three replicates (n = 30); different letters on each bar indicate a statistically significant difference between treatments (Tukey's HSD, at the 0.05 probability level).

Active substance **2** has the molecular formula $C_{10}H_{10}O_4$, as established using ESI-MS at m/z 195.0660 $[M + H]^+$ (calcd. for $C_{10}H_{11}O_4$, 195.0657). The spectrum data of ^1H NMR (400 MHz, CD_3OD) presented δ_H: 7.59 (d, J = 16.1 Hz, 1 H, H-3), 7.18 (d, J = 1.8 Hz, 1 H, H-9), 7.07 (dd, J = 8.2, 1.8 Hz, 1 H, H-5), 6.81 (d, J = 8.2 Hz, 1 H, H-6), 6.32 (d, J = 16.1 Hz, 1 H, H-2), 3.90 (s, 3 H, H-10). Comparison of these spectral analysis data with published

data identified the substance as 3-methoxy-4-hydroxycinnamic acid [43]. This compound is commonly known as ferulic acid (Figure 4A).

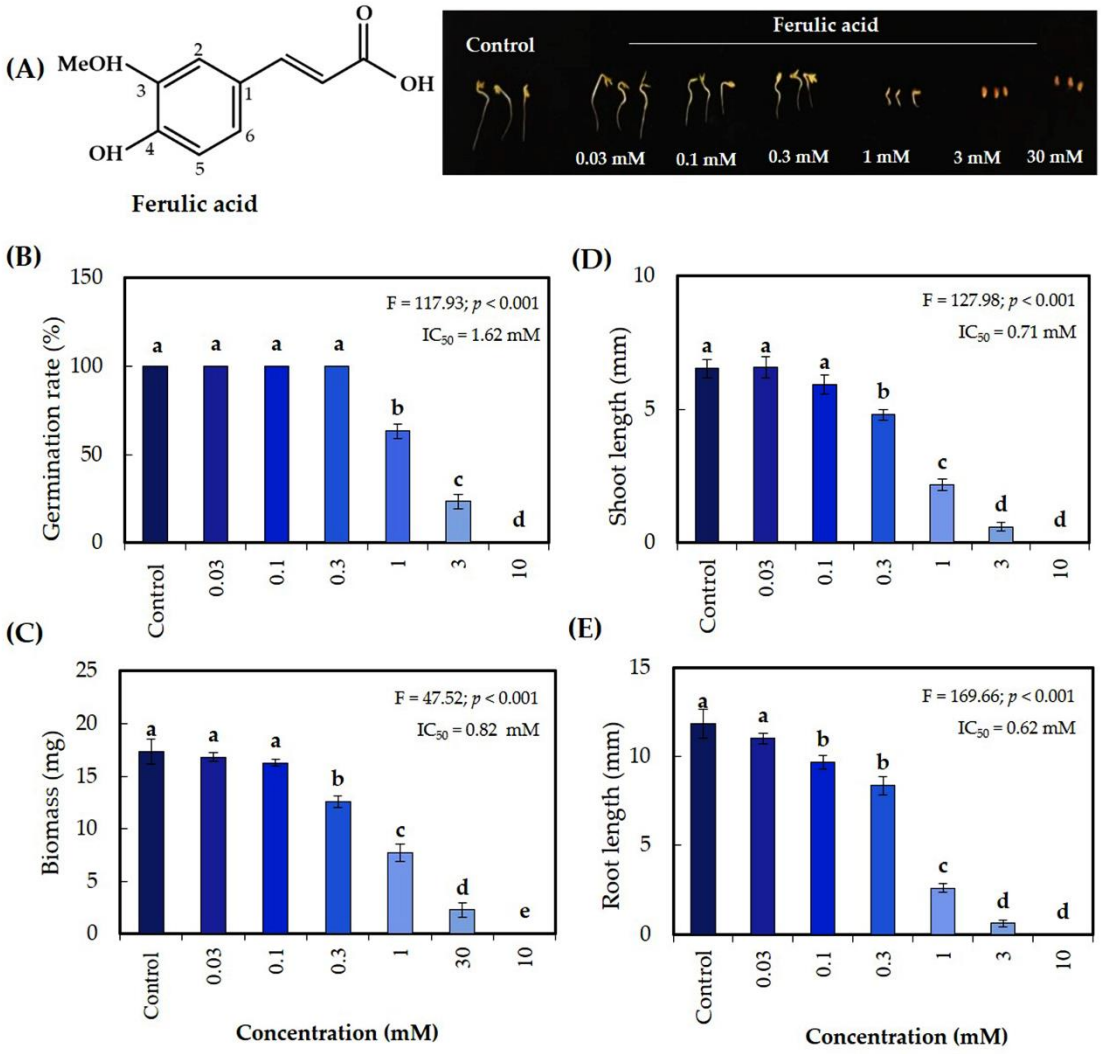

Figure 4. Growth inhibitory effects on *Lepidium sativum* exposed to ferulic acid isolated from the *S. garrettiana* leaf extracts. (**A**) *L. sativum* seedlings after 48 h of treatment, (**B**) seed germination, (**C**) dry biomass, (**D**) shoot length, and (**E**) root length. Values are the mean (±S.D.) of three replicates (n = 30); different letters on each bar indicate a statistically significant difference between treatments (Tukey's HSD, at the 0.05 probability level).

Vanillic acid and ferulic acid are phenolic compounds. Vanillic acid is a benzoic acid derivative [44], while ferulic acid is a cinnamic acid derivative [45]. Both compounds have been detected widely in plant species such as *Saccharum officinarum* L. [46], *Lantana camara* L. [47], and *Triticum aestivum* L. [48]. They are considered potential anti-inflammatory compounds [49,50], with microbial activity [51] and antioxidant activities [52,53]. Our findings suggest that the leaves of *S. garrettiana* are a source of vanillic acid and ferulic acid, which have phytotoxic effects on plant growth.

3.4. Biological Activity of the Compounds Isolated from the S. garrettiana Leaf Extract against L. sativum

The phytotoxic activity of vanillic acid and ferulic acid was determined against *L. sativum*. The inhibitory potential of the two compounds significantly affected the seed germination, seedling growth, and dry biomass of *L. sativum* ($p \leq 0.05$, Figures 3 and 4).

The degree of inhibition of the *L. sativum* growth parameters by both compounds increased with increased concentration. The seed germination, shoot length, root length, and dry biomass of *L. sativum* were inhibited by 23.4, 53.6, 57.0, and 37.0%, respectively, in response to 1 mM of vanillic acid. Based on the IC_{50} values, the order of inhibition efficiency was root length (0.82 mM) > shoot length (0.93 mM) > dry biomass (1.81 mM) > germination (7.14 mM) (Figure 2). In contrast, 1 mM of ferulic acid inhibited the seed germination, shoot length, root length, and dry biomass by 26.7, 76.7, 78.2, and 44.3%, respectively. The IC_{50} values showed that the degree of inhibition of the growth parameters was root length (0.62 mM) > shoot length (0.71 mM) > dry biomass (0.82 mM) > germination (1.62 mM) (Figure 3). Furthermore, a 0.3 mM mixture of vanillic acid and ferulic acid had synergistic effects against *L. sativum* (Figure 5). The inhibitory effect on the *L. sativum* roots by the mixture of the compounds was 1.15 and 1.41 times more than vanillic acid and ferulic acid alone, respectively. No significant inhibitory effect was observed on the seed germination or shoot length.

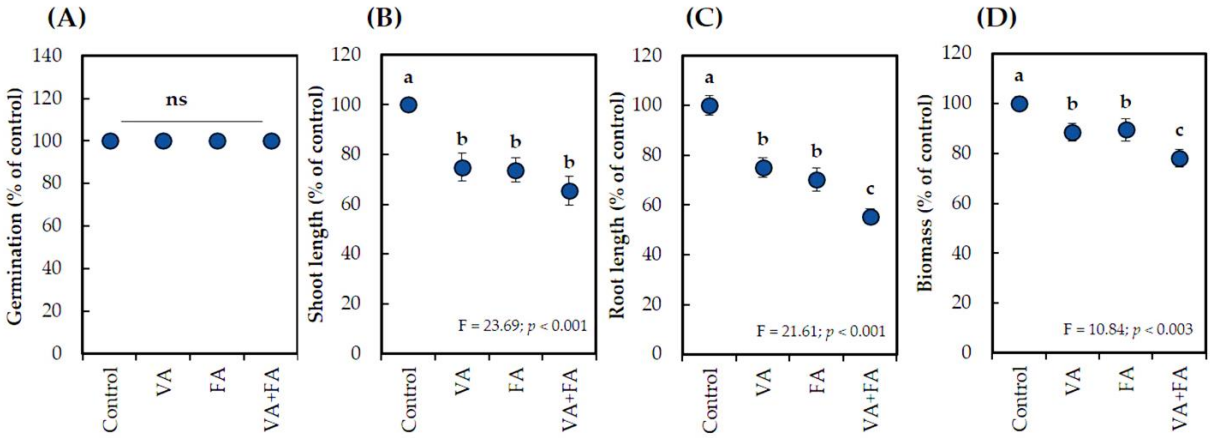

Figure 5. Growth inhibitory effects of a mixture of vanillic acid (VA) and ferulic acid (FA) at a concentration of 0.3 mM on (**A**) seed germination, (**B**) shoot length, (**C**) root length, and (**D**) dry biomass of *L. sativum.* Values are the mean (±S.D.) of three replicates (n = 30); different letters on each bar indicate a statistically significant difference between treatments (Tukey's HSD, at the 0.05 probability level).

Plant seed germination and seedling growth (shoot length, root length, and plant biomass) are critical steps in the growth and development of plant species, showing the significance of high allelopathic activity [54,55]. In this study, vanillic acid and ferulic acid appeared to alter seed germination and seedling growth and significantly reduced the dry biomass of *L. sativum*. These results support early studies reporting that both compounds possess potent phytotoxicity [56,57]. Moreover, our results showed that the percentage of *L. sativum* seed germination was the least affected parameter and that the root length of *L. sativum* was the most inhibited by both compounds. Therefore, the results indicated that these compounds had higher potential for inhibiting seedling growth than seed germination in *L. sativum*. The greater sensitivity of root growth to compounds is because radicles have more permeable tissue than other organs [58]. Thus, roots are less protected by the cuticle than shoots, leading to a higher accumulation of phytotoxic compounds in root tissues. Both compounds possess phytotoxic properties that cause excessive reactive oxygen species (ROS) and oxidative stress in vitro [59,60], which may be one reason for their growth inhibitory activity. The accumulation of ROS and changes in the antioxidant system of plants lead to membrane lipid peroxidation and the impaired structure and function of the entire cell membrane, thereby inhibiting plant growth [61–63]. Talukdar [64] also reported that an aqueous extract of *Lantana camara* L. inhibits the root development and growth of *Lathyrus sativus* L. due to increased lipid peroxidation and membrane damage. Although both compounds in this study initially appeared to have

similar activities, the inhibition levels by ferulic acid were higher than those by vanillic acid for all growth parameters, particularly root growth. These findings suggest that both compounds may have a more active analog via the same mechanism or that these structurally related molecules have different modes of action. Both compounds have the same structure, except for the functional group at the C-1 position (Figures 3A and 4A). Different compositions and concentrations of phenolic compounds may be responsible for differences in activity, making the mode of action unique [16]. Therefore, we inferred that the functional group at the C-1 position may be key to the phytotoxic activity of phenolic acids. However, we have not studied the phytotoxic effects of either compound on *L. sativum* seedlings at the molecular level; thus, the effects on cell division and elongation, as well as on growth regulation, must be determined in future studies.

Moreover, the mixture of vanillic acid and ferulic acid (0.3 mM) had greater inhibitory effects than the individual compounds (Figure 5), suggesting synergistic activity that strongly reduced the root growth of *L. sativum*. This finding agrees with the results of Rial et al. [65] and Tena et al. [66], who found a synergistic effect of allelopathic activity in a mixture of phytotoxic compounds. A higher inhibitory effect of a mixture of compounds compared with the individual compounds suggests that allelopathy is influenced by the type of compound combination used, concentration interactions, and the sensitivity of receptor plant species [67,68]. Therefore, the effects of a mixture of these compounds on different indicator plants should be investigated to examine whether they are potential new herbicides.

4. Conclusions

Aqueous methanol extracts of *S. garrettiana* leaf exhibited a significant growth inhibitory effect on the test plants at extract concentrations $\geq$ 3 mg DW equivalent extract mL^{-1} and increasing the extract concentration increased the inhibition. These results indicate that extracts of *S. garrettiana* leaves might contain potent phytotoxic substances. The bioassay-directed fractionation of the *S. garrettiana* leaf extracts resulted in the isolation of vanillic acid and ferulic acid. Both compounds tended to have similar effects on the growth of *L. sativum*, particularly on the root growth. It was also observed that the level of inhibition by ferulic acid against all the growth parameters was greater than that by vanillic acid. In addition, a low concentration of a mixture of vanillic acid and ferulic acid had a synergistic inhibitory effect on the *L. sativum* roots. Our results indicate that the phytotoxic compounds in the *S. garrettiana* leaf extracts are potential candidates for developing natural herbicides. Future studies should determine the combined effects of these two compounds on their mode of action at the molecular level in target plants.

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