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RESEARCH ARTICLE

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Isolation and biological activities of 3-hydroxy-4(1H)-pyridone

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ABSTRACT

3-Hydroxy-4(4H)-pyridone (3,4-DHP), a degraded product of mimosine [β -[N-(3-hydroxy-4-oxypyridyl)]- α -aminopropionic acid], is known to cause goiters, loss of hair, and infertility in animals, but limits of 3,4-DHP on separation and purification have prevented efforts on investigating other toxicity and biological properties of 3,4-DHP. By this study, a novel and simple isolation of 3,4-DHP was developed either from *Leucaena* leaves using an ion-exchanged resin or mimosine degraded in high temperature (110°C, 6 h). The inhibition of mimosine on the growth of barnyardgrass was approximately fourfold higher ($IC_{50} = 0.04 \text{ mg g}^{-1}$) than that of 3,4-DHP ($IC_{50} = 0.15 \text{ mg g}^{-1}$). In general, the antifungal activity of mimosine is much stronger than that of 3,4-DHP, but it differs depending on the kind of fungi. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of 3,4-DHP, in contrast with the growth inhibitory activity, is about fourfold stronger [$EC_{50} = 2.4 \text{ mg g}^{-1}$ gallic acid equivalent (GAE)] than that of mimosine [$EC_{50} = 10.3 \text{ mg g}^{-1}$ GAE]. This study is the first to report on the herbicidal, antifungal, and antioxidant activities of 3,4-DHP.

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KEYWORDS

Mimosine; 3, 4-DHP; inhibition; antifungal; antioxidant activity; degradation

Introduction

Mimosine [β -[N-(3-hydroxy-4-oxypyridyl)]- α -aminopropionic acid] is a non-protein amino acid, and is a major compound in all plant parts of Mimosaceae, which include *Leucaena* (*Leucaena leucocephala*), *Leucaena glauca*, and other legumes belonging to *Mimosa* spp. (Xuan et al. 2006). Structurally, it is an analog of dihydroxyphenylalanine with a 3-hydroxy-4-pyridone ring instead of a 3,4-dihydroxyphenyl ring (Figure 1). Despite *Leucaena* having high protein content and high annual yield, the presence of mimosine and condensed tannin has limited the wide use of this plant as animal feed (Chanchay and Poosaran 2009; Tan et al. 2011; Soltan et al. 2013). This compound can cause alopecia, growth retardation, cataract, and infertility in animals (Xuan et al. 2006). Mimosine has general antimitotic activity that blocks the cell cycle in the large G_1 phase (Khanna & Lavin 1993) and inhibits DNA synthesis, which prevents the formation of the replication fork by altering deoxyribonucleotide metabolism (Gilbert et al. 1995). This amino acid may act as a tyrosine analog which incorporates biologically vital proteins and in turn causes hair loss (Crouse et al. 1962).

Mimosine can be degraded to 3,4-DHP [3-hydroxy-4(4H)-pyridone] (Figure 1) by microorganisms in the rumen (Jones & Lowry 1984; Allison et al. 1992; Dominguez-Bello & Stewart 2006). In vivo, the amount of dietary mimosine cannot be completely degraded to 3,4-DHP, as it is dependent on the adaption of ruminal microorganisms to mimosine (Ruiz-Gonzalez et al. 2011; Arjona-Alcocer et al. 2012; Contreras-Hernandez et al. 2013; Ruz-Ruiz 2013; Rodriguez et al. 2014). Mimosine can also be degraded by bacteria in the rhizome nodule of *Leucaena*, by endogenous enzymes in the *Leucaena* plants (Smith & Fowden 1966; Lowry et al. 1983; Lyon 2006), or by hydrolytic enzymes of *Leucaena* psyllids

(jumping plant lice) (Kamada et al. 1996). Although 3,4-DHP is known to be toxic, it is suggested to have lower toxicity than mimosine (Tawata 1990), but it also causes goiters, loss of hair, and reduced productivity when fed to animals (Crouse et al. 1962; Hegarty et al. 1964).

Except for these effects of 3,4-DHP towards animals, other biological activities such as herbicidal and antifungal activities and antioxidant capacity of this compound have not been known. Compared with other plants, the antioxidant property of *Leucaena* or other *Mimosae* has been investigated sporadically. Benjakul et al. (2014) reported the antioxidant activities in *Leucaena* seeds and observed that the antioxidative activities were dependent on extracting solvents. The antioxidant activities in *Mimosa pudica* were also investigated (Genest et al. 2008; Parmar et al. 2015), but how much mimosine contributed to the antioxidant property in *Leucaena* and *M. pudica* has not been studied. Since mimosine can also be degraded to 3,4-DHP by rumen organisms (Jones & Lowry 1984; Allison et al. 1992; Dominguez-Bello & Stewart 2006), it also needs to clarify the antioxidant capacities of this compound to help clarify how it influences animal health and the quality of meat and milk. Regarding the chemical structures of mimosine and 3,4-DHP, they both have a single OH in the benzene ring which may possess promising antioxidant properties that need to be investigated.

Spears and Weiss (2008) and Chauhan et al. (2014) reported that antioxidant dynamics in animal feeds contribute effectively to animal health and quality of meat and milk. As *Leucaena* leaves can be provided as feedstocks, to understand the antioxidant activities of mimosine and its degraded product 3,4-DHP can provide more evaluable information on the use of this plant as animal feed. No toxicity on human consumption of mimosine has been reported (Poonam & Pushpa

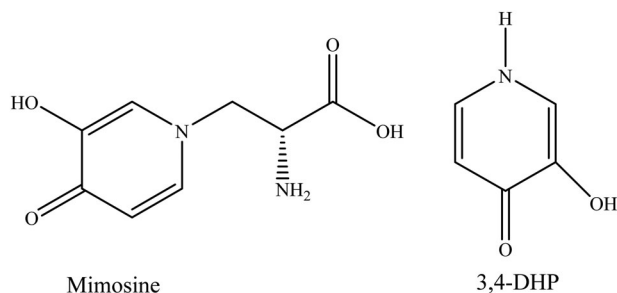


Figure 1. Chemical structures of mimosine and 3,4-DHP.

1995), and in Okinawa, Japan, dried *Leucaena* leaves are served as tea. In addition to the antioxidant capacity of *Leucaena* and its chemical components such as phenols (Prasad 1995) and tannins, mimosine and 3,4-DHP may also help enhance the value of *Leucaena* tea consumption.

The determination of 3,4-DHP was done using analytical instruments such as colorimetric method (Allison et al. 1990) that used a mixture of ferric chloride reagents to detect mimosine, 3,4-DHP, and 2,3-DHP visually, and high performance liquid chromatography (HPLC) (Tangendjaja & Wills 1980; Acamovic et al. 1982; Soedarjo et al. 1994). Lalitha et al. (1993) determined mimosine and 3,4-DHP by a spectrophotometric method using azodye formed with *p*-nitroaniline. The degradation of mimosine to 3,4-DHP by bacteria and enzymes was described as mentioned above; however, no research on the degradation of mimosine to 3,4-DHP under temperature and chemical factors has been so far conducted.

In this study, a novel and simple isolation of 3,4-DHP from *Leucaena* leaves and degradation of mimosine by high temperature using an oil bath method is described. It is the first report to evaluate the herbicidal and antifungal activities and antioxidant capacity of 3,4-DHP.

Materials and methods

Mimosine

The mimosine used in this study was purified in the Department of Subtropical Bioscience and Biotechnology, Faculty of Agriculture, University of the Ryukyus, Japan, by a simple method introduced by Tawata (1990) with some modifications. *Leucaena* leaves were boiled and the solution was filtered. Ultra-filtration was conducted at 4 atm at 30°C, 700 rpm equipped with a Filtron membrane. A column packed with acid form Amberlite IRA (technical grade) was employed and the resin was then washed with 2N NH₄OH with the pH adjusted to 4.5–5.0. The structure of mimosine and its molecular weight (mol wt = 198) were confirmed using Electron-Impact Ionization Mass Spectrometry and Nuclear Magnetic Resonance as described in Tawata (1990) and Xuan et al (Xuan et al. 2006).

Chemicals

Chemicals were of high grade and purchased from Wako Chemical Company, Japan.

HPLC analysis

Mimosine and 3,4-DHP were measured at 280 nm using a Shimadzu HPLC (SCL-10A VP, Shimadzu Co., Kyoto,

Japan) coupled with a UV–VIS detector (SPD-20A Shimadzu). Separations were achieved on a column (Jasco, C18 150.0 × 4.60 mm). The mobile phase employed was a mixed solution of 10 mM potassium-dihydrogen phosphate, 10 mM phosphoric acid, and acetonitrile (45:45:10), and finally, 0.1% sodium 1-octanesulfonate was added to the mixture as the surface active agent. The flow rate was 1.0 mL min⁻¹.

Isolations of 3,4-DHP

From Leucaena leaves: an amount of 200 g young leaves at vegetative stage was obtained from 3-year-old *Leucaena* trees grown in campus of the University of the Ryukyus, Okinawa, Japan were collected in July–August 2010, temperature 28–32°C, and transferred to the laboratory. The leaves were boiled at 15 min in 1 L distilled water, cooled by ice, and filtered. The supernatant was immersed for one night with an ion-exchange resin (200 g) and washed using 200 ml of 80% ethanol. The ethanol was completely evaporated by a rotary evaporator and added with 20 ml of HCL 0.1 N. The mixture was shaken for 1 d at room temperature (about 25°C), filtered, and adjusted to 1000 ppm for HPLC analysis.

From degradation of mimosine by high temperature: 5 mg of mimosine purified from *Leucaena* leaves as mentioned above was used. It was adjusted to 1000 ppm by using a solvent of 0.1 N HCL and kept in a glass tube. The tube was placed in an oil bath maintained at 110°C for 6 h. The solvent was cooled to room temperature and filtered by a 25 µm filter and injected in an HPLC for analysis.

Isolation of 3,4-DHP by column chromatography

The solvents were immersed in an ion-exchange resin for one night and washed using 200 ml of 80% ethanol in a column (30 cm in length and 3 cm in diameter) to separate mimosine and 3,4-DHP. pH was adjust from 4.5 to 5 to crystallize mimosine and 3,4-DHP. In addition, at each 1–6 h interval, the quantity of mimosine degraded into 3,4-DHP was determined.

Traces of mimosine and 3,4-DHP were confirmed by a colorimetric assay as described in Allison et al. (1992) by using a ferric chloride reagent [0.6 g FeCl₃ × 6H₂O in 1 mL HCL 1% (v/v) solution in distilled water]. Mimosine and 3,4-DHP were detected visually in purple color, but no trace of 2,3-DHP was detected (blue color).

Quantification of mimosine and 3,4-DHP

The amounts of mimosine and 3,4-DHP were quantified by HPLC based on peak area measurements, which were reported to calibration curves of the corresponding mimosine and 3,4-DHP standards (10, 50, 500, and 1000 ppm).

Herbicidal activity

Seeds of barnyardgrass (*Echinochloa crus-galli* Vasing), a noxious paddy weed, were used for bioassay. Empty and undeveloped seeds were discarded after they floated in tap water. The remaining seeds were then air-dried and hermetically stored at –20°C. These seeds were sterilized with 1% sodium hypochlorite for 30 min and rinsed many times with distilled water before use. The germination percentage

of each of these plants was randomly checked, and all were >80%. Twenty seeds of *E. crus-galli* were sown evenly in a Petri dish (9 cm in diameter) lined with filter paper and moistened with 8 mL of each concentration of 1, 10, 50, and 100 ppm of mimosine and 3,4-DHP. Five replicates of all treatments were placed in an incubator (25°C, 4000 lx, with an 8/16 h day/night cycle, humidity: 75%) using a completely random design. Treatments with distilled water only were the controls. After 7 d, lengths of shoots and roots were measured. Inhibition (% of controls) of each concentration was calculated and expressed as half maximal inhibitory concentration (IC₅₀).

Antifungal activity

Colletotrichum gloeosporioides, *Colletotrichum acutatum*, and *Sclerotinia sclerotiorum* are noxious plant fungi. *C. gloeosporioides* causes anthracnose, a serious foliar disease of mango in South and Southeast Asia, South Africa, southern and middle America, France, and Hawaii (Kullnig-Gradinger et al. 2001). *C. acutatum* causes the most destructive fungal disease, anthracnose, of lupin species worldwide (Cesar et al. 2015). *S. sclerotiorum*, known as white mold, can affect hundreds of plant species, including many important crops (Abawi & Grogan 1979).

The fungal strains of *C. gloeosporioides*, *C. acutatum*, and *S. sclerotiorum* were used in this test. Antifungal activity was determined by using the method described by Masika and Afolayan (2002). Fungal cultures were maintained on potato dextrose agar (PDA) and were recovered for testing by sub-culturing on fresh medium for 3 d. The prepared PDA plates containing mimosine and 3,4-DHP dissolved in water at concentrations of 1, 10, 50, and 100 ppm with three replicates were inoculated with plugs obtained from the actively growing margin of the fungus plates and incubated at 25°C. Controls contained distilled water only. After 4 d, the antifungal activities of mimosine and 3,4-DHP were determined. Inhibition (% of controls) of each concentration was calculated and expressed as half maximal inhibitory concentration (IC₅₀).

1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The radical scavenging activity was evaluated as described previously (Kähkönen et al. 1999). An aliquot of 2 ml of the ethanol solution of mimosine and 3,4-DHP of each concentration of 1, 10, 50, and 100 ppm was mixed with 1 ml of 0.5 mM DPPH ethanol solution and 2 ml of 0.1 M sodium acetate buffer (pH 5.5). After shaking, the mixture was incubated at room temperature in the dark for 30 min, following which the absorbance was measured at 517 nm using a Shimadzu UV-160A spectrometer. Ethanol was used as the negative reference. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the formula of Son and Lewis (2002) as follows:

$$\% \text{ radical scavenging activity} = \left[\frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \right] \times 100,$$

where A_{control} is the absorbance of the control (DPPH solution without the test sample) and A_{test} is the absorbance of the test sample (DPPH solution plus antioxidant). Means were expressed as half maximal effective concentration (EC₅₀).

Statistical analysis

All treatments were arranged in a completely randomized design with three replications and repeated twice. Data of the two repetitions in each trials were subjected to two-way analysis of variance, with significant differences between means identified by general linear models (GLM) procedures using SAS version 6.12 (1997) with $P < .05$ adopted as the criterion of significance. Differences between means were declared significant at $P < .05$ using Tukey's procedure for multiple comparisons. Mean of herbicidal and antifungal and antioxidant bioassays was expressed as IC₅₀ and EC₅₀, respectively, to compare the effects between mimosine and 3,4-DHP. Therefore, the variable of dose effects was removed from the statistical model.

Results and discussion

Quantity of 3,4-DHP in *Leucaena* leaves

By HPLC, mimosine and 3,4-DHP were detected at 4.46 and 7.65 min, respectively (Figure 2, Table 1). Quantities of the two compounds in dried leaves of *Leucaena* were each 2.50% and 0.07%.

Degradation of mimosine to 3,4-DHP at high temperature

Figure 3 shows that the amount of mimosine degraded to 3,4-DHP was 2.1% after 15 min at high temperature (110°C), but increased rapidly after 1 h (16.9%). The quantity of 3,4-DHP after 2, 4, and 6 h was correspondingly 28.4%, 50.2%, and 69.3%, respectively. The optimum degradation of mimosine to 3,4-DHP was approximately 70%, as the amount of 3,4-

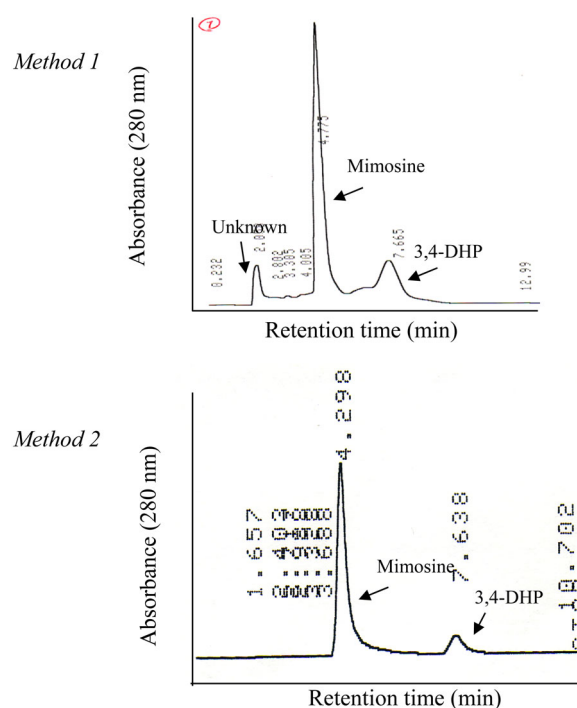


Figure 2. Mimosine and its degraded product 3,4-DHP in the HPLC profile. Method 1: From young *Leucaena* leaves; Method 2: By oil bath. HPLC (Shimadzu) solvents: 10 mM KH₂PO₄; 10 mM H₃PO₄; acetonitrile = 45 : 45:10 (v/v), 0.1% sodium 1-octanesulfonate; Column: Jasco, C18 150 × 4.60 mm; Flow rate: 1 ml min⁻¹; and Wave length: 280 nm.

Table 1. Quantities of mimosine and 3,4-DHP in *Leucaena* leaves.

Compounds	Retention time (min)	Quantity (% of dried leaves)
Mimosine	4.46 ± 0.03a	2.50 ± 0.35a
3,4-DHP	7.65 ± 0.01b	0.07 ± 0.01b

Notes: Values are means ± SE. Different letters in bars indicate significantly different ($P < .05$) using Tukey's procedure.

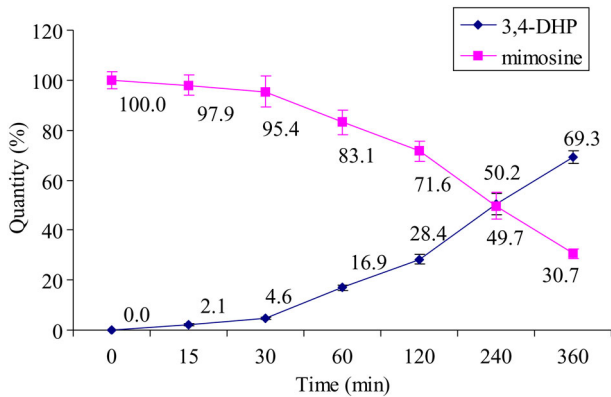


Figure 3. Correlative percentage of mimosine degraded to 3,4-DHP by high temperature. Bar values are mean ± SE (standard errors).

DHP did not increase after 7–10 h (data not shown) at a similar temperature of 110°C.

Herbicidal activity

A lower value of IC_{50} indicates a stronger activity. It is therefore observed that the inhibition of mimosine against the growth of barnyardgrass was about fourfold (0.04 mg g^{-1}) stronger than that of 3,4-DHP (0.15 mg g^{-1}) (Figure 4).

Antifungal activity

In general, inhibition of the growth of fungi by mimosine was stronger than that of 3,4-DHP, but the inhibitory magnitude differed depending on the kind of fungi (Figure 5). For *C. gloeosporioides* and *S. sclerotiorum*, the suppressive effect of mimosine was approximately threefold stronger than

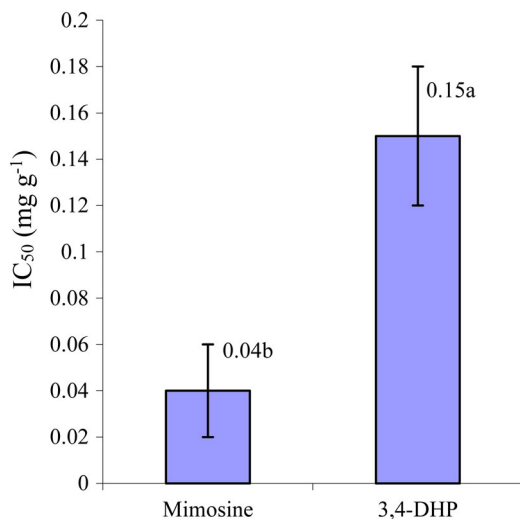


Figure 4. Inhibitory concentration (IC_{50}) of mimosine and 3,4-DHP on the growth of barnyardgrass (mg/ml). Different letters in bars indicate significantly different ($P < .05$) using Tukey's procedure. Bar values are mean ± SE.

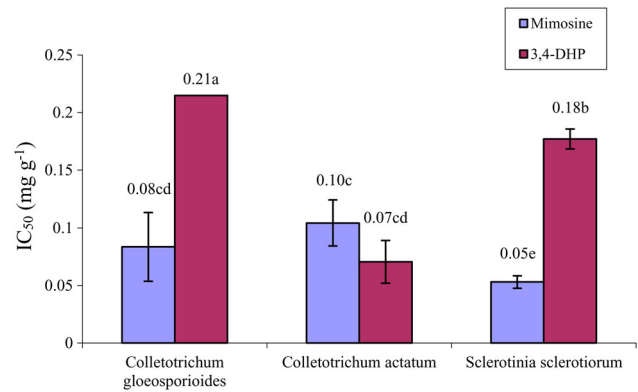


Figure 5. Inhibitory concentration (IC_{50}) of mimosine and 3,4-DHP on fungal growth (mg/ml). Different letters in bars indicate significantly different ($P < .05$) using Tukey's procedure. Bar values are mean ± SE.

that of 3,4-DHP; however, the antifungal activity was not significantly different from that of *C. acutatum* (Figure 5).

Antioxidant activity

Similar to IC_{50} , a lower value of EC_{50} of antioxidant activity reveals stronger capacity. Data in Figure 6 show that the DPPH scavenging activity of 3,4-DHP was markedly higher than that of mimosine. The antioxidant capacity of 3,4-DHP (2.4 mg g^{-1} gallic acid equivalent (GAE)) was >fourfold higher than that of mimosine (10.3 mg g^{-1} GAE).

Researches on the degradation of mimosine to 3,4-DHP have been reported by microorganisms in the rumen, bacteria in rhizome nodules, endogenous enzymes in *Leucaena* plants, or hydrolytic enzymes of *Leucaena* psyllids (jumping plant lice) (Smith & Fowden 1966; Lowry et al. 1983; Jones & Lowry 1984; Kamada et al. 1996; Dominguez-Bello & Stewart 2006; Lyon 2006). This study finds that by HCl hydrolysis, mimosine can be degraded to 3,4-DHP, but it was in low content (0.07% of dried leaves). Without HCl hydrolysis, the amount of 3,4-DHP in *Leucaena* leaves converted by endogenous enzymes may be in much lower quantity as compared to the content of 0.07% caused by HCl hydrolysis, and requires elaboration. At high temperature of 110°C, the degradation of mimosine to 3,4-DHP increased rapidly

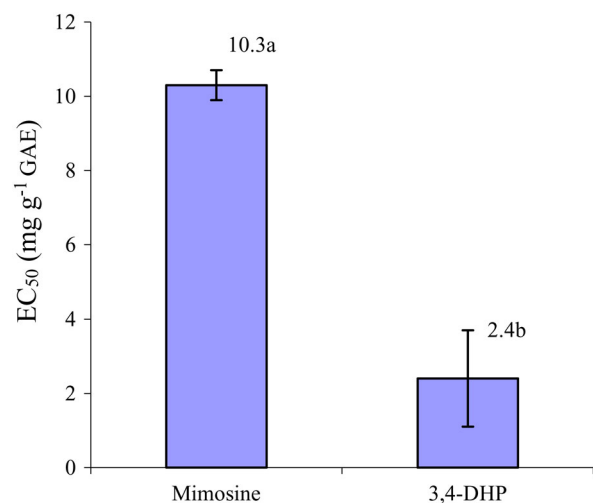


Figure 6. DPPH scavenging activity of mimosine and 3,4-DHP. Different letters in bars indicate significantly different ($P < .05$) using Tukey's procedure. Bar values are the effective concentration (EC_{50}) of GAE ± SE.

corresponding to applied times and approximately 70% of mimosine was degraded to 3,4-DHP (Figure 3). At 110°C, we also examined the degradation of mimosine after 7–10 h, but the quantity of 3,4-DHP did not increase as compared to that of the 6 h trial. It needs to be examined whether greater degradation of mimosine to 3,4-DHP may be obtained by increasing the temperature, but possibly temperature higher than 110°C may also cause the degradation of 3,4-DHP to 2,3-DHP or other by products. In this study, traces of mimosine and 3,4-DHP (purple color) were detected following a colorimetric method as reported in Allison et al. (1992) and repeated by Aung et al. (2011), but no trace of 2,3-DHP (blue color) was detected in either by HCl hydrolysis or at high temperature (110°C) (data not shown).

The quantity of mimosine in *Leucaena* young leaves was 2.66% of dry weight (Xuan et al. 2006) and it was similar in quantity as observed in this study (2.5% dry weight of *Leucaena* leaves) (Table 1). The actual amount of mimosine in the young leaves in this study may be higher, but by HCl hydrolysis, a certain quantity of mimosine was degraded to 3,4-DHP (0.07% dry weight of *Leucaena* leaves, Table 1). The amount of mimosine in *Leucaena* leaves in this study agreed with previous reports that in non-hybridized *Leucaena*, mimosine accounts for 2–5% of dry weight (Chou & Kuo 1986; Adeneye 1991), and the level of concentration could increase to 10% in young leaves (Brewbaker & Hylin 1965).

The herbicidal activity of mimosine on the growth of vegetables and weeds was examined and was reported to be dose dependent (Chou & Kuo 1986; Prasad & Subhashini 1994). Mimosine also shows selective effects against certain bacteria and fungal growth; of them, some bacteria were inhibited (Soedrho & Borthakur 1998), whereas the growth of several bacteria was promoted by either mimosine or 3,4-DHP (Soedarjo & Borthakur 1996; Tawata et al. 2005). Xuan et al. (2013) reported that at 100 ppm, the herbicidal activity of mimosine was stronger than that of the synthesized propionate derivatives of mimosine (80–90% growth of *Brassica rapa*), but they exerted a rather lower antifungal capacity than the propionates (10–79% of inhibition).

The toxicity of mimosine, which causes problems in the growth and development of animals, is ascribed to the presence of –OH and –O in the pyridine ring which is known to suppress iron-containing enzymes and compete with tyrosine (Vickery & Vickery 1981). In this study, the herbicidal and antifungal activities of mimosine were found to be much stronger than that of 3,4-DHP, suggesting that these activities are rather dependent on the α -amino and carboxyl groups of mimosine, which were absent in the structure of 3,4-DHP, than the structure of the pyridine ring. Therefore, the development of herbicides and fungicides from mimosine should concentrate on the synthesis of different derivatives of the α -amino and carboxyl groups. It is concluded that the herbicidal and antifungal capacities and antioxidant activity of mimosine and 3,4-DHP react in a reversed way, of which the inhibition on growth of weeds and fungi relies on the presence of α -amino and carboxyl groups, but antioxidant and possible pharmaceutical properties may be much dependent on the presence of –OH and –O of the pyridine ring. It was reported that the C = C in COOH favorably stabilizes the specific radical species to enhance the radical scavenging activities, while the presence of the single OH in the *ortho* position of the COOH group disfavors the activities (Chen

et al. 2015). The carboxyl group may be the reason why the DPPH scavenging activity of mimosine was much lower than that of 3,4-DHP (Figure 4).

As 3,4-DHP shows a DPPH scavenging activity >fourfold stronger than mimosine, it should be considered carefully to exploit 3,4-DHP as an antioxidant source as this compound causes goiters, loss of hair, and infertility in animals (Crouse et al. 1962; Allison et al. 1992). The presence of nitrogen in the pyridine ring, as well as the presence of –OH and –O, is known to suppress iron-containing enzymes and compete with tyrosine (Xuan et al. 2013). Mimosine has been reported to inhibit the activity of cyclin E-associated kinases in human breast cancer cells and suppress the proliferation of human lung cancer cells, and the non-protein amino acid is suggested to be a useful agent for the study of cell cycle control (Chang et al. 1999). As 3,4-DHP reveals much stronger antioxidant activity than mimosine, it may possess interesting pharmaceutical properties, but it needs further elaboration.

The objectives of this study include the examination of whether mimosine and 3,4-DHP can be used as novel antifungal reagents; however, only three plant fungi were tested, but further experiments on ruminal fungi should also be conducted. As these plant fungi are examined in aerobic conditions at 25°C, but ruminant fungi are anaerobic and the incubation temperature should be at 39–40°C with pH at 6.8, therefore mimosine and 3,4-DHP may have different influences on ruminant fungi. Rumen anaerobic fungi actively colonize plant cell walls and may account for up to 8–12% of the microbial biomass in rumen (Rezaeian et al. 2004). It was reported that ruminant fungi effectively take part in fiber digestion in ruminants (Dey et al. 2004; Lee et al. 2004). These fungi secrete high levels of very active fiber-degrading enzymes (cellulases, hemicellulases, xylanases, avicelases, glycosidase, etc.) (Williams et al. 1994; Lee et al. 2001).

Conclusion

In this study, 3,4-DHP can be easily purified from *Leucaena* leaves by using an ion-exchange resin or mimosine by using high temperature of an oil bath. The trace of 2,3-DHP in visual blue color was not detected, showing that at high temperature (110°C) and by HCl hydrolysis, 3,4-DHP was not degraded to 2,3-DHP. Since 3,4-DHP can be easily isolated, many other toxicological, biological, and pharmaceutical properties of this compound can be further examined. Though the herbicidal and antifungal activities of 3,4-DHP were not stronger than that of mimosine, the fourfold stronger antioxidant capacity of 3,4-DHP than mimosine is promising and requires critical investigation; but its use as an antioxidant reagent is limited as it causes toxicity against animal growth and fertility.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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