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# Allelopathy and Allelopathic Substances in the Leaves of *Metasequoia glyptostroboides* from Pruned Branches for Weed Management

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**Abstract:** *Metasequoia glyptostroboides* Hu et W.C. Cheng, known as a living fossil species, is planted in parks, gardens, and streets in many temperate regions worldwide. Adequate branch pruning is necessary to grow the plants in these locations, and pruning generates a large amount of waste. In this study, allelopathic activity of pruned-branch waste was investigated to search for beneficial applications of the waste. The leaves of *M. glyptostroboides* obtained from pruned branches were extracted, and the extracts showed growth-inhibitory activity on four weed species, namely, *Vulpia myuros*, *Lolium multiflorum*, *Echinochloa crus-galli*, and *Phleum pretense*. The inhibition was extract-concentration dependent. The roots of *P. pretense* were the most sensitive, and the coleoptiles of *E. crus-galli* were the least sensitive to the extracts among all roots and coleoptiles of these weed species. Two allelopathic substances in the extracts were isolated and identified as umbelliferone and (+)-rhododendrol. Both compounds showed inhibitory activity on the growth of *V. myuros*, although the inhibitory activity of (+)-rhododendrol was much greater than that of umbelliferone. The leaves may also contain some other allelopathic substances. These allelopathic substances, including umbelliferone and (+)-rhododendrol, may work as growth-inhibitory substances of leaf extracts. Therefore, the leaves of *M. glyptostroboides* obtained from pruned branches are allelopathic and potentially useful for weed control in certain agricultural settings such as foliar spray and soil additive, to decrease synthetic herbicide application in crop production pursuant to developing ecofriendly agriculture.

**Keywords:** allelopathy; fossil tree; decomposition; phytotoxicity; pruning waste; weed management



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## 1. Introduction

*Metasequoia glyptostroboides* Hu et W.C. Cheng, known as a living fossil species, is a perennial deciduous conifer, and the sole species of the genus *Metasequoia* of Cupressacea family. The species was first found as fossils from the Cretaceous period (145–166 million years ago) in several strata in the northern hemisphere [1–3]. Its living plants were later discovered in the 1940s in Southeast China, and the species has remained unchanged for millions of years compared with the characteristics of the fossils [2,3]. *M. glyptostroboides* has survived under conditions of tremendous geological, climate, and ecological changes, and countless attacks of pathogens and herbivores from generation to generation [4,5].

The species grows up to approximately 50 m in height, with pyramidal shape and approximately 2 m in trunk diameter. The leaves are opposite and 1–3 cm long, bright green, and fall in autumn (Figure 1). Its morphological characteristics, such as arrangement of the cataphylls, leaves, strobili, and cone scales are different from other species of the Cupressacea family [6–8]. Regeneration properties, such as seed production ability,

germination rate, and seedling survival rate, are considerably different even within the species [9]. However, it is not clear the reason for the longevity of the species.

**Figure 1.** *M. glyptostroboides*.

More than 300 compounds were identified in the leaves, seeds, and barks of *M. glyptostroboides* in many chemical classes such as fatty acids, flavonoids, terpenoids, lignans, norlignans, and other phenolic compounds, and over 20 compounds are unique to the species [10]. Terpenoids are the first major group of those compounds in *M. glyptostroboides* and flavonoids are the second major group. The essential oil and the extracts of the species showed anti-bacterial and anti-fungal activity [11,12]. Diterpenoids and their derivatives, sugiol, taxodone, taxoquinone, and totrarol, also showed anti-bacterial and anti-fungal activity [13–17]. In addition, the comparison between compounds in the wood fragments of the species buried deeply in the soil for 53 million years and those in fresh wood pieces showed the species has also remained unchanged during those years at molecular levels [18]. Therefore, some of those compounds found in the species may play in a role in defense functions against pathogenic fungal attacks.

*Metasequoia glyptostroboides* thrives as an ornamental tree in parks and gardens, and has become one of the major street trees in many temperate regions worldwide because of its fast growth property and beautiful shapes [19–22]. However, adequate maintenance such as the removing of branches is necessary as a garden and street tree [23,24]. Pruning generates a large amount of waste because of the fast growth property of the species. This waste could be managed and utilized more efficiently. It seems, therefore, worthwhile to discover beneficial applications of pruning waste to decrease economic and environmental concerns.

Allelopathy is the interaction between host plant species and neighboring plant species through certain compounds called allelochemicals [25]. Allelochemicals are produced and stored in some plant parts, and released into the vicinity of the host plants by rainfall leachates and volatilization from the plant parts, root exudation, and decomposition process of their residues. The released allelochemicals suppress the germination and growth of the competitive plants near the host plants [26–29]. The residues and extracts of several plant species and their plant parts such as leaves and roots showed significant weed-control ability as soil additives and mulch because of their allelopathic potential [30–33]. Thus, allelopathy of plants can be applied for weed management options in several agriculture settings to decrease the application of the commercial herbicide. The objective of the

present research was the investigation of the allelopathic property of the leaves from pruned branches of *M. glyptostroboides*, and the identification of allelopathic substances in the waste to search for beneficial applications of the waste, pursuant to developing sustainable agriculture.

## 2. Materials and Methods

### *2.1. Plant Material*

*M. glyptostroboides* leaves were sampled from pruned branches at the Kagawa University on July 2018, and kept at −20 °C soon after collection, till extraction. Four weed species—*Vulpia myuros* (L.) C.C. Gmel., *Lolium multiflorum* Lam., *Echinochloa crus-galli* (L.) P. Beauv., and *Phleum pretense* L.—were used to determine allelopathic activity. Cress (*Lepidium sativum* L.) was used as a bioassay plant for the isolation process of the allelopathic substance because of its stable germination rate and easier handling property.

### *2.2. Extraction and Determination of Allelopathic Activity*

*M. glyptostroboides* leaves (60 g fresh weight) were cut into small pieces using scissors, and extracted by soaking in 80% (*v*/*v*) aqueous methanol (500 mL) for 48 h. After filtration of the extract with a filter paper (No. 2; Toyo, Tokyo, Japan), the resulting residue was extracted again by soaking in methanol (500 mL) for 48 h and filtered. These two filtered extracts were combined and concentrated to be aqueous solution in a rotary evaporator at 40 °C.

The leaf extract was dissolved with methanol, and an aliquot of the methanol solution was applied onto a sheet of filter paper (No. 2) in a Petri dish (2.8 cm i.d.). The final concentrations in the Petri dishes were adjusted to the extracts obtained from 1, 3, 10, 30, 100, 300, and 1000 mg leaves per mL. The solution in the Petri dish was completely evaporated in a fume hood, and Tween 20 solution (0.6 mL; 0.05%, Nacalai, Kyoto, Japan) was poured onto the filter paper. Seeds of *V. myuros*, *L. multiflorum*, *E. crus-galli*, and *P. pretense* were incubated on the moisten filter paper for 48 h in the dark at 25 °C, and the uniform germinated seeds were selected. Ten germinated seeds of each species were then separately placed onto the filter paper. After the incubation for 48 h in the dark at 25 °C, the length of the roots and coleoptiles of these weed species was measured using a ruler. The percentage length of the roots and coleoptiles of extract-treated seedlings was determined against the length of control roots and coleoptiles. Control treatment was handled with exactly the same procedures, without the extracts. Methanol was applied onto a sheet of filter paper in a Petri dish and methanol in the Petri dish was completely evaporated. After adding the Tween 20 solution onto the filter paper, 10 germinated seeds of each species were separately placed.

The extracts and/or fractions obtained during the separation steps described in Section 2.3. were evaporated, and dissolved with methanol. Then, an aliquot of the methanol solution was applied onto a sheet of filter paper in a Petri dish. After the evaporation of methanol in the Petri dish, Tween 20 solution was added onto the filter paper, and 10 cress seeds were directly placed onto the moisten filter paper. Other procedures were carried out as described above.

### *2.3. Separation of M. glyptostroboides Extract*

*M. glyptostroboides* (1 kg fresh weight) leaves were extracted by soaking in 80% (*v*/*v*) of 4 L aqueous methanol for 48 h, and the residues was extracted again by soaking in 4 L of methanol for 48 h. Two filtrates were combined and concentrated to obtain aqueous solution as described above. The aqueous solution was then adjusted to pH 7.0 with 1 M phosphate buffer and partitioned three times with ethyl acetate. Resulting ethyl acetate fraction was evaporated to dryness, and subjected to silica gel (90 g, silica gel 60, 70–230 mesh; Merck) chromatography. The chromatography was eluted with 9 solvents. This included 7 mixtures of *n*-hexane: ethyl acetate (80:20, 70:30, 60:40, 50:50, 40:60, 30:70, and 20:80; *v*/*v*, 100 mL each), ethyl acetate (100 mL), and methanol (200 mL). Allelopathic

activity of all separated fractions was determined using a cress bioassay as described in the Section 2.2. The activity was found in two fractions eluted with the solvents of *n*-hexane and ethyl acetate mixtures, 40:60 (active fraction A) and 30:70 (active fraction B).

### *2.4. Isolation of Allelopathic Substances from Active Fractions A and B*

After the evaporation of solvents, active fractions A and B were separately subjected to Sephadex LH-20 (80 g, Sigma-Aldrich, Burlington, VT, USA) chromatography and eluted with mixtures of water: methanol (20:80, 30:70, 40:60, 50:50, 40:60, 30:80, and 10:90; *v*/*v*, 100 mL each) and methanol (100 mL). Activity was detected in the fractions eluted with the mixtures of 40:60 for active fraction A, and 60:40 for active fraction B.

Active fraction A was then subjected to an ODS cartridge (YMC-Dispo SPE; YMC Ltd., Kyoto, Japan) eluted with mixtures of water: methanol (70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90; *v*/*v*, 30 mL each), and methanol (60 mL). Activity was detected in the fraction eluted with the mixture of 80:20. The active fraction was finally purified by a reverse-phase HPLC (10 mm i.d. × 500 cm, ODS-AQ; YMC Ltd., Kyoto, Japan; detection at 220 nm) eluted with 25% aqueous methanol (flow rate; 1.5 mL). Activity was found in a peak fraction eluted between 194 and 196 min, yielding an active compound **1**. Chemical structure of the compound was characterized by the analyses of HRESI-MS and $^{1}$H-NMR spectrum (400 MHz, $CD_3OD$).

Active fraction B was also subjected to an ODS cartridge as described above. Activity was detected in the fraction eluted with the mixture of 70:30. The active fraction was finally purified by a reverse-phase HPLC eluted with 35% aqueous methanol, and activity was found in a peak fraction eluted between 126 and 128 min, yielding an active compound **2**.

### *2.5. Allelopathic Activity of Compounds* **1** *and* **2**

Isolated compounds **1** and **2** were dissolved with methanol, and an aliquot of the methanol solution was applied to a sheet of filter paper in a Petri dish. After evaporation of methanol in the Petri dish, the filter paper was moistened with 0.6 mL of Tween 20. Then, 10 germinated seeds of *V. myuros* were placed on the filter paper in Petri dishes and grown for 48 h. The concentrations of the compounds **1** and **2** in the Petri dishes were 10, 30, 100, 300, 1000 and 3000 mM and 1, 3, 10, 30, 100 and 300 mM, respectively. All other procedures were carried out as described in Section 2.2.

### *2.6. Statistical Analysis*

The determination of the allelopathic activity by weed species was repeated 4 times using a completely randomized design, with 10 plants for each determination. Cress bioassay was repeated 3 times using a completely randomized design, with 10 plants for each determination. These data were analyzed by a one-way ANOVA and post hoc with Tukey's HSD test ($p < 0.05$). $IC_{50}$ values were obtained using GraphPad Prism 6.0.

## 3. Results

### *3.1. Allelopathic Activity of M. glyptostroboides Leaves Obtained from Pruned Branches*

The leaf extracts of *M. glyptostroboides* suppressed the root and coleoptile growth of four weed species, *V. myuros*, *L. multiflorum*, *E. crus-galli*, and *P. pretense*, at concentrations greater than 10–30 mg leaf equivalent extract per mL (Figure 2). The extract obtained from 100 mg of *M. glyptostroboides* leaves suppressed the root growth of *V. myuros*, *L. multiflorum*, *E. crus-galli*, and *P. pretense* by 2.2%, 7.4%, 14.3%, and 2.4% of control root growth, respectively, and suppressed the coleoptile growth of *V. myuros*, *L. multiflorum*, *E. crus-galli*, and *P. pretense* by 4.9%, 14.8%, 41.5%, and 3.7% of control coleoptile growth, respectively. Table 1 shows the $IC_{50}$ values of the leaf extracts of *M. glyptostroboides*, which caused 50% growth inhibition on the roots and coleoptiles of these weed species. $IC_{50}$ values of the four weed roots ranged between 12.1 and 39.2 mg leaf equivalent extract per mL, and those of the four coleoptiles ranged between 21.4 and 71.8 mg leaf equivalent extract per mL.

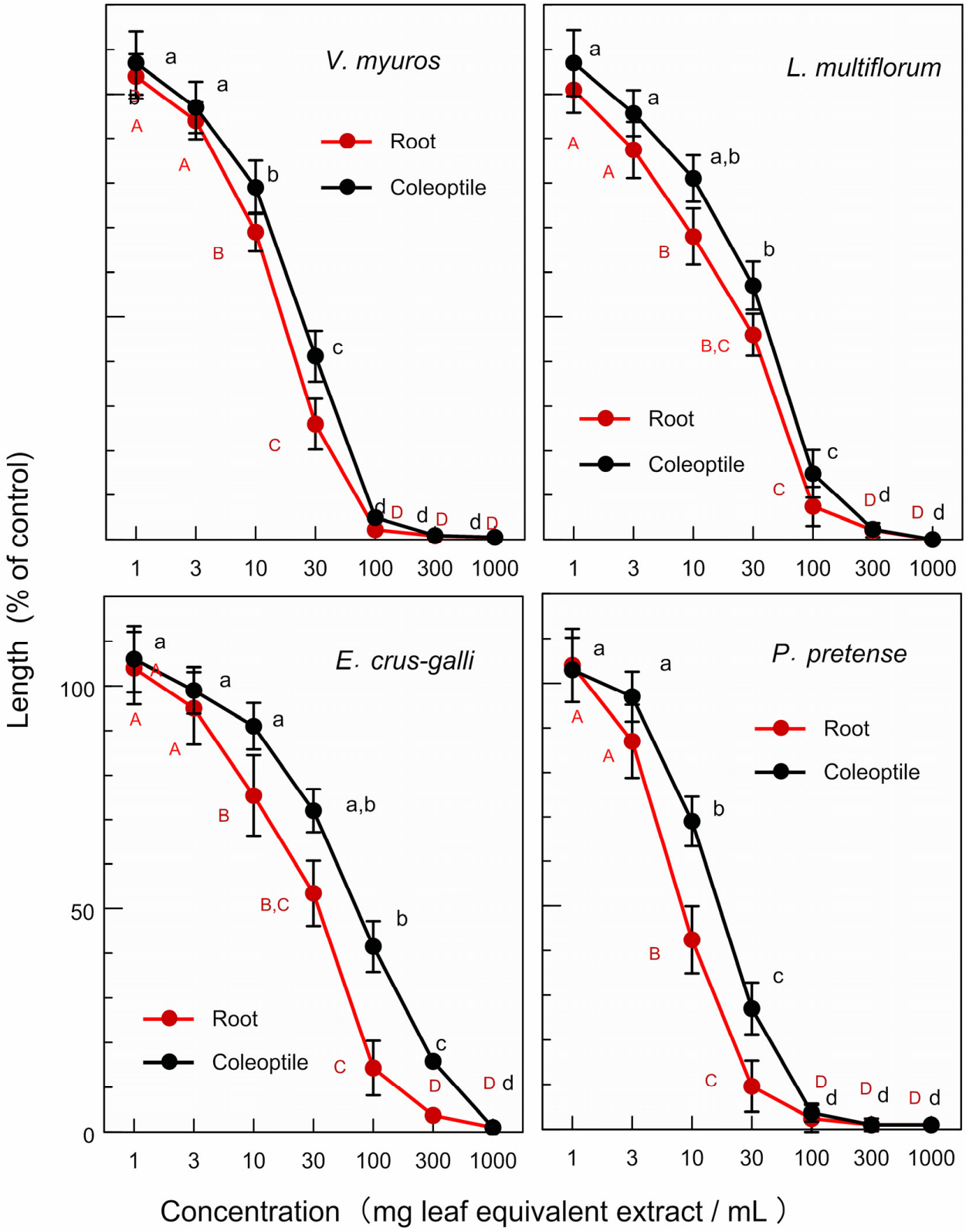


**Figure 2.** Effects of *M. glyptostroboides* leaf extracts on the growth of roots and coleoptiles of *V. myuros*, *L. multiflorum*, *E. crus-galli*, and *P. pretense*. Concentration (mg leaf equivalent extract/mL) indicates the concentration of tested sample corresponded to the extracts from 1, 3, 10, 30, 100, 300, and 1000 mg leaves. Means ± SE from 4 independent experiments with 10 seedlings for each determination are shown. Different letters in the same panels indicate significant differences (Tukey's HSD test, $p \leq 0.05$).

**Table 1.** $IC_{50}$ values of *M. glyptostroboides* leaf extracts on the growth of roots and coleoptiles of the bioassay plant species. The values (mg fresh weight equivalent extract per mL) were determined with GraphPad Prism.

| Test Plant | Root | Coleoptile |
|---|---|---|
| *Vulpia myuros* | 22.7 | 28.9 |
| *Lolium multiflorum* | 31.3 | 38.1 |
| *Echinochloa crus-galli* | 39.2 | 71.8 |
| *Phleum pretense* | 12.1 | 21.4 |

The extracts also suppressed cress roots and hypocotyls at concentrations greater than 30 mg leaf equivalent extract per mL (Figure 3). The extract obtained from 100 mg of *M. glyptostroboides* leaves suppressed the root and hypocotyl growth of cress by 4.6% and 7.5% of control root and hypocotyl growth, respectively. $IC_{50}$ values of cress roots and hypocotyls were 26.8 and 29.4 mg leaf equivalent extract per mL.

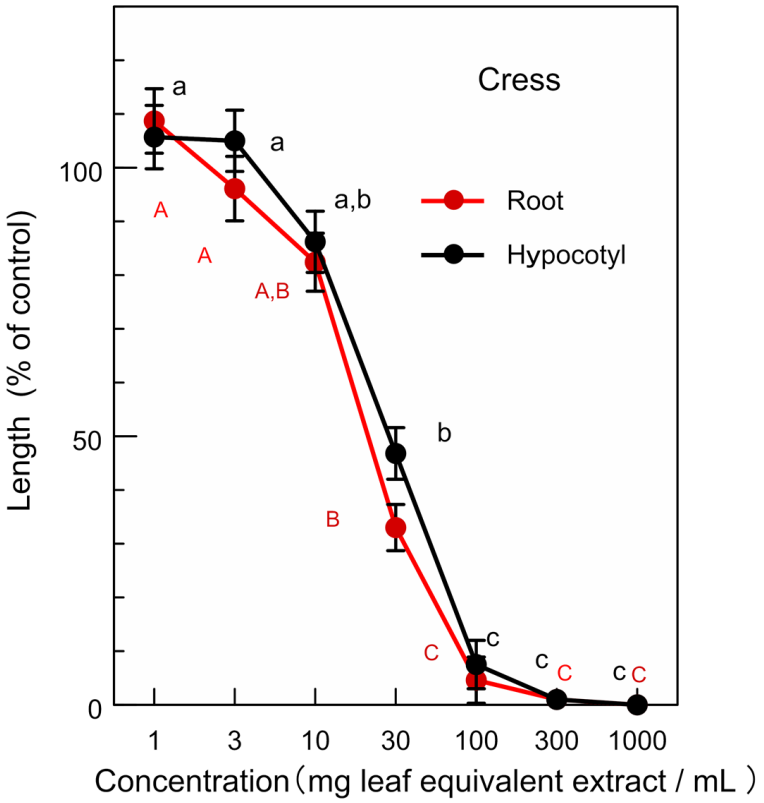


**Figure 3.** Effects of *M. glyptostroboides* leaf extracts on the growth of roots and hypocotyls of cress. Means ± SE from 3 independent experiments with 10 seedlings for each determination are shown. Other details as for Figure 2.

### *3.2. Separation and Identification of Allelopathic Substances*

The leaf extracts of *M. glyptostroboides* were separated by silica gel chromatography, and the activity of all separated fractions was determined by a cress bioassay. Allelopathic activity was found in two fractions: active fraction A and active fraction B. At concentration of 1000 mg fresh weight equivalent extract $mL^{-1}$, active fraction A suppressed the growth of the cress roots and hypocotyls by 14.6% and 15.3% of the control root and hypocotyls, respectively, and active fraction B suppressed the growth of the cress roots and hypocotyls by 4.2% and 8.8% of the control roots and hypocotyls, respectively. Active fractions A and B were further separated using the Sephadex LH-20 and ODS cartridge. Finally, two active compounds **1** and **2** were isolated by HPLC from active fractions A and B, respectively.

The molecular formula of compound **1** was $C_9H_6O_3$ by HRESI-MS. The $^1$H-NMR spectrum of the compound as measured in acetone-$d_6$ showed the presence of three aromatic proton signals at $\delta_H$ 7.51 (1H, d, $J$ = 8.5), 6.84 (1H, dd, $J$ = 8.5, 2.3), and 6.75 (1H, d, $J$ = 2.3), and two olefinic proton signals at $\delta_H$ 7.86 (1H, d, $J$ = 9.5) and 6.16 (1H, d, $J$ = 9.5). Considering these spectrum data with published data in the literature [34], the chemical structure of compound **1** was determined to be umbelliferone (Figure 4). Compound **2** was identified as (+)-rhododendrol (Figure 4) by its HRESI-MS, $^1$H-NMR spectrum, and specific rotation, as in our previous report [35].

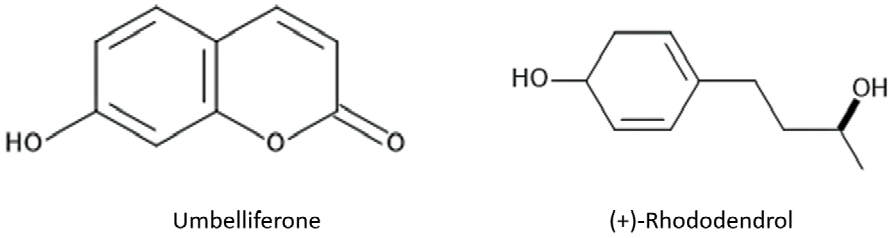


**Figure 4.** Chemical structures of umbelliferone and (+)-rhododendrol.

### *3.3. Allelopathic Activity of Isolated Compounds*

Umbelliferone and (+)-rhododendrol significantly suppressed the growth of *V. myuros* roots and coleoptiles at concentrations greater than 100 and 10 mM, respectively (Figure 5). At 100 mM application of the compounds, umbelliferone suppressed the root and hypocotyl growth by 61.4% and 87.4% of the control root and coleoptile growth, respectively, and (+)-rhododendrol suppressed the root and hypocotyl growth by 6.8% and 8.4% of the control root and coleoptile growth, respectively. $IC_{50}$ values of *V. myuros* root and coleoptile, respectively, were 257 mM and 366 mM for umbelliferone and 18.7 mM and 31.4 mM for (+)-rhododendrol.

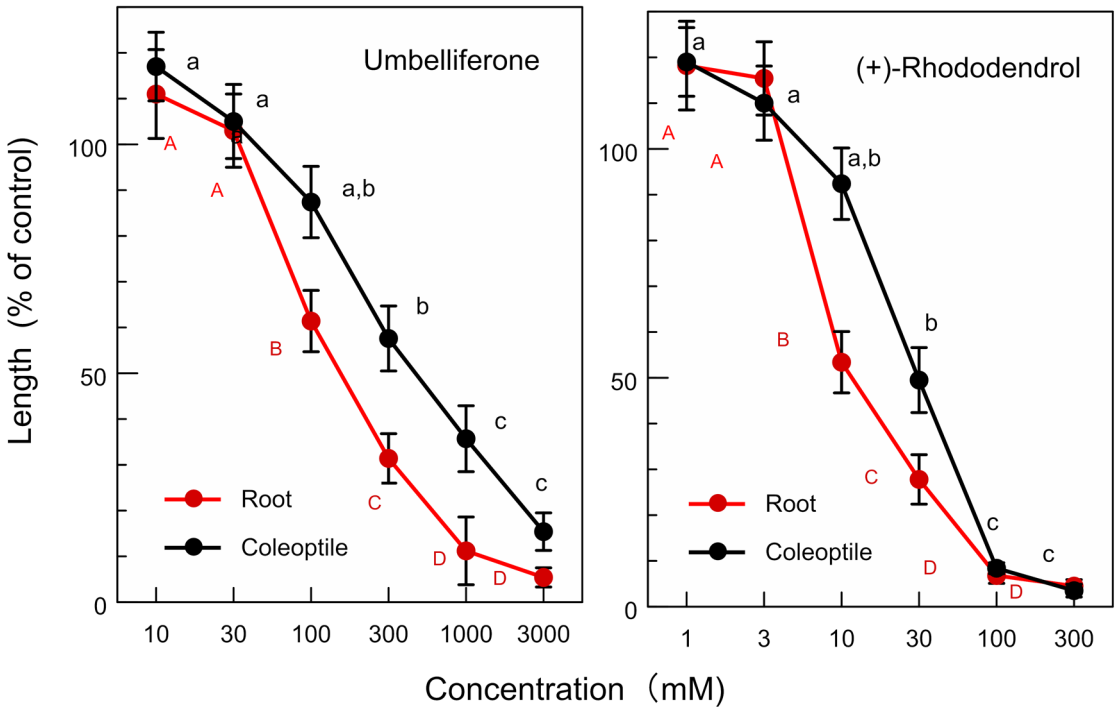


**Figure 5.** Effects of umbelliferone and (+)-rhododendrol on root and coleoptile growth of *V. myuros*. Means ± SE from 3 independent experiments with 10 seedlings for each determination are shown. Different letters on the symbols in the same panels indicate significant differences (Tukey's HSD test, $p \leq 0.05$).

## 4. Discussion

*M. glyptostroboides* are planted in parks, gardens, and streets as ornamental trees, and pruning of the branches for the maintenance of the tree generates a large amount of waste [22–25]. Development of beneficial applications of pruning waste is necessary to decrease economic and environmental concerns. The leaves of *M. glyptostroboides* were obtained from pruned branches, and allelopathic activity of the leaf extracts were determined with four weed species (Figure 2). The extracts significantly suppressed the growth of roots and coleoptiles of all weed species, with extract-concentration dependence. Considering $IC_{50}$ values (Table 1), the roots of *P. pretense* were the most sensitive to the extracts among all roots and coleoptiles, and the coleoptiles of *E. crus-galli* were the least sensitive among them. Many plants were reported to produce allelochemicals and store them in certain plant parts [26–29]. Therefore, the inhibitory activity of *M. glyptostroboides* leaf extracts suggests that the leaves may contain some allelopathic substances.

The leaf extract of *M. glyptostroboides* was separated by silica gel and Sephadex LH-20 columns, ODS cartridge, and HPLC, and two active compounds **1** and **2** were isolated as described in Sections 2.3 and 2.4. During the separation steps, all fractions obtained by the separations were determined by a cress bioassay, and the active fraction was applied to the next chromatographic separation. The germination rate of cress seeds was over 90% at 48 h after sowing, while the germination rate of *V. myuros*, *L. multiflorum*, *E. crus-galli*, and *P. pretense* was 35–60% at 48 h after sowing. Thus, the selection of the uniform germination seeds of these weed species is necessary to obtain reliable data, and takes some time for selection, while selection is not necessary for the cress seeds because of their stable germination rate. Comparing $IC_{50}$ values of cress with four weed species, the sensitivity of cress to the extracts was not low and not high (Figures 2 and 3, Table 1). Therefore, cress seeds were selected as a bioassay species during separation steps of the extracts without germination procedure. The separation steps generate many isolated fractions, for which allelopathic activity needed to be determined.

The chemical structures of the compounds **1** and **2** were identified as umbelliferone and (+)-rhododendrol based on their spectrum data and published data [34,35]. Both compounds significantly inhibited the growth of roots and coleoptiles of *V. myuros* (Figure 5). Considering $IC_{50}$ values of the compounds, the inhibitory activity of (+)-rhododendrol was 11–14 times greater than that of umbelliferone. *V. myuros* was selected as the test weed species to determine the inhibitory activity of both compounds because its sensitivity to the extracts of *M. glyptostroboides* was not the highest or lowest among the four weed species (Table 1).

Umbelliferone (7-hydroxycoumarin) is a derivative of coumaric acid, and synthesized through the shikimate pathway [36]. The compound showed anti-fungal activity and anti-oxidant activity [37,38]. The compound also showed growth-inhibitory activity on the seedlings of lettuce (*Lactuca sativa* L.), alfalfa (*Medicago sativa* L.), and *Festuca rubra* L. [38,39]. (+)-Rhododendrol (4-[(3R)-3-hydroxybutyl]phenol) is a phenolic compound and has been isolated from Rhododendron genus species [40]. The compound showed inhibitory activity on tyrosinase in animal and human cells, and was once developed for cosmetics as a melanin-production inhibitor [41,42]. The compound also showed growth-inhibitory activity on cress seedlings [43]. In addition, (+)-rhododendrol has recently isolated from the fallen leaves of *M. glyptostroboides* and showed growth-inhibitory activity on *L. multiflorum* as an allelopathic agent [35].

Many secondary metabolites such as fatty acids, flavonoids, terpenoids, lignans, and norlignans were identified in the leaves, seeds, and barks of *M. glyptostroboides* [10]. Among them, flavonoids—quercetin, catechin, and epicatechin—were reported to show growth-inhibitory activity of several plant species [44–48]. Those compounds also inhibited the nitrification process, which is an important step in the nitrogen cycle in soil [49]. α-Pinene was also identified in the leaves and reported to show allelopathic activity on the growth of *Solanum elaeagnifolium* Cav. [50].

Conventional agriculture relies on synthetic herbicide to control weeds. However, excessive application of herbicides increases the potential to develop resistant weeds [51–53]. Therefore, an alternative method based on natural products is now a most demanding issue. The application of leaf extracts and their soaking water obtained from several plant species such as *Solidago canadensis* L, *Imperata cylindrica* (L.) Beauv., *Leucaena leucocephala* (Lam.) de Wit., *Tithonia diversifolia* (Hemsl). A. Gray, and *Lantana camara* L. as foliar spray and/or irrigation water significantly suppressed the germination and growth of several weed spaces in laboratory, greenhouse, and field conditions [54–58]. Soil mixture with the leaves of *I. cylindrica*, *L. leucocephala*, and *L. camara* L. also significantly suppressed the germination and growth of some weed species in greenhouse and field conditions [59–62]. Leaf mulch of *L. leucocephala* covered on soil surfaces inhibited the germination and growth of *Vigna unguiculata* (L.) Walp., and the root nodulation of *V. unguiculate* [63]. Those investigations indicate that the leaves of some plant species possess inhibitory activity on the germination and growth of certain weed species, and probably contain extractable allelochemicals. Some of those allelochemicals may also be liberated into the soil during their decomposition processes, causing growth-inhibitory activity. Therefore, some allelopathic plants are applicable for weed control in ecofriendly agriculture.

The present investigation suggests that the leaves of *M. glyptostroboides* have inhibitory activity on the growth of *V. myuros*, *L. multiflorum*, *E. crus-galli*, and *P. pretense*. Umbelliferone and (+)-rhododendrol were isolated and identified in the extracts as allelopathic active substances. Both allelopathic substances showed growth-inhibitory activity on *V. myuros*. When the leaves of *M. glyptostroboides* were incorporated into the soil, certain allelopathic substances in the leaves could be liberated into the field soil through their decomposition process [26–29], and these liberated compounds may cause growth suppression on some weed species. The extracts of the leaves can also apply as foliar spray to suppress the growth of some weed species.

## 5. Conclusions

The extracts of *M. glyptostroboides* leaves obtained from pruned branches significantly suppressed the growth of four weed species, *V. myuros*, *L. multiflorum*, *E. crus-galli*, and *P. pretense*. The allelopathic substances causing the inhibitory activity of the extracts were isolated and identified as umbelliferone and (+)-rhododendrol. The leaves may also contain some other allelopathic substances. Therefore, the extracts and leaves of *M. glyptostroboides* obtained from pruned-branch waste are potentially useful for weed control in certain agricultural settings such as foliar spray for extracts, mulch, and soil additives for leaves, to reduce synthetic herbicide application in crop production and to develop ecofriendly agriculture. However, the allelopathic activity of the leaves and extracts in field conditions should be evaluated.

**Author Contributions:** Conceptualization, H.K.-N.; methodology, T.T. and H.K.-N.; software K.M. and C.S.; validation, T.T. and H.K.-N.; formal analysis, K.M. and C.S.; investigation, K.M., C.S. and S.T.; data curation, T.T. and H.K.-N.; writing H.K.-N.; visualization, H.K.-N.; supervision, H.K.-N. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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