

RESEARCH ARTICLE

Assessment of allelopathic potential of *Dalbergia cochinchinensis* Pierre and its growth inhibitory substance

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ABSTRACT

In forestry systems, many types of forest trees possess allelopathic properties and release a wide variety of allelochemicals that influence the growth and development of surrounding species. *Dalbergia cochinchinensis*, a forest tree distributed in Southeast Asia, is reputed to possess several biological properties and contain several secondary compounds. However, there have been no studies on the allelopathy of *D. cochinchinensis*. Therefore, *D. cochinchinensis* leaf extracts were examined for allelopathic potential. The present study showed that *D. cochinchinensis* extracts significantly inhibited the seedling growth of six test plant species: timothy, Italian ryegrass, barnyard grass, cress, alfalfa, and lettuce. Concentrations of the *D. cochinchinensis* extracts negatively correlated with the shoot length ($r = -0.50$ to -0.89) and root length ($r = -0.65$ to -0.89) of all the test plant species. The extracts were then purified using several chromatographic steps and the growth inhibitory substance was isolated. The chemical structure of the substance was identified through spectroscopic analysis as 3,4-dihydroxybenzoic acid (protocatechuic acid). Protocatechuic acid at a concentration higher than 3 mM significantly inhibited the growth of cress seedlings, whereas barnyard grass seedlings were inhibited at concentrations higher than 0.3 mM. As the protocatechuic acid concentration increased, the seedling growth of cress and barnyard grass was significantly reduced. I_{50} values showed the effectiveness of protocatechuic acid against both test plant species was apparently greater on root growth than shoot growth. Results of this study suggest that protocatechuic acid isolated from *D. cochinchinensis* might be responsible for its inhibitory effects.

Keywords: Allelopathy; *Dalbergia cochinchinensis*; Inhibitory activity; Protocatechuic acid; Sustainable agriculture

INTRODUCTION

In agriculture, different kinds of synthetic herbicide have been widely used for their easy availability and efficacy in controlling weeds. However, excessive and repeated use of synthetic herbicides has had negative effects such as soil sickness, environmental pollution, and an increase in the number of herbicide-resistant biotypes in weed species (Duary, 2008; Awan et al., 2015; Hasanuzzaman et al., 2020). These findings indicated that synthetic herbicides are not sustainable for agricultural production over long periods of time. Thus, many researchers have focused on searching for environmentally safer and unique compounds that are more effective for weed management (Amb and Ahluwalia, 2016; Bari and Kato-Noguchi, 2017; Poonpaiboonpipat and Poolkum, 2019; Sakamoto et al., 2019).

In this regard, allelopathy is considered to be practicable and ecologically balanced for weed management in sustainable agriculture (Bhowmik and Inderjit, 2003; Jabran et al., 2015; Dafaallah et al., 2019). The forest systems have a diverse range of natural forest species. Some of forest species have been possessed the allelopathic activity against other organisms that make remarkable impacts on the components of ecosystem including plant community (Nishimura and Mizutani, 1995; Wardle et al., 1998; Bertin et al. 2003). Allelopathy is a phenomenon in which plants release secondary metabolites or allelochemicals into the environment in several ways such as volatilization, root exudation, leaching, and decomposition of plant residues (Rice, 1984; Koocheki et al., 2013). Those allelochemicals interfere with the establishment and growth of neighboring plants (Souto et al., 1994; Souto et al., 2001; Latif et al.,

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2017). For example, the foliage leachate of *Eucalyptus* species containing a large number of allelopathic substances markedly decreased the growth and abundance of understory and other nearby species (Ziaebrahimi et al., 2007, Ruwanza et al., 2015). *Melia dubia* Cav. has also been reported to decrease in the growth, biomass and fruit yield of chilli (*Capsicum frutescens* L.) and eggplant (*Solanum melongena* L.) and its allelopathic substances were identified as phenolic acids and their derivatives, alkaloids, methyl ketones (volatile allelochemical) (Parmar et al., 2019). Therefore, forest species are considered as one of the attractive candidates to search the allelopathic substances for the development of bioherbicide for sustainable weed management in the future.

Dalbergia cochinchinensis Pierre (Thailand rosewood) is a forest tree belonging to the Fabaceae family and is widely distributed in Southeast Asia. This plant is an important deciduous tree in agroforestry systems of the tropics with much ecological and economic importance. The cores of this plant are used in traditional folk medicine to treat blood-stasis and tumor (Palasuwan et al., 2005). Additionally, different biological properties of *D. cochinchinensis* have been documented such as anti-androgen activities, anti-ageing, antimicrobial, and antioxidants (Kuroyanagi et al., 1996; Pathak et al., 1997; Peterson and Dwyer, 1998; Palasuwan et al., 2005). It has also been reported to contain several compounds such as flavonoids, terpenes, benzoic acids, and volatile components (Shirota et al., 2003; Zhong et al., 2013; Liu et al., 2016; Xiang et al., 2018). Several researchers have been suggested that some plants that have medicinal values may possess phytotoxic properties with phytotoxic substances (Boonmee et al., 2018; Bari et al., 2019; Rob et al., 2020). In addition, some *Dalbergia* species have been shown to possess allelopathic activity that suppressed the growth and yield of target species (Singh et al., 1999; Singh et al., 2006). Based on medicinal properties and phytotoxic potential of *Dalbergia* species, it is possible that *D. cochinchinensis* may also contain inhibitory substances with allelopathic activity. Therefore, this present study aimed to determine the allelopathic activity of *D. cochinchinensis* on the seedling growth of six test plant species and to isolate and identify its inhibitory substances under laboratory condition.

MATERIALS AND METHODS

Plant material

Dalbergia cochinchinensis leaves were freshly collected from Phitsanulok Province, Thailand. All collected leaves were washed thoroughly using distilled water. The leaves were immediately dried under shade and then pulverized using an electric grinder. The ground powder was subsequently stored in a tightly closed plastic container and kept in the refrigerator at 2°C for further experiments.

Test plant species

Six plant species were used as the receptor plants to determine the allelopathic activity: timothy (*Pheleum pratense* L.), Italian ryegrass (*Lolium multiflorum* Lam.), and barnyard grass (*Echinochloa crus-galli* (L.) P. Beauv.) were selected as monocotyledonous plants, and cress (*Lepidium sativum* L.), alfalfa (*Medicago sativa* L.), and lettuce (*Lactuca sativa* L.) were selected as dicotyledonous plants. Those plant species were selected on the basis of their known growth behavior, higher sensitivity to allelopathic substances and common weed characteristics (Kato-Noguchi et al., 2016; Islam et al., 2017). The seeds of the monocotyledonous plants were allowed to germinate in distilled water and then transferred to dark condition at 25°C until the root of each seedling emerged 1 mm in length prior to starting the bioassay experiments.

Extract preparation

The leaf powder (100 g) of *D. cochinchinensis* was soaked in 70% (v/v) aqueous methanol (500 mL) and kept in the dark at room temperature (25 ± 2°C). After soaking for two days, the liquid was poured through a Buchner funnel with filter paper (No. 2; Toyo Roshi Kaisha, Tokyo, Japan). The residue was immediately re-soaked in cold methanol (500 mL) and kept in the same condition as described above for one day, and then filtered. The organic solvents of the combined filtrate were removed under reduced pressure until dryness. The crude residue was diluted with methanol to obtain concentrations from 0.001 to 0.3 g D.W. equivalent extract/mL.

Treatment of the test plant species with the *D. cochinchinensis* extracts

The growth bioassay of the extracts was determined using the method described by Islam et al. (2019). An aliquot of the *D. cochinchinensis* extracts at assay concentrations was added to a Petri dish each containing a sheet of filter paper (No. 2) and dried in a laminar flow cabinet. Then, the filter paper was moistened with Tween 20 solution. Seeds (n=10) of the dicotyledonous plants or sprouted seeds (n=10) of the monocotyledonous plants were placed in a Petri dish and then incubated in darkness at 25°C. The treatments were designed in a completely randomized manner, each with six replications. After two days incubation, the growth parameters (shoot and root length) were measured for each seedling. The percentage of seedling growth was calculated using the following formula:

$$\% \text{ of seedling growth length} = \frac{\text{growth length of treated seedling}}{\text{growth length of control seedling}} \times 100$$

Isolation and identification of the inhibitory compound

Dalbergia cochinchinensis extracts were prepared using the same process as described above. The crude residue was suspended in distilled water and then adjusted to a pH of 7.0 using 1 M phosphate buffer. The aqueous residue was transferred to a separating funnel for liquid-liquid partition with ethyl acetate (equal volume, v/v), and divided into ethyl acetate and aqueous fractions. The biological activity of both fractions was determined using cress and barnyard grass bioassay. The ethyl acetate fraction was then evaporated to dryness after overnight soaking in anhydrous sodium sulfate as a further purification step. After every purification step, all fractions obtained from each step were tested for their growth inhibitory activity against cress seedlings as described above. The residue was then chromatographed through a column of silica gel (60 g, silica gel 60, 70-230 mesh; Nacalai Tesque, Kyoto, Japan), and eluted with stepwise gradient mixtures of *n*-hexane: ethyl acetate (9:1 to 2:8 (v/v), and methanol for final elution. The active fraction was further subjected to a column of Sephadex LH-20 (50 g; GE Healthcare, Uppsala, Sweden).

The mobile phase was used as stepwise water: methanol mixtures (8:2 to 2:8 (v/v), and methanol. The active fraction (60% aqueous methanol) obtained by Sephadex LH-20 was then transferred to reversed-phase C₁₈ cartridges (1.2×6.5 cm; YMC Co. Ltd., Kyoto, Japan). The cartridge was eluted step by step with water: methanol mixtures (9:1 to 2:8 (v/v), and methanol. The active fraction (10% aqueous methanol) obtained by reversed-phase C₁₈ cartridge was then dried for final purification by HPLC. The column (250 × 4.6 mm I.D., particle size 5 μm, ODS-3; Inertsil, Tokyo, Japan) was eluted with 10% aqueous methanol, 0.5 mL per min flow rate. Peaks eluted were detected by absorbance at 220 nm. The diagram of the isolation procedure is shown in Fig.1. Finally, the chemical structure of the active substance was identified using HRESIMS, ¹H NMR (400 MHz, CD₃OD), and ¹³C NMR (100 MHz, CD₃OD) spectra.

Determination of the phytotoxic activity of the identified inhibitory substance

Five concentrations (0.3 to 30 mM) were prepared by dissolving and diluting the identified inhibitory substance

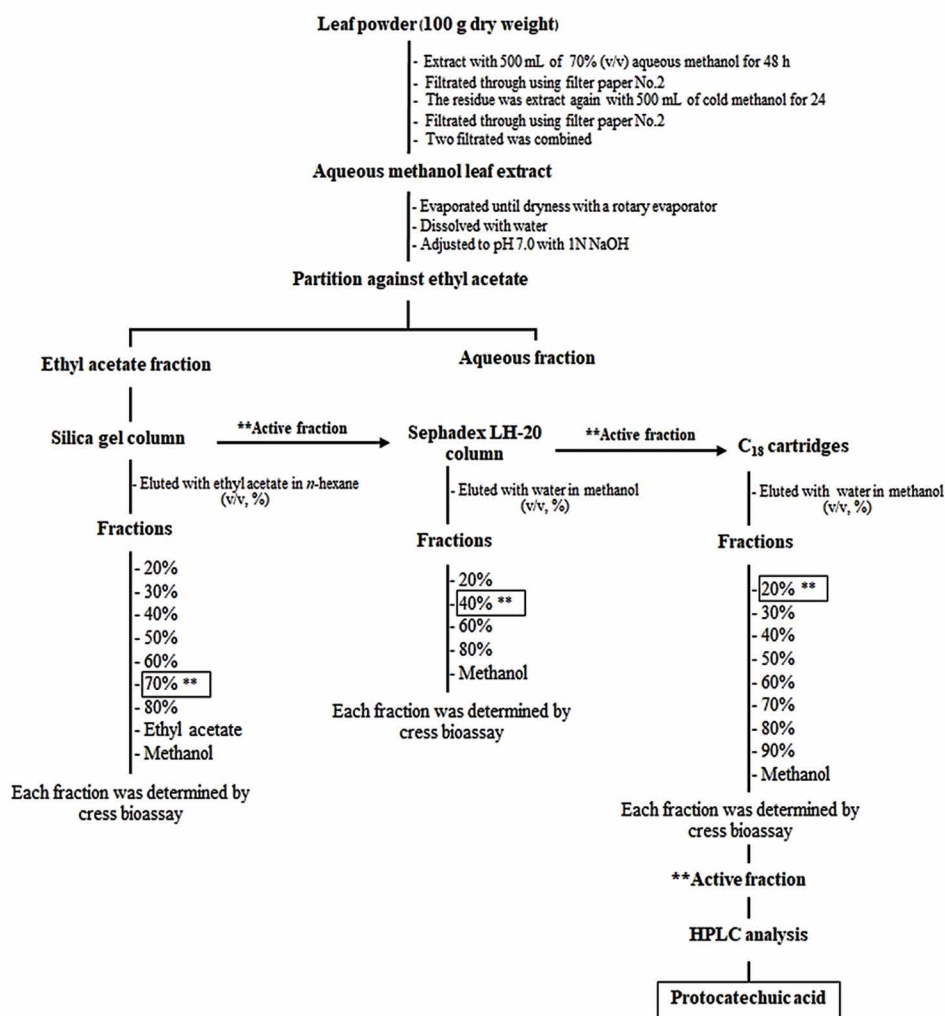


Fig 1. Procedure for the isolation and purification of protocatechuic acid from *D. cochinchinensis* leaf extracts.

with methanol. Each concentration was determined by cress and barnyard grass bioassay as described above. The treatments were designed in a completely randomized manner, each with three replications. The growth parameters (shoot and root length) were measured for each seedling. The percentage of seedling growth was calculated using the formula as described above.

Statistical analysis

Significant differences of the shoot and root growth between treatment and control were analyzed using Tukey’s test with SPSS version 16.0 (Figs. 2-4). The Student’s t-test was used to analyze significant differences between the shoot and root growth (Fig. 5 and 6). The concentration required for 50% inhibition of each test plants was calculated using GraphPad

Prism 6.0 (Fig. 5 and 6). The relationship between seedling growth of the test plants and the extract concentrations was analyzed using two-tailed Pearson correlation test (Fig. 2).

RESULTS

Allelopathic potential of the *D. cochinchinensis* extracts

The seedling growth of all the target plant species was affected by the *D. cochinchinensis* extracts and varied with the amount of the extracts applied as shown in Fig. 2. At 0.03 g D.W. equivalent extract/mL, the shoot length of cress and lettuce was completely inhibited (100%), whereas Italian ryegrass, alfalfa, timothy, and barnyard grass were inhibited to 13.8, 16.8, 53.7, and 84.8% of control length,

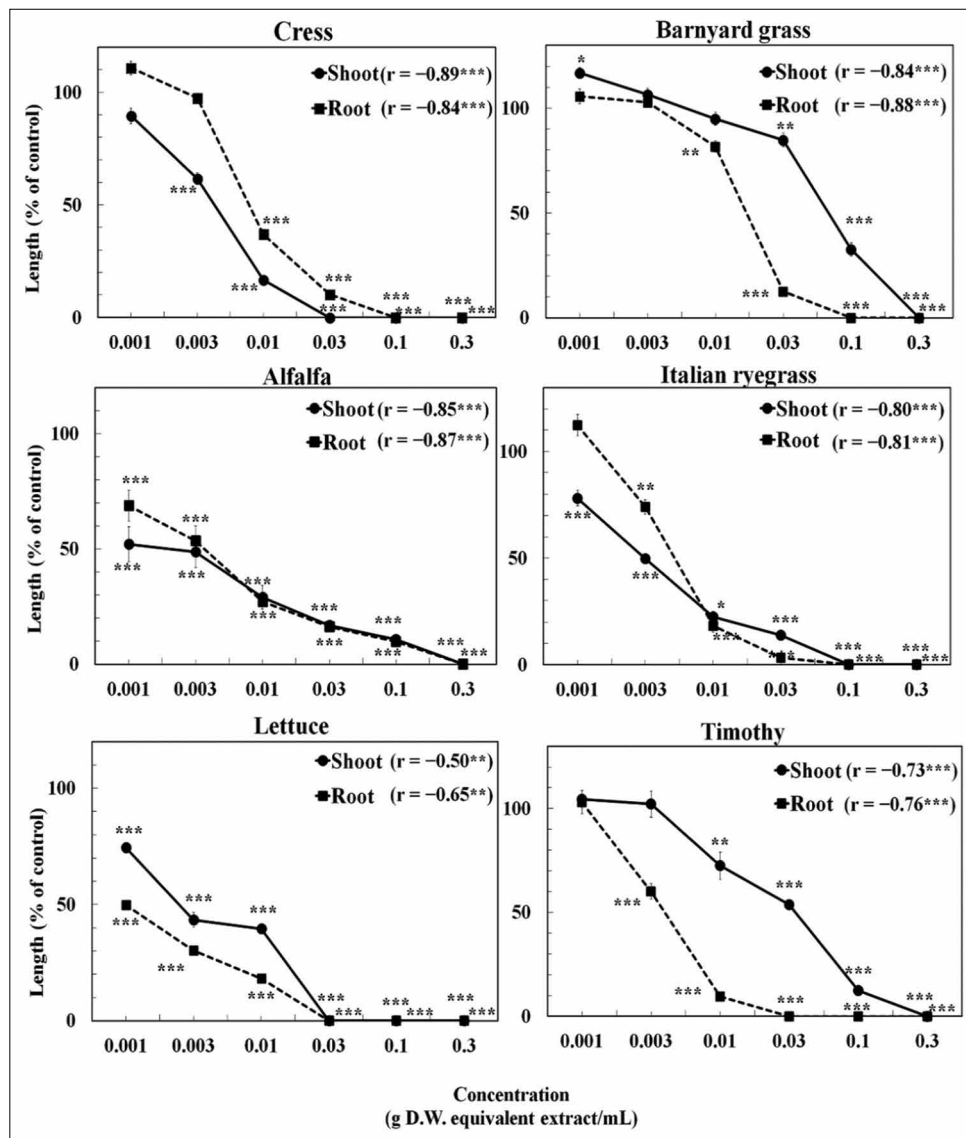


Fig 2. Effect of *Dalbergia cochinchinensis* leaf extracts on the seedling growth of six test plant species with different concentrations. The bars on each experiment show mean ± SE with six replications (n=60). The asterisks represent a statistically significant comparison between extract concentration and control according to Tukey’s test: *p<0.05, **p<0.01, and ***p<0.001. Correlation coefficient (r); asterisks represent statistical significance: **p<0.01 and ***p<0.001 by the two-tailed Pearson correlation test.

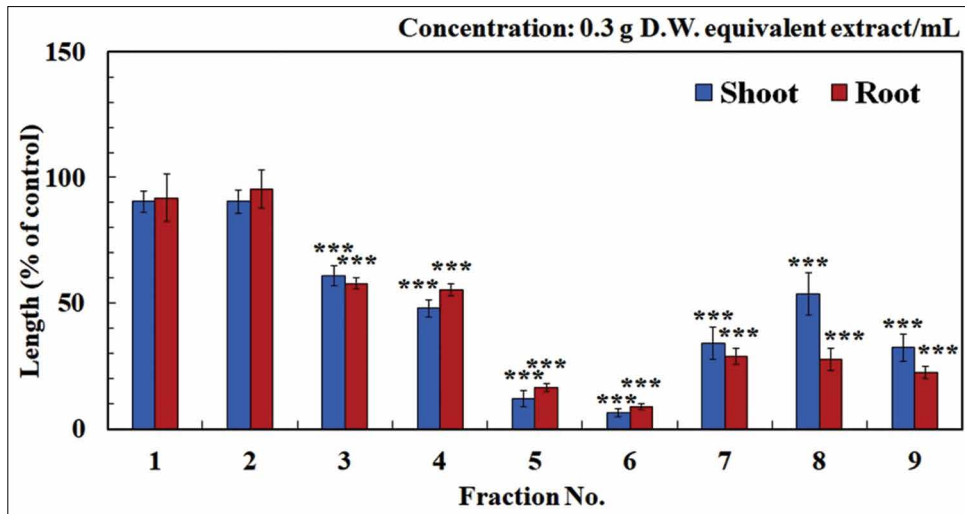


Fig 3. Effect of fractions obtained from silica gel column eluted with ethyl acetate in *n*-hexane (v/v) on the seedling growth of cress. The bars on each experiment show mean \pm SE with three replications (n=30). The asterisks represent a statistically significant between treatment and control according to Tukey's test: ***p<0.001.

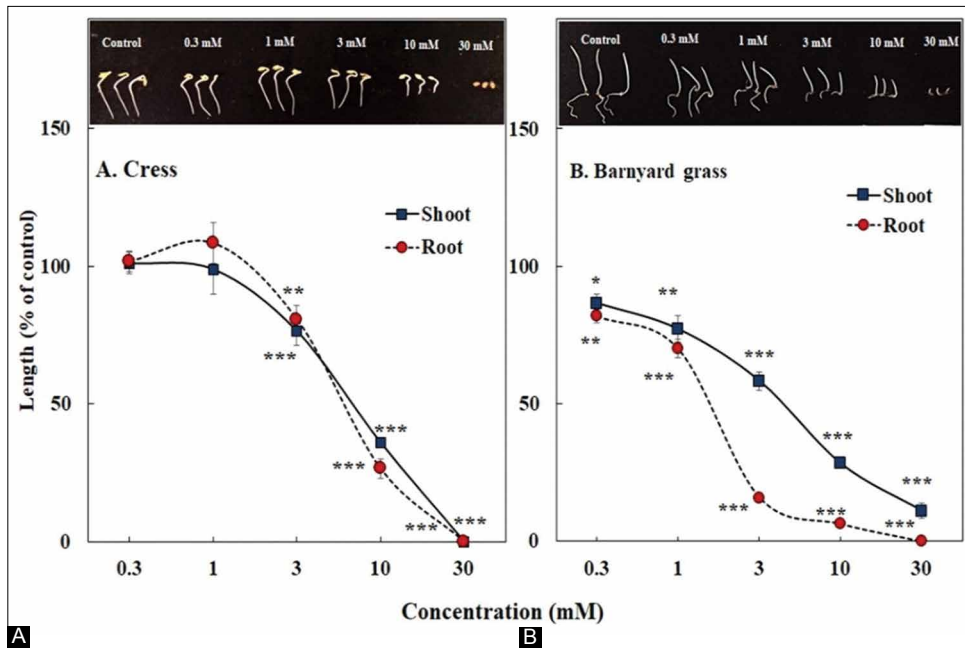


Fig 4. Effect of protocatechuic acid isolated from the *Dalbergia cochinchinensis* leaf extracts on the seedling growth of A. cress and B. barnyard grass. The bars on each experiment show mean \pm SE with three replications (n=30). The asterisks represent a statistically significant between treatment and control according to Tukey's test: *p<0.05, **p<0.01, and ***p<0.001.

respectively. At the same concentration, the root length of timothy roots was completely inhibited (100%), whereas lettuce, Italian ryegrass, cress, barnyard grass, and alfalfa roots were inhibited to 0.8, 3.3, 10.2, 12.6, and 16.3% of control length, respectively. At the highest concentration (0.3 g D.W. equivalent extract/mL), the shoot and root length of all the test plant species were completely inhibited (100%). In addition, the results showed a strong negative correlation between extract concentration and shoot length ($r = -0.50$ to -0.85) and root length ($r = -0.60$ to -0.89) of the test plant species (Fig. 2). That means the degree

of inhibitory effect on all the test plant species increased as the concentration increased.

The difference in I_{50} values between each test plant species is shown in Fig. 5, with the lettuce seedlings being the most susceptible to the *D. cochinchinensis* extracts compared with the other plant seedlings. The barnyard grass seedlings were the least susceptible to the *D. cochinchinensis* extracts. Furthermore, the effectiveness of the *D. cochinchinensis* extracts against all the test plant species was apparently greater on the roots than their

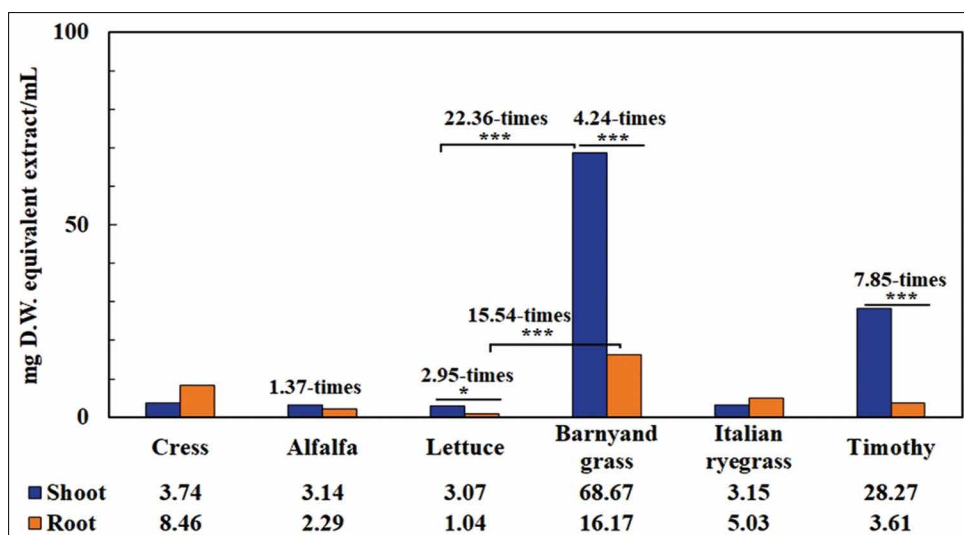


Fig 5. The concentration required for 50% inhibition (I_{50} values) of seedling growth of the six test plant species by the *Dalbergia cochinchinensis* leaf extracts. The asterisks represent a statistically significant comparison between the shoot growth and root growth of each test plant species according to Student's t-test: * $p < 0.05$ and *** $p < 0.001$.

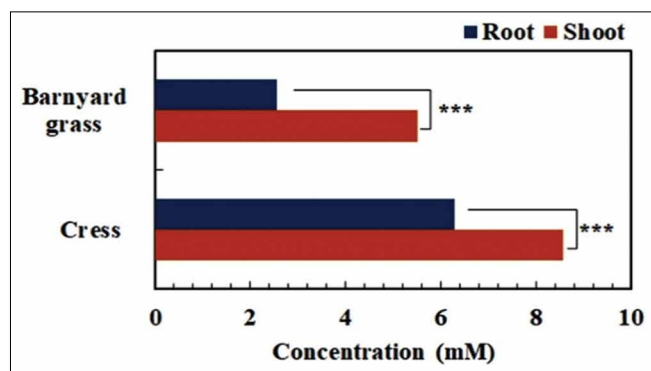


Fig 6. The concentration required for 50% inhibition (I_{50} values) of the seedling growth of cress and barnyard grass by protococatechuic acid isolated from the *Dalbergia cochinchinensis* leaf extracts. The asterisks represent a statistically significant comparison between the shoot and root length of each test plant species according to Student's t-test: *** $p < 0.001$.

corresponding shoots, except for the cress and Italian ryegrass seedlings.

Isolation and identification of the inhibitory substance

The bioassay results of the separated fractions obtained from the silica gel column showed significant inhibition on the cress seedlings (Fig. 3). Fraction 6 (70% ethyl acetate in *n*-hexane) showed the highest inhibition on the shoot and root length of cress with 6.4 and 8.8% of control length, respectively. Therefore, fraction 6 obtained from the silica gel column was repeatedly purified through a series of chromatography and HPLC steps. Finally, the chemical structure of the inhibitory substance was identified by spectroscopic analysis. The molecular formula was determined as $C_7H_6O_4$, which was identified by HRESIMS at m/z 153.0167 [M-H]⁻ (calcd for $C_7H_5O_4$, 153.0188, $\Delta = -2.1$ mmu). The proton NMR spectrum (400 MHz, CD_3OD)

showed δ_H 7.32 (d, $J = 2.6$ Hz, 1 H, H2), 7.42 (dd, $J = 8.6, 2.6$ Hz, 1 H, H6), 6.79 (d, $J = 8.6$ Hz, 1 H, H5). The carbon NMR spectrum (100 MHz, CD_3OD as internal standard) showed δ_C 170.3 (C-7), 151.5 (C-4), 146.1 (C-3), 123.9 (C-6), 123.2 (C-1), 117.7 (C-5), 115.7 (C-2). Based on the spectroscopic data and comparing with published data, the substance was identified as 3,4-dihydroxy benzoic acid, which is known as protococatechuic acid (Fig. 7) (Chang et al., 2009).

Biological activity of the identified inhibitory substance

The inhibitory activity of protococatechuic acid isolated from *D. cochinchinensis* was tested against cress and barnyard grass seedlings (Fig. 4 and 6), and showed significant inhibitory activity against both test plant species. The level of inhibition depended on the concentration and plant species. At 10 mM protococatechuic acid, the shoot and root length of cress was inhibited to 35.9 and 26.5% of control length, respectively (Fig. 4A), whereas the barnyard grass seedlings were inhibited to 28.3 and 6.3% of control length, respectively (Fig. 4B). At the highest concentration (30 mM), the seedling growth of both test plant species was completely inhibited, except for barnyard grass shoots, which were inhibited to 11.1% of control length. The I_{50} values for the shoot and root length of cress were 7.56 and 5.28 mM, respectively, and those of barnyard grass were 5.52 and 2.57 mM, respectively (Fig. 6). Comparing the I_{50} values, the root length of both test plant species was more inhibited by protococatechuic acid compared with their shoot length.

DISCUSSION

The present study showed that *D. cochinchinensis* significantly inhibited the seedling growth of all the test plant species

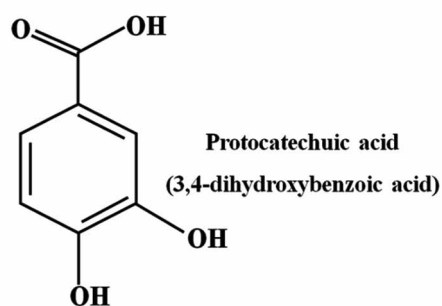


Fig 7. The chemical structure of protocatechuic acid isolated from the *Dalbergia cochinchinensis* leaf extracts.

in both a species- and concentration-dependent manner (Fig. 2 and 5). Therefore, our results indicated that the *D. cochinchinensis* extracts have allelopathic potential, which led to the isolation and identification of the inhibitory substance in *D. cochinchinensis*.

The inhibitory substance was isolated from *D. cochinchinensis* and identified by spectroscopic analysis as 3,4-dihydroxybenzoic acid, commonly known as protocatechuic acid (Fig. 3). Protocatechuic acid has been detected in many plant spices, such as *Euterpe oleracea* (Pacheco-Palencia et al., 2008), *Viburnum foetidum*, *Houttuynia cordata*, *Perilla ocimoides* (Seal et al., 2016), *Hibiscus sabdariffa* (Hassan and Švajdlenka, 2017), and *Veronia peregrina* (Polechońska et al., 2019). In addition, it is well known that protocatechuic acid is one of the biologically active components in medicinal plants (Ellnain-Wojtaszek, 1997; Herrmann and Nagel, 1989; Ali et al., 2005). Different biological and biological activities of protocatechuic acid have been extensively studied including its antioxidant, antibacterial, anticancer, anti-inflammatory, antihyperglycemic, and antiapoptotic activities (Kakkar and Bais, 2014; Semaming et al., 2015; El-Sonbaty et al., 2019). However, although protocatechuic acid has been found in many other plants, this study is the first to report on protocatechuic acid isolated from *D. cochinchinensis*.

Based on the results of the molecular bioassay, the seedling growth of cress and barnyard grass was significantly inhibited by protocatechuic acid (Fig. 4 and 6). Our results are in agreement with Beninger et al. (2004), Wu et al. (2007), Shang et al. (2017), and Fu et al. (2019), who reported that protocatechuic acid has phytotoxic effects and can influence the growth of plants at various stages. However, the relationship between chemical structure and mechanism of allelopathy of protocatechuic acid is not well understood. Accordingly, the -OH group at the *meta* position in protocatechuic acid may be responsible for its inhibitory activity. Gao et al. (2011) reported that protocatechuic acid (-OH group at the *meta* position) exhibited high inhibitory activity (4-fold higher activity)

compared with vanillic acid (-OCH₃ group at the *meta* position). On the other hand, the -COOH group may be another reason for the decreased activity of protocatechuic acid because of the electron-withdrawing properties of the -COOH group in protocatechuic acid influencing the biological activity of the hydroxybenzoic acid derivatives (Rice-Evans et al., 1996; Velika and Kron, 2012).

These findings are in accordance with those of Chou and Leu (1992), Wu et al. (2007), and Fu et al. (2019), who reported that the level of activity depends on the target species and the concentration of protocatechuic acid. In view of the results described above, *D. cochinchinensis* has allelopathic properties, which might be due to protocatechuic acid contributing to its inhibitory activity.

CONCLUSION

This study is the first to report on the allelopathic activity of protocatechuic acid identified from *D. cochinchinensis*. Protocatechuic acid displayed different inhibitory activity on the tested plants proportional to the extract concentrations. These results suggest that protocatechuic acid may contribute to the growth inhibitory activity of *D. cochinchinensis*.

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Authors' contributions

Ramida Krumrsri carried out the experiments, analyzed the data and wrote the manuscript. Arihiro Iwasaki and Kiyotake Suenaga contributed to the determination of chemical structures of the compounds. Hisashi Kato-Noguchi designed the experiments, supervised the data analysis and contributed greatly to the writing of the manuscript.

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