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Allelopathic Activity of a Novel Compound, 5,6-Dihydrogen-11α-*O*-acetyl-12β-*O*-tigloyl-17β-marsdenin, and a Known Steroidal Glycoside from the Leaves of *Marsdenia tenacissima* (Roxb.) Moon

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Abstract: Medicinal plants are rich sources of bioactive substances that can be used to develop environmentally friendly weed control alternatives. *Marsdenia tenacissima* (Roxb.) Moon is a traditional medicinal plant well known for its pharmacological activities and several bioactive compounds. However, its allelopathy and related substances have not been reported. Hence, the present study was conducted to explore the allelopathic potential and substances from *M. tenacissima* leaves. Aqueous methanol extracts of *M. tenacissima* showed significant inhibitory activities against the growth of cress (*Lepidium sativum* L.) and Italian ryegrass (*Lolium multiflorum* Lam.). The extracts were purified through various chromatography steps, and two allelopathic substances were isolated and determined by spectral data to be steroidal glycoside 1 (5,6-dihydrogen-11α-*O*-acetyl-12β-*O*-tigloyl-17β-marsdenin), a novel compound, and steroidal glycoside 2 (5,6-dihydrogen-11α,12β-di-*O*-tigloyl-17β-marsdenin). Both compounds significantly inhibited the growth of cress seedlings. Steroidal glycoside 1 showed 1.6- and 4-times greater growth inhibitory potential against the cress shoots and roots than steroidal glycoside 2. The concentrations needed for 50% growth inhibition of the cress seedling shoots and roots were 0.46 and 0.03 mM for steroidal glycoside 1, respectively, and 0.74 and 0.12 mM for steroidal glycoside 2, respectively. Therefore, these results suggest that steroidal glycosides 1 and 2 may be responsible for the allelopathy of the *M. tenacissima* leaves.

Keywords: *Marsdenia tenacissima*; allelopathic substance; medicinal plant; steroidal glycoside

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1. Introduction

Weeds compete with cultivated crops for natural resources, resulting in crop yield losses ranging from 45 to 95% [1,2]. Herbicides are powerful tools for controlling weeds. However, improper and long-term use of herbicides not only negatively affects human and environmental health but also cause an increase in herbicide-resistant weeds [3,4]. To avoid these effects, investigating allelochemicals from potent allelopathic plants has become crucial in the search for weed control alternatives to synthetic herbicides [5]. Bioactive secondary metabolites with allelopathic effects on other plants are termed allelochemicals, which can be found in different parts of plants: roots, leaves, stems, bark, fruits, flowers, and seeds [6]. These allelochemicals obtained from plants belong to diverse chemical groups like alcohols, fatty acids, phenolics, flavonoids, terpenoids, and steroids [7,8]. When released into the environment, allelochemicals can inhibit the germination, growth, and development of adjacent plants by disrupting physiological mechanisms including photosynthesis, respiration, membrane permeability, cell extension, cell division, and water and nutrient uptake [8,9]. In addition, allelochemical-based bioherbicides are less

contaminative to soil and water, as well as less harmful to bio-ecosystems and human health, because they do not remain active in the environment for long periods of time [8]. Thus, allelochemicals obtained from plants are a promising source of environmentally safe bioherbicides for effective weed control [8,10]. Many researchers have reported on the allelopathic potential and substances of various medicinal plants. For example, Kato-Noguchi et al. [11] discovered that two novel compounds, nimbolide B and nimbic acid B, from the leaves of *Azadirachta indica* have strong allelopathic effects on the growth of cress (*Lepidium sativum* L.) and barnyard grass (*Echinochloa crus-galli* (L.) P.Beauv.); Kyaw et al. [12] showed the phytotoxic potential of *Dregea volubilis* leaf extracts and the phytotoxic activity of its two compounds, dehydrovomifoliol and loliolide, on cress (*Lepidium sativum* L.) and Italian ryegrass (*Lolium multiflorum* Lam.); Krumsri et al. [13] reported on the allelopathic activity of two phenolic compounds, vanillic acid and ferulic acid, which were separated from *Senna garrettiana* leaves, on cress (*Lepidium sativum* L.). Based on these data, it is possible that medicinal plants may contain bioactive substances that could exert allelopathic activity.

The Apocynaceae family consists of a diverse variety of medicinal plants [14]. Its sub-family Asclepiadaceae comprises 250 genera and 2000 species of herbs and shrubs. Notably, many researchers have reported that several plant species in this family possess various phytochemicals, pharmacological activities [15–18], and potential allelopathic substances [12,19–22]. However, the allelopathic activity of many species in this family remains to be exploited.

Marsdenia tenacissima (Roxb.) Moon (family, Asclepiadaceae) is a twining shrub distributed in the tropical and subtropical parts of China, India, Myanmar, Bangladesh, Cambodia, Laos, Nepal, Sri Lanka, Thailand, Vietnam, and Indonesia [23]. This species is found in dry and moist deciduous forests, having an annual rainfall of between 1000 and 1500 mm, and below an altitude of 2000 m [24]. Leaves are opposite, 7–15 cm long and 7–10 cm wide, with a circle of heart or broadly ovate form, shortly acuminate, softly hairy on both sides, and petioles are 5–9 cm long [25]. Inflorescences are greenish yellow, numerous, and large, arranged in many branched, axillary cymes, with pedicels long and hairy (Figure 1). The roots are cylindrical and yellow to buff [26].

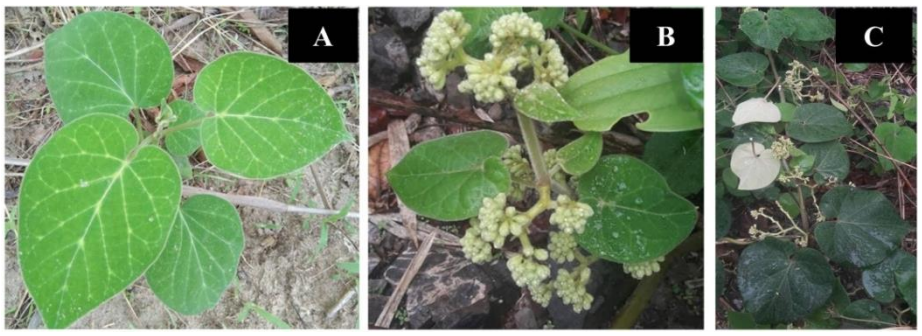

Figure 1. (**A**) Leaf, (**B**) inflorescence, and (**C**) aerial part of *Marsdenia tenacissima*.

Marsdenia tenacissima has been widely used as a traditional household remedy in Myanmar, India, Laos, and China [26,27]. This plant possesses many pharmacological activities such as anti-cancer, anti-tumor, anti-inflammatory, anti-diarrheal, and immunomodulatory [15,28]. Different parts of the *M. tenacissima* plant, especially the roots, have been used in folk medicine to treat various ailments like pneumonia, cancer, fever, cough, vomiting, tumors, diabetes mellitus, heart disease, postpartum milk impassability in women, and gonorrhea [25,29]. The leaf extracts contain benzoic acid, gallic acid, pyrogallol, salicylic acid, trans-cinnamic acid, and vanillic acid [30]. Around 196 phytochemicals, including 155 steroids, triterpenes, phenolic compounds, and organic acids, have been found in different parts of *M. tenacissima* [24]. Many researchers have extensively studied the phytochemical and pharmacological activities of this plant. However, to date, there is no information on the allelopathic activity and allelopathic substances of this plant. Therefore,

the objective of this research was to determine the allelopathic activity and to identify the allelopathic substances from leaf extracts of *M. tenacissima*.

2. Materials and Methods

2.1. Plant Material

Fresh leaves of *M. tenacissima* were collected from Khin-U Township, Shwe Bo district, Sagaing Division Region, Myanmar (22°49′4″ N and 95°48′12″ E) during July–August 2020. The collected leaves were dehydrated in the shade, chopped into small pieces, and ground to a fine powder using an electric grinder. The leaf powder was then kept in plastic bags in a refrigerator at 2 °C until extraction. Two plant species, cress (*Lepidium sativum* L.) and Italian ryegrass (*Lolium multiflorum* Lam.), were chosen as test plants for growth bioassays.

2.2. Extraction and Growth Bioassay

Leaf powder (500 g) was extracted with 70% (*v/v*) aqueous methanol (3 L) for 48 h in the dark. The extract was filtered through a single layer of filter paper (No. 2, 125 mm; Toyo Ltd., Tokyo, Japan). After filtration, the extract residue was re-extracted with methanol (3 L) for 24 h and filtered. The two filtrates were then mixed and evaporated to obtain crude extracts under reduced pressure at 40 °C. The crude extracts were dissolved in methanol, and six different bioassay concentrations (1, 3, 10, 30, 100, and 300 mg dry weight (DW) equivalent extract/mL) were prepared. An aliquot of the extract (600 µL) and control (only methanol) were added to sheets of filter paper (No. 2; Toyo Ltd.) in 28 mm Petri dishes. The methanol in the Petri dishes was then evaporated in a draft chamber. The dried filter papers were then moistened with 0.6 mL of a 0.05% aqueous solution of polyoxyethylene sorbitan monolaurate (Tween 20; Nacalai Tesque, Inc., Kyoto, Japan). Subsequently, ten seeds of cress and ten sprouted seeds of Italian ryegrass (germinated in darkness at 25 °C for 36 h) were incubated at 25 °C for 48 h. After incubation, seedling length was measured and compared with the length of the control seedlings to calculate the percentage of seedling growth. A bioassay experiment was conducted a completely randomized design (CRD) with three replications (10 seedlings/replication, $n = 30$), and repeated two times ($n = 60$).

2.3. Purification of the Active Substances

Leaf powder of *M. tenacissima* (3.5 kg) was extracted and filtered as described above. A cress bioassay was used to assess the biological activity of the separated fractions in each purification step, as described above. The aqueous methanol extracts were evaporated using a rotary evaporator at 40 °C to obtain an aqueous residue. The aqueous residue was then adjusted to pH 7.0 using 1 M phosphate buffer and partitioned six times with an equal volume (150 mL/time) of ethyl acetate. The ethyl acetate fraction was subjected to a column of silica gel (60 g, silica gel 60, 70–230 mesh; Nacalai Tesque) and eluted with 20, 30, 40, 50, 60, 70, and 80% ethyl acetate in *n*-hexane (*v/v*; 150 mL per step), ethyl acetate (150 mL), and methanol (300 mL). Inhibitory activity was detected in fraction 8 (F_8), which was eluted with ethyl acetate (150 mL). After evaporation of this fraction, the residue was further purified using a column of Sephadex LH-20 (100 g; GE Healthcare, Uppsala, Sweden) and eluted with 20, 40, 60, and 80% aqueous methanol (*v/v*; 150 mL per step) and methanol (300 mL). The most active fraction F_2, which was eluted with 40% aqueous methanol, was then evaporated to obtain a crude residue. The residue was dissolved in 20% (*v/v*) aqueous methanol and loaded onto a reverse-phase C_{18} cartridge (1.2 × 6.5 cm; YMC, Kyoto, Japan) and eluted with 20, 30, 40, 50, 60, 70, 80, and 90% (*v/v*) aqueous methanol (15 mL per step) and methanol (30 mL per step). The active fraction was then obtained by elution with 70% (*v/v*) aqueous methanol (F_6) and evaporated using a rotary evaporator to obtain a crude residue. After that, the active substances in this fraction were purified by using reverse-phase HPLC, (500 × 10 mm I.D. ODS AQ-325; YMC Ltd.) at a flow rate of 1.5 mL/min with 70% aqueous methanol and detected at 220 nm wavelength and 40 °C oven temperature.

Biological activity was found in the peak fractions eluted at the retention times of 159–165 and 270–282 min, yielding two active compounds. These two active peak fractions were purified again using reverse-phase HPLC (4.6 × 250 mm I.D., S-5 μm, Inertsil® ODS-3; GL Science Inc., Tokyo, Japan) at a flow rate of 0.8 mL/min with 70 and 65% aqueous methanol. Inhibitory activity of the active peaks was detected at retention times of 85–92 min for compound **1** and 118–125 min for compound **2**. The chemical structures of compounds **1** and **2** were then characterized by ESIMS, ^{1}H-NMR spectrum (400 MHz, CD_3OD), HMBC, NOESY, ^{13}C-NMR spectra (100 MHz, CDC_{l3}), and optical rotation. ^{1}H NMR chemical shifts were assigned using a combination of data from COSY and HMQC experiments. Similarly, ^{13}C-NMR chemical shifts were assigned based on HMBC and HMQC experiments. HR-ESIMS spectra were obtained on an LCT Premier XE time-of-flight (TOF) mass spectrometer. A JASCO DIP-1000 polarimeter was used to quantify optical rotations. UV spectra were taken on a JASCO V730-BIO Spectrophotometer. A Bruker ALPHA instrument was used to record IR spectra. All NMR spectral data were recorded on JEOL JNM-ECX400 and JNM-ECS400 spectrometers for ^{1}H (400 MHz) and ^{13}C (100 MHz).

2.4. Biological Activity of Compounds **1** *and* **2**

Compounds **1** and **2** were dissolved in 2 mL methanol. The assay concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, and 6 mM were then prepared and added to sheets of filter paper (No. 2, 28 mm; Toyo) in 28 mm Petri dishes. These Petri dishes were placed in a draft chamber to evaporate the methanol. After drying, the filter papers were moistened with 0.6 mL Tween 20. To examine the biological activities of compounds **1** and **2**, the assay experiment was conducted in a CRD design with three replications (10 seedlings/replication, $n = 30$), as described above. Then, 10 seeds of cress were placed on the filter papers in the Petri dishes and incubated under dark conditions at 25 °C for 48 h. The shoot and root length of cress were measured to calculate the percentage of seedling growth.

2.5. Statistical Analysis

The bioassay experiments were performed using a completely randomized block design (CRD) with three replicates and repeated two times. The results are presented as mean ± standard error (SE). ANOVA of all data was carried out using SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA), and significant differences between the control and sample treatments were determined using Tukey's test at a significance level of 0.05. The *M. tenacissima* extracts and the related compound concentrations required for 50% growth inhibition (I_{50}) of the shoots and roots of cress and Italian ryegrass were calculated using Graph Pad Prism® Ver. 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Plant Growth Inhibitory Activity of the Marsdenia tenacissima Leaf Extracts

The leaf extracts of *M. tenacissima* suppressed the seedling growth of cress and Italian ryegrass at concentrations greater than 3 to 10 mg DW equivalent extract/mL ($p \leq 0.05$) (Figure 2). The leaf extracts obtained from 10 mg of *M. tenacissima* suppressed the shoot growth of cress completely and Italian ryegrass by 31.99% compared with control, whereas the root growth of cress and Italian ryegrass was inhibited by 8.2 and 11.8%, respectively, compared with control. Moreover, the extract concentration of 30 mg DW equivalent extract/mL completely inhibited the shoot and root growth of the two tested plants compared with control.

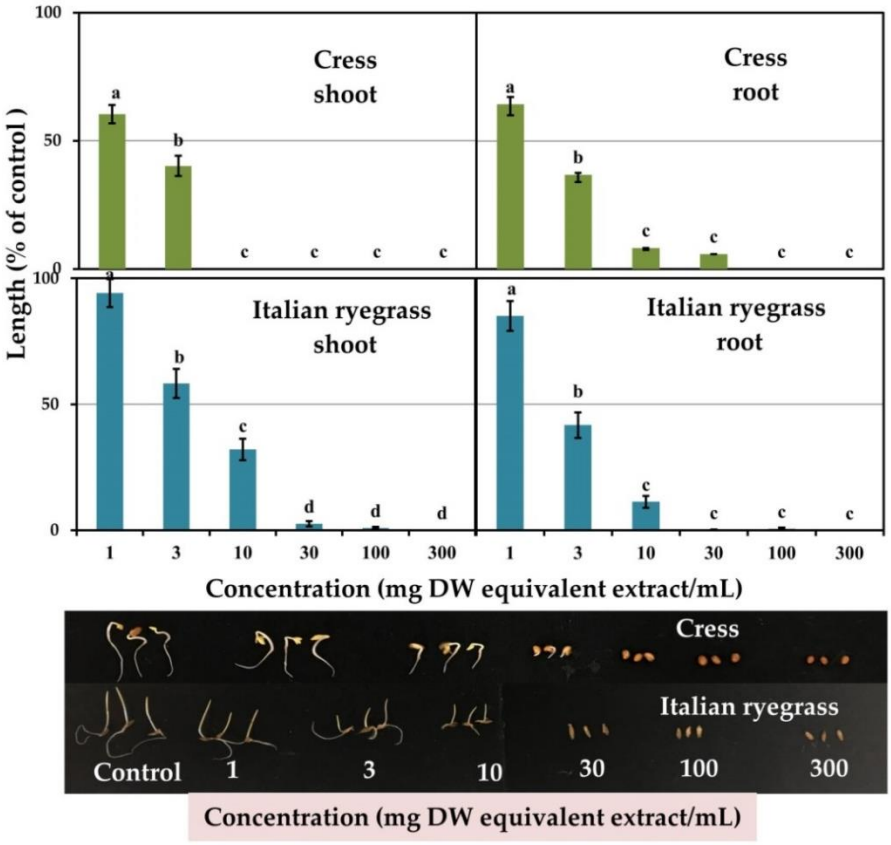

Figure 2. Inhibitory effects of aqueous methanol extracts of *Marsdenia tenacissima* on the seedling growth of cress and Italian ryegrass with six concentrations. The bars on each experiment show mean ± SE. Different letters indicate significant differences among the treatments (Tukey's HSD, $p < 0.05$).

The I_{50} values of the *M. tenacissima* extracts for the shoot and root growth of cress and Italian ryegrass varied from 1.7 to 4.6 mg DW equivalent extract/mL, respectively (Table 1). The I_{50} values of the *M. tenacissima* extracts for the shoot growth of cress (I_{50} = 1.5) were not significantly different compared with its root growth (I_{50} = 1.7), whereas the root growth of Italian ryegrass (I_{50} = 2.6) was significantly less than its shoot growth (I_{50} = 4.6) ($p < 0.05$). The values of the correlation coefficient ranged from −0.74 to −0.81 ($p < 0.01$) between the seedling growth of the test plant species and *M. tenacissima* extract concentrations, showing a negative correlation (Table 1).

Table 1. I_{50} values (mg DW equivalent extract/mL) and correlation coefficient (r) of the shoot and root growth of the test plant species by the aqueous methanol extracts of *Marsdenia tenacissima*.

Test Plant Species	I_{50} Value (mg DW Equivalent Extract/mL)		Correlation Coefficient (r)	
	Shoot	Root	Shoot	Root
Cress	1.5 c	1.7 c	−0.80 ***	−0.81 ***
Italian ryegrass	4.6 a	2.6 b	−0.74 ***	−0.76 ***

Different letters indicate significant difference between the treatments according to Tukey's HSD test ($p < 0.05$), and asterisks in correlation coefficient denote statistical significance: *** $p < 0.001$.

3.2. Isolation and Investigation of the Allelopathic Substances from the Leaf Extracts of M. tenacissima

The leaf extracts of *M. tenacissima* were separated through partitioning into ethyl acetate and aqueous fractions. Both fractions showed significant inhibitory effects on the seedlings growth of cress compared with control ($p < 0.05$, Figure 3). However, the

ethyl acetate fraction had more significant inhibitory potential than the aqueous fraction at both concentrations. Thus, the ethyl acetate fraction was separated through a series of chromatography steps: silica gel, Sephadex LH-20, and reverse-phase C_{18} cartridges and HPLC. Finally, two active substances were isolated and characterized by reverse-phase HPLC and spectral data analysis.

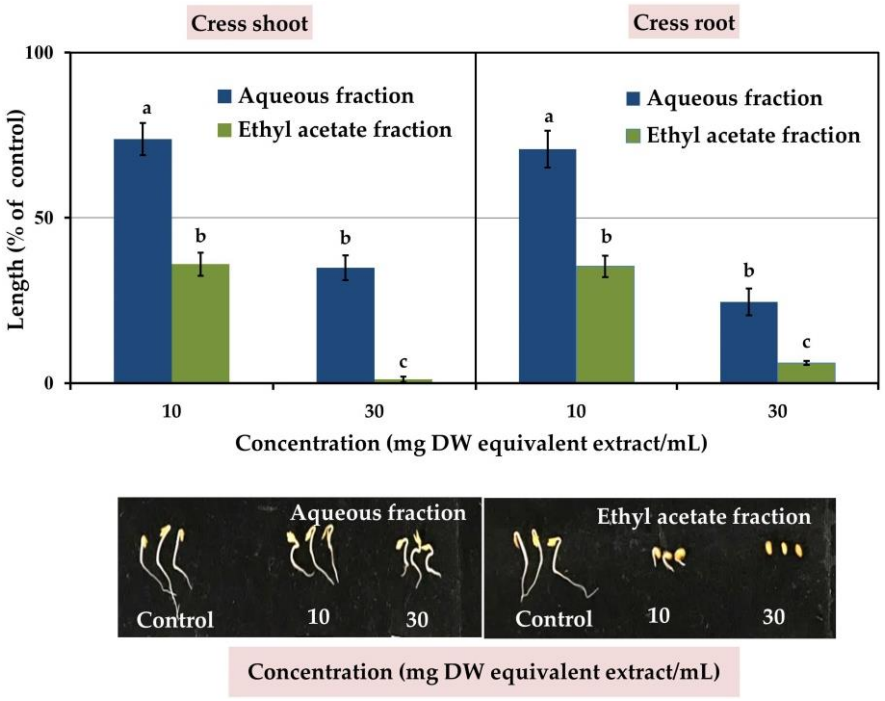

Figure 3. The inhibitory effect of the aqueous and ethyl acetate fractions obtained from *Marsdenia tenacissima* on the shoot and root growth of cress in partition. The bars on each experiment express mean ± SE with three replicates ($n = 30$). Different letters indicate significant differences among the treatments (Tukey's HSD, $p < 0.05$).

The molecular structure of compound **1** was determined as $C_{42}H_{66}O_{15}$ by using HR-ESIMS *m/z* 833.4306 $[M + Na]^+$ (calcd for $C_{42}H_{66}O_{15}Na$ 833.4299) (Figure 4). The specific rotation of the compound showed $[\alpha]_D^{23} = +33$ (c 0.15, CH_3OH); IR (neat) 3468, 2934, 1744, 1698, 1650, 1448 cm^{-1}. The ^1H and ^{13}C-NMR data is summarized in Table 2.

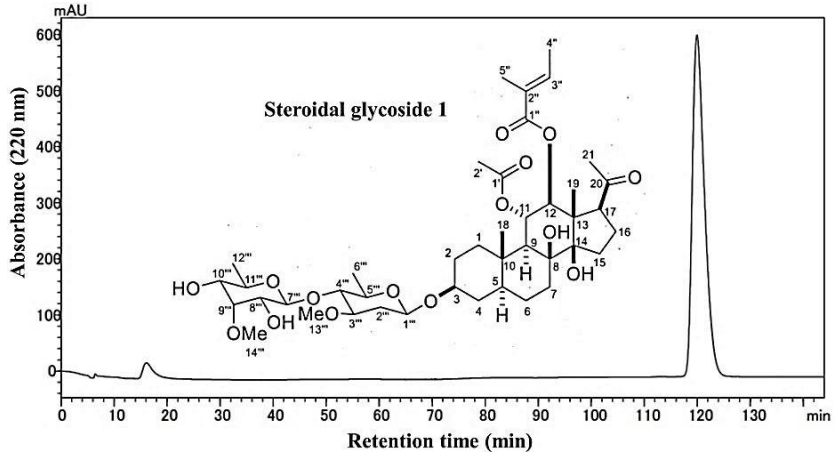

Figure 4. HPLC chromatogram and molecular structure of compound **1**.

Table 2. ^{1}H and ^{13}C-NMR data of compound **1** in CD_3OD.

Position	δ_C [a]	δ_H (*J* in Hz) [b]	Selected COZY	Selected HMBC
1a	40.4	1.20, m	2a	
1b		1.14, m	2b	
2a	30.3	1.78, m	1a, 1b, 3	
2b		1.24, m	3	
3	78.1	3.64, m	2a, 2b, 4a, 4b	
4a	35.7	1.67, m	3, 5	
4b		1.38, m	3, 5	
5	46.5	1.16, m	4a, 4b, 6a	
6a	25.9	1.67, m	5, 7a	
6b		1.12, m	7b	
7a	3.61	1.98, m	6a	
7b		1.35, m	6b	
8	85.2			
9	51.3	1.65, m	11	
10	38.9			
11	72.5	5.82, t (10.8)	9, 12	
12	79.4	4.95, d (10.8)	11	1″
13	56.4			
14	86.3			
15a	32.0	2.22, m	16	
15b		1.89, m		
16	25.4	2.04, m	15a, 17	
17	60.1	2.95, dd (8.3, 6.0)	16	20
18	13.3	1.06, s		1, 5, 9, 10
19	14.0	1.23, s		12, 13, 14, 17
20	216.7			
21	32.0	2.13, s		20
1′	171.8			
2′	21.7	1.83, s		
1″	169.3			
2″	129.2			
3″	140.4	6.93, m	4″, 5″	
4″	14.6	1.83, brs	3″	2″, 3″
5″	12.1	1.85, brs	3″	1″, 2″
1‴	98.6	4.64, m	2‴ a, 2‴ b	3
2‴ a	37.9	2.22, m	1‴, 3‴	
2‴ b		1.34, m	1‴, 3‴	
3‴	80.6	3.37, m	2‴ a, 2‴ b, 4	
4‴	84.0	3.17, m	3‴, 5‴	
5‴	72.5	3.38, m	4‴, 6‴	
6‴	18.8	1.35, d (6.1)	5‴	
7‴	102.2	4.72, d (8.1)	8‴	4‴
8‴	73.6	3.30, m	7‴, 9‴	
9‴	84.0	3.62, m	8‴, 10‴	
10‴	75.0	3.18, m	9‴, 11‴	
11‴	71.2	3.65, m	10‴, 12‴	
12‴	18.2	1.23, d (6.3)	11‴	
13‴	57.4	3.40, s		3‴
14‴	62.5	3.60, s		9‴

[a] Measured at 100 MHz. [b] Measured at 400 MHz.

The molecular structure of compound **2** was determined as $C_{45}H_{70}O_{15}$ by using HR-ESIMS at *m/z* 873.4659 $[M + Na]^+$ (calcd for $C_{45}H_{70}O_{15}Na$ 873.4612) (Figure 5). The ^{1}H NMR spectrum (400 MHz, C_5D_5N) showed δ_H values of 7.06 (qq, *J* = 6.0, 1.4 Hz, 1 H, H-3‴), 6.98 (qq, *J* = 6.0, 1.4 Hz, 1 H, H-3″), 6.50 (t, *J* = 10.8 Hz, 1 H, H-11), 5.44 (d, *J* = 10.1 Hz, 1 H, H-12), 5.34 (d, *J* = 8.1 Hz, 1 H, H-7‴), 4.84 (d, *J* = 9.7 Hz, 1 H, H-1‴), 4.18 (dq, *J* = 10.0, 6.5 Hz 1 H, H-11‴), 4.10 (t, *J* = 2.9 Hz, 1 H, H-9‴), 3.89–3.94 (m, 2 H, H-3, 8‴), 3.84 (s, 3 H, H-14‴), 3.60–3.67 (m, 4 H, H-3‴, 4‴, 5‴, 10‴), 3.52 (s, 3 H, H-13‴), 3.28 (dd, *J* = 9.7, 4.8 Hz, 1 H, H-17),

2.44 (m, 1 H, H-2‴a), 6.50 (t, *J* = 10.8 Hz, 1 H, H-11), 2.18–2.36 (m, 4 H, H-1a, 7a, 15a, 16a), 2.16 (s, 3 H, H-21), 1.90–2.13 (m, 5 H, H-2b, 6a, 9, 15b, 16b), 1.89 (s, 3 H, H-5′), 1.82 (s, 3 H, H-5″), 1.72–1.79 (m, 3 H, H-2a, 4a, 2‴b), 1.70 (brs, 3 H, H-6‴), 1.66 (brs, 3 H, H-4″), 1.62 (brs, 3 H, H-4′), 1.61 (brs, 3 H, H-18), 1.70 (brs, 3 H, H-6‴), 1.56 (d, *J* = 6.3 Hz, 3 H, H-12‴), 1.53 (s, 3 H, H-19), 1.43–1.52 (m, 2 H, H-4b, 7b), 1.27 (m, 1 H, H-1b), 1.10–1.21 (m, 2 H, H-5, 6b). The specific rotation of the compound showed $[\alpha]_D^{26}$ = +52 (c = 0.225, CH_3OH).

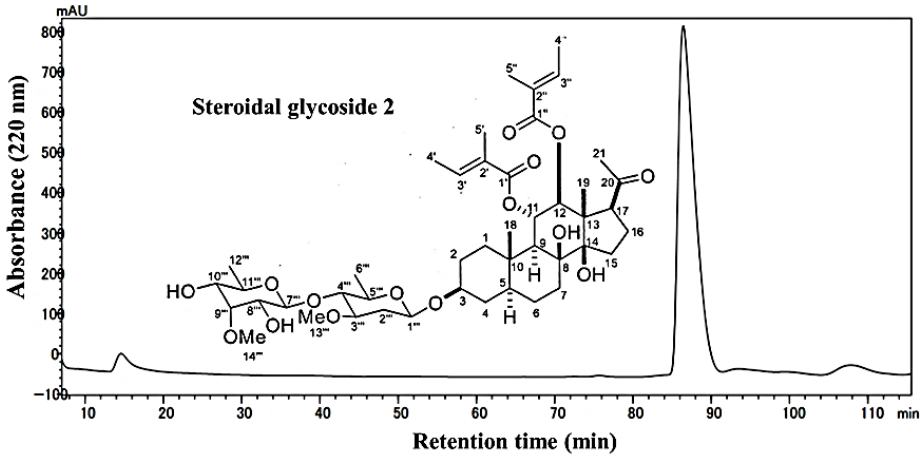

Figure 5. HPLC chromatogram and molecular structure of compound **2**.

3.3. Allelopathic Activity of Compounds **1** *and* **2**

The two compounds significantly inhibited the seedling growth of cress, and the degree of inhibitory activity increased with increasing concentration. Compound **1** significantly inhibited the cress shoots and roots at concentrations greater than 0.3 and 0.03 mM, respectively ($p < 0.05$) (Figure 6). On the other hand, compound **2** significantly suppressed the cress shoots and roots at concentrations greater than 1 and 0.1 mM, respectively ($p < 0.05$) (Figure 7).

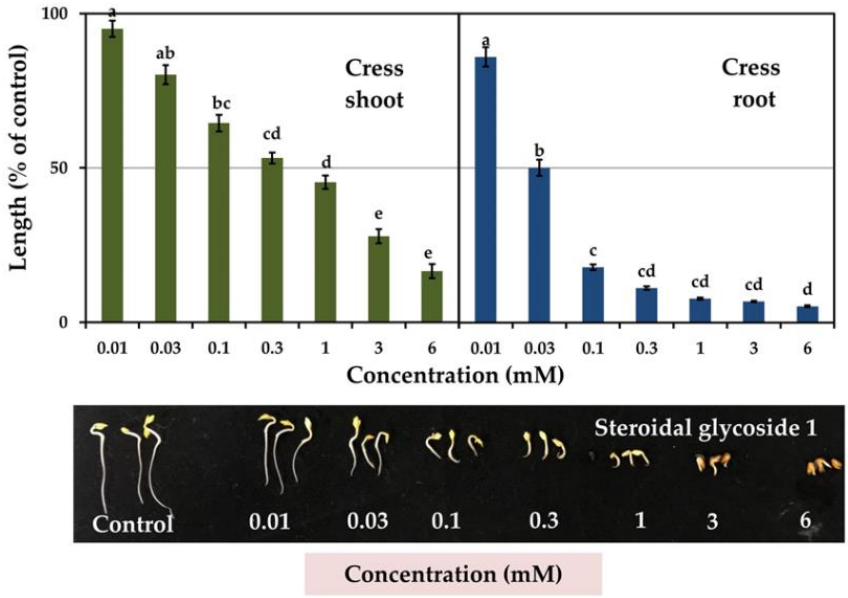

Figure 6. Inhibitory activity of compound **1** on the shoot and root growth of cress. The bars on each experiment express mean ± SE with three replicates (*n* = 30). Different letters indicate significant differences among the treatments (Tukey's HSD, $p < 0.05$).

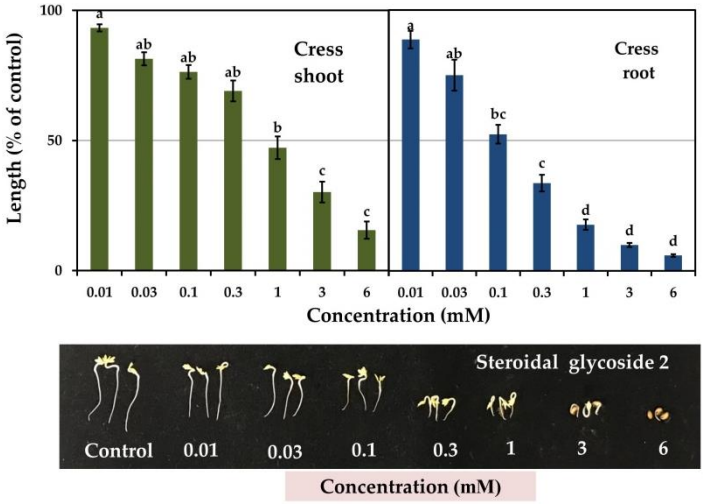

Figure 7. Inhibitory activity of compound **2** on the shoot and root growth of cress. The bars on each experiment express mean ± SE with three replicates (n = 30). Different letters indicate significant differences among the treatments (Tukey's HSD, $p < 0.05$).

The I_{50} values of the shoot and root growth of cress were 0.46 and 0.03 mM for compound **1**, respectively, and 0.74 and 0.12 mM for compound **2**, respectively ($p < 0.05$) (Table 3). Compound **1** against root growth (I_{50} = 0.03 mM) was >4 times more potent than compound **2** (I_{50} = 0.12 mM), and the effect of compound **1** on the shoot growth (I_{50} = 0.46 mM) was >1.6 times stronger than compound **2**. In addition, a negative correlation coefficient was found between the seedling growth of cress and the concentrations of compounds **1** and **2**, and this correlation coefficient varied from −0.69 to −0.83 ($p < 0.01$) (Table 3).

Table 3. I_{50} values (mM) and the correlation coefficient (r) of the shoot and root growth of cress by compound **1** and compound **2**.

Test Plant		I_{50} Value (mM)		Correlation Coefficient (r)	
		Compound 1	Compound 2	Compound 1	Compound 2
Cress	Shoot	0.46 b	0.74 a	−0.83 ***	−0.69 ***
	Root	0.03 c	0.12 c	−0.82 ***	−0.79 ***

Different letters indicate significant difference between the treatments according to Tukey's HSD test ($p < 0.05$), and asterisks in correlation coefficient denote statistical significance: *** $p < 0.001$.

4. Discussion

The aqueous methanol extracts of the *M. tenacissima* leaves significantly inhibited the seedling growth of cress and Italian ryegrass (Figure 2). A strong negative correlation was observed between the concentration of the *M. tenacissima* extracts and the shoot and root growth of the test plant species (Table 1), showing that the inhibitory effect depended on concentration. In addition, the I_{50} values of the shoot and root growth of the two test plants varied, indicating that the inhibitory activity of the *M. tenacissima* extracts was species-dependent (Table 1). Similar results of concentration- and species-dependent inhibitory activity for extracts of *Garcinia xanthochymus*, *Nephrolepis cordifolia*, *Albizia richardiana*, *Clerodendrum indicum*, and *Afzelia xylocarpa* have also been reported [31–35]. Thus, the growth inhibitory activity of the *M. tenacissima* leaf extracts against the tested plants suggests that its leaf extracts may contain allelopathic substances.

Based on a comparison of the ^{1}H and ^{13}C-NMR spectroscopic signals of compound **1** with marsdenoside M [36] and tenacissoside L and M [37], which were isolated from the stem of *M. tenacissima*, the aglycone moiety of the compound had a polyoxypregnan-20-one skeleton. Therefore, the structure of compound **1** was elucidated based on the spectroscopic

data of C_{21} steroidal glycosides. The spectroscopic signals of the pregnane skeleton were assigned through analysis of ^{1}H-^{1}H COSY, HMQC, and HMBC spectra (Figure 8). ^{1}H and ^{13}C-NMR spectra showed signals for three tertiary methyl groups at δ_H 1.23 (s), 1.83 (s), and 1.06 (s), with the related carbon (C) signals at δ_C 14, 13.3, and 21.7, respectively (Table 2). We also found three secondary methyl groups at δ_H 1.35 (d, J = 6.1 Hz), 1.83 (brs), and 1.23 (d, J = 6.3 Hz), with the resonance C at δ_C 18.8, 102.2, and 18.2; two methoxyl groups at δ_H 3.40(s) and 3.60 (s), with the corresponding C signals at δ_C 57.4 and 62.5; and one methyl of an acetyl (Ac) group at δ_H 2.13 (s), with the resonance C signal at δ_C 32.0. An NMR study (^{1}H and ^{13}C-NMR, COSY, and HMQC) and a consideration of the molecular formula indicated that compound **1** includes one carbonyl carbon of a ketone group, two acyl groups, five methyls, seven methylenes, six methines (three oxygenated), four quaternary carbons (two oxygenated), and two olefinic carbons, as well as two sugar units.

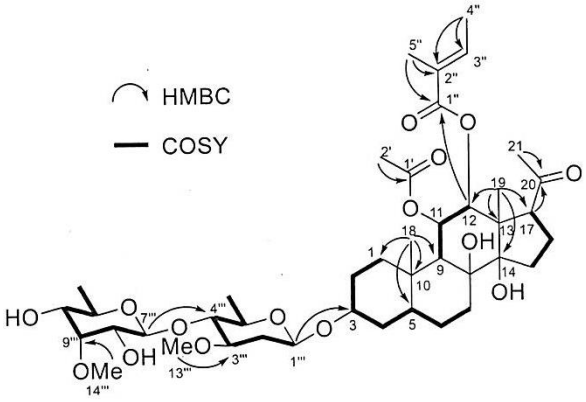

Figure 8. COSY and HMBC correlations of compound **1**.

In the HMBC spectrum, steroidal glycoside 1 showed a rotation system from H-1 to H-4 and H-9 to H-12, as well as correlations between H-6/H-7, H-15/H-16, and H-20/H-21, which might be clarified by the four-ring skeleton of a pregnane derivative. The HMBC correlations from H-18 to C-1, C-5, C-9, and C-10, and H-19 to C-12, C-13, C-14, and C-17 indicate the two angular methyl groups were connected to C-10 and C-13, respectively (Figure 8). One more methyl signal of δ_H 1.83 (s), together with two carbon resonance signals at δ_C 171.8 and 21.7, suggested the existence of one acetyl (Ac) group on the aglycon of compound **1**. This group was attached at the C-11 position on the basis of HMBC correlations from δ_H 5.82 (t, J = 10.8 Hz, H-11) to δ_C 171.8 (C-1′ of Ac) (Figure 8). The tiglogy (Tig) group was identified through a series of proton signals at δ_H 6.93 (m), 1.83 (brs), and 1.85 (brs), with carbon resonance signals at δ_C 169.3, 129.2, 140.4, 14.6, and 12.1 in the ^{13}C-NMR spectrum. The Tig group was attached at the C-12 position on the long-range of HMBC correlations from δ_H 4.95 (d, J = 10.8 Hz, H-12) to δ_C 129.2 (C-1″ of Tig). Moreover, the correlation from the protons H-11 to H-8 and H-12 to H-9 revealed that the acetyl group at C-11 was in α-orientation and the tiglogy group at C-12 was in β-orientation in the NOESY spectrum. In addition, NOESY correlation between H-12 and C-17 (δ_H 2.95, dd) indicated that the C-17 side-chain was in β-orientation and the carbonyl carbon of the β-linked methyl ketone at C-17 appeared near δ_C 216.7. Previous studies [38,39] reported similar findings where the C-17 side-chain and β-linked methyl ketone at C-17 were observed near δ_C 217 and δ_C 217.2, respectively.

In the ^{1}H-NMR spectrum, the two anomeric protons (δ_H 4.64 (m), 4.72 (d, J = 8.1 Hz)) indicated that the two β-linkage sugars were attached to the aglycone of compound **1**. Furthermore, the two secondary methyl groups (δ_H 1.35 (d, J = 6.1 Hz), 1.23 (d, J = 6.3 Hz)) and two methoxyl groups (δ_H 3.40 (s), 3.60 (s)) indicated the presence of 6-deoxy-3-methoxy sugars. On the basis of the ^{1}H-^{1}H COSY, HMQC, and HMBC spectra, proton spin systems and carbon resonances of each sugar were fully assigned (Table 2). The two sugar units were then identified as oleandropyranosyl (Ole) and 6-deoxy-3-O-methyl-allopyranose (Allo) [40,41]. The two β-linked deoxy-sugar units were shown by the anomeric proton

signals (Figure 8). Moreover, the long-range correlations from H-3 of the aglycone (δ_H 3.64) to the carbon C-1‴ of Ole (δ_C 98.6) and H-7‴ of the Allo (δ_H 4.72) to the carbon C-4‴ of Ole (δ_C 84.0) in the HMBC spectrum indicated that the sugar chain was attached at C-3 and the two sugars were linked through the 1→4 position, respectively. Therefore, the structure of compound **1** was determined to be a novel compound (steroidal glycoside 1) and defined as 3-*O*-[6-deoxy-3-*O*-methyl-β-allopyanosyl(1→4)-β-oleandropyranosyl]-5,6-dihydrogen-11α-*O*-acetyl-12β-*O*-tigloyl-17β-marsdenin.

The spectral data of compound **2** was consistent with compound **6** (C_{21} steroidal glycoside-$C_{45}H_{70}O_{15}$) in a previous study [41]. The ^{1}H-NMR spectrum of compound **2** comprised two secondary methyl groups (δ_H 1.70 (brs), 1.56 (d, *J* = 6.3 Hz)), two methoxyl groups of deoxy-sugars (δ_H 3.52 (s), 3.84 (s)), and two anomeric protons (δ_H 4.84 (d, *J* = 7.9 Hz), 5.34 (d, *J* = 8.1 Hz)). The anomeric proton signals indicated that each sugar in compound **2** possessed β-linked deoxy-sugars. ^{1}H and ^{13}C-NMR spectral data confirmed that compound **2** had a similar aglycone moiety to compound **1**, except for a tigloyl group (Tig-1) at the C-11 position in compound **2** instead of the acetyl (Ac) group in compound **1**. Based on the long-range HMBC correlation, the positions of two Tig groups were confirmed by a series of proton signals from δ_H 6.50 (t, *J* = 10.8 Hz, H-11) to resonance C signals at δ_C 167.0 (C-1′ of Tig-1) and from δ_H 5.44 (d, *J* = 10.1 Hz, H-12) to δ_C 168.0 (C-1″ of Tig-2), and the substitution position at C-11 was in α-orientation and at C-12 was in β-orientation. After analyzing the 2D NMR data, we found that compound **2** (Figure 5) possesses the same sugar unit as compound **1**. Therefore, the structure of compound **2** was determined to be steroidal glycoside 2 and defined as 3-*O*-[6-deoxy-3-O-methyl-β-allopyanosyl(1→4)-β-oleandropyranosyl]-5,6-dihydrogen-11α,12β-di-*O*-tigloyl-17β-marsdenin.

According to previously reported spectral data [42], our two isolated compounds (steroidal glycosides 1 and 2) belong to a group of C_{21} steroidal glycosides. Although a wide range of pharmacological activities (including cytotoxic, anticancer, anti-inflammatory, and antitumor) of different C_{21} steroidal glycosides have been documented [37,43–47], no allelopathic activities of C_{21} steroidal glycosides have been reported until now. Thus, this report is the first on the allelopathic activity of steroidal glycosides 1 and 2 from *M. tenacissima* leaves.

In this study, steroidal glycosides 1 and 2 significantly suppressed the shoot and root growth of cress seedlings (Figures 6 and 7). The I_{50} values also showed that steroidal glycoside 1 has more growth inhibitory activity than steroidal glycoside 2 (Table 3). The different inhibitory activities of steroidal glycosides 1 and 2 might be due to the different chemical structures and substituent groups in the C-11 and C-12 positions of the C_{21} steroidal glycosides [48]. Our findings indicate that *M. tenacissima* leaves possess allelopathic activity, and steroidal glycosides 1 and 2 may contribute to its allelopathy. Therefore, the allelopathic activity of *M. tenacissima* leaves and its two allelopathic substances may lead to developing new environmentally safe bioherbicides for weed management. Accordingly, *M. tenacissima* leaves could be used as a soil-additive resource instead of synthetic chemical herbicides for environmentally friendly weed control in agriculture.

5. Conclusions

The leaf extracts of *M. tenacissima* showed significant allelopathic potential against the seedling growth of cress and Italian ryegrass. Two allelopathic substances (compounds **1** and **2**) were isolated and determined as steroidal glycoside 1 (3-O-[6-deoxy-3-O-methyl-β-allopyanosyl(1→4)-β-oleandropyranosyl]-5,6-dihydrogen-11α-O-acetyl-12β-O-tigloyl-17β-marsdenin), a novel compound, and steroidal glycoside 2 (3-O-[6-deoxy-3-O-methyl-β-allopyanosyl(1→4)-β-oleandropyranosyl]-5,6-dihydrogen-11α,12β-di-O-tigloyl-17β-marsd-enin). These two compounds significantly suppressed the shoot and root growth of cress. The growth inhibitory activities of those compounds may be responsible for the allelopathic activity of *M. tenacissima* leaves. However, it is necessary to determine the concentrations of those compounds and their mode of action in soil to clarify the contribution of the allelopathic activities. Thus, our findings suggest that *M. tenacissima* leaves may be considered as

a promising candidate for a soil-additive resource to reduce the dependency on synthetic chemical herbicides for controlling weeds in sustainable agriculture.

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