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Determination of Allelopathic Potential in Mahogany (*Swietenia macrophylla* King) Leaf Litter Using Sandwich Method

Arnia Sari Mukaromah[1,*], Yekti Asih Purwestri[1], and Yoshiharu Fujii[2]

[1]Faculty of Biology, Universitas Gadjah Mada, Jalan Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia
[2]Departement of International Environmental and Agricultural Science, Tokyo University of Agriculture and Technology, Tokyo 183-0057, Japan

Abstract

The sandwich method is a reliable screening bioassay that can be utilized to investigate allelopathic activity of leaf litter leachates. Screening the allelopathic potential of mahogany (*Swietenia macrophylla* King) leaf litter in plant–plant interaction using the sandwich bioassay method has not been reported. The research objectives were to determine and categorize allelopathic potential of *S. macrophylla* leaf litter using the sandwich bioassay method, and to determine specific activity (EC_{50}) of *S. macrophylla* leaf litter. The results showed that *S. macrophylla* leaf litter exhibited strong allelopathic activity when compared with 46 leaf litter species and was included in the top ten of allelopathic leaf litter species. Increasing *S. macrophylla* leaf litter concentration was concomitant with inhibition of radicle lettuce seedling growth compared with the control. According to the linear regression analysis, the effective concentration (EC_{50}) of *S. macrophylla* was estimated to be 3.25 mg D.W. eq. mL^{-1} and was considered to have strong growth-inhibitory activity on lettuce radicle elongation. The results suggest the possibility of allelopathic potential of leaf litter in plant–plant interaction under *S. macrophylla* trees.

Keywords: Allelopathy, EC_{50}, leaf litter, mahogany, Sandwich Method

Introduction

Allelopathy is one of the interactions that have contributed to inducing biological phenomena in the environment. Allelopathy is described as chemical interactions between plant–plant, plant–microorganism, and plant–animal, whether stimulatory or inhibitory of growth and development (Rice, 1984; Einhelling, 1995; Blum, 2011; Gniazdowska and Bogatek, 2005). The major constituent of allelochemicals are secondary metabolites that influence vegetation community patterns and succession, germination of seeds or fungi spores, the nitrogen cycle, crop productivity, and plant protection (Einhelling, 1995; Gniazdowska and Bogatek, 2005). Allelochemicals have been applied in sustainable weed management and agricultural pest management because they are environmentally friendly, biodegradable, and can reduce the utilization of herbicide. In the field, allelopathic plants have been applied as cover and smother crops, mulching, intercropping, crop rotation, green manure, and natural pesticide (Farooq *et al*., 2011; Jabran *et al*., 2015; Singh *et al*., 2003).

Mahogany (*Swietenia macrophylla* King), a tropical timber tree species with high-quality wood, has been cultivated in Indonesia since it was introduced in 1870 (Whitemore, 2003; Krisnawati *et al*., 2011). *S. macrophylla* is another famous member of the Meliaceae family besides *Azadirachta indica*. *S. macrophylla* has great potential applications, such as the protection of slopes, water catchment, avenue, furniture and cabinet making (Krisnawati *et al*., 2011), compost fertilizer (Nugroho, 2014), antimicrobial, anti-inflammatory, antioxidant, antimutagenic, anticancer, antidiabetic, antidiarrhoeal, antiviral, antimalarial, hypolipidemic activities (Moghadamtousi *et*

***Corresponding author:**
Arnia Sari Mukaromah
Faculty of Biology, Universitas Gadjah Mada, Jalan Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia
E-mail: arnia.sari.m@mail.ugm.ac.id

al., 2013) and heavy metal phytoremediation (Fan *et al*., 2011). Meanwhile, mahogany leaf litter is one of the source of potent allelopathic substances which can inhibit sunflower (*Helianthus annuus* L.) seed germination and phenolic compound produced throughout decomposition of *S. macrophylla* leaf litter inhibit acacia (*Acacia mangium* Wild.) seedling growth (Muhartini, 1987; Tambaru, 1998). Germacrene D and γ-himachalene of *S. macrophylla* leaves may play important role in attracting mahogany shoot borer (*Hypsipyla grandella*) to oviposit on mahogany leaves (Soares *et al*., 2003). Furthermore, ethanol extracts of *S. macrophylla* leaves reveal acaricidal activity to *Varroa destructor* mites inside colonies of honeybees (El Zalabani *et al*., 2012).

Central Java and West Java have contributed as much as 60% of the total number of mahogany trees in Indonesia, and have produced abundant leaf litter that has not been maximally used (Krisnawati *et al*., 2011; Nugroho, 2014). Mahogany that has a diameter of 29 cm, height of 15 m and canopy thickness of 4.5 m produces 1296.94 g/m^2/year of leaf litter, with the highest leaf litter production occurring in May–June (dry season) at 89.24 g/m^2/week (Nugroho, 2014). Allelochemicals are transferred to the environment by leaching from leaves or other aerial parts, volatile emissions, root exudation, and decomposition of plant residue (Weir *et al*., 2004).

Preliminary studies can be utilized to indicate allelopathic activity. Several preliminary approaches have been developed such as growing the receiver plant with plant residues or watering the receiver plant with leaf leachate (Duke, 2015). However, the previous preliminary studies need extraction procedure, and suitable for a small number of samples. The sandwich method are very useful allelopathic screening for leaf leachate under laboratory conditions (Fujii *et al*., 2003). The sandwich method is a reliable, fast, easy bioassay and can screen a large number of samples from leaf litter leachates. It has screened more than 852 species (Fujii *et al*., 2003; Fujii *et al*., 2004; Appiah *et al*., 2015; Mardani *et al*., 2016; Morikawa *et al*., 2012). Nevertheless, to date, screening of the allelopathic potential of *S. macrophylla* leaf litter in plant–plant interaction using the sandwich bioassay method and investigation of growth-inhibitory activity have not been reported. The objectives of this research were to determine and categorize allelopathic potential of *S. macrophylla* leaf litter using the sandwich bioassay method, and to determine specific activity (EC_{50}) of *S. macrophylla* leaf litter.

Materials and Methods

Plant materials

S. macrophylla leaf litter were collected from Bawen village, Semarang regency, Central Java, Indonesia, in the end of August 2015, and then identified in the Laboratory of Plant Systematics at the Faculty of Biology, Universitas Gadjah Mada, Indonesia. Comparing leaf litter species were collected from Tsukuba Botanical Garden, Koishikawa Botanical Garden, Yumenoshima Botanical Garden, Jindai Botanical Garden, and Tokyo University of Agriculture and Technology, Fuchu campus, on February 11 to March 2, 2016. Each sample was placed into a separate paper bag and used for laboratory studies in the Laboratory of International Agro-Biological Resources and Allelopathy at Tokyo University of Agriculture and Technology, Japan.

Sandwich method

The sandwich method procedure was adopted from Fujii *et al*. (2003), Fujii *et al*.(2004), and Morikawa *et al*.(2012). The amount of leaf litter sample (either 10 mg or 50 mg) was placed into a six well (~10 cm^2 area per well) multi-dish plate (36 mm × 18 mm, Nalge Nunc Int.) with specifity (each well in upper row for 10 mg and lower row for 50 mg). Agar powder (Nacalai Tesque, Kyoto, Japan, with a gelling temperature of 30–31°C) was prepared as 0.75% (w/v) and autoclaved at 115°C for 15 min (Sanyo, Japan). The autoclaved agar was cooled down to ca. 45°C in a water bath. The first layer of autoclaved agar (5 mL) was added to each well using a pipette (Gilson Co. Ltd., Villiers-le-Bel,

France). After gelatinization, the second layer of autoclaved agar (5 mL) was added to each well and gelatinized for 30–60 min at room temperature. Five seeds of lettuce (*Lactuca sativa* L. var. Legacy; Takii Company, Kyoto, Japan) were sown in the surface of the second layer of agar and limited of sowing time could prevent contamination. The multi-dish was covered and sealed with a cellophane adhesive tape. Furthermore, it was wrapped with aluminum foil, and incubated in an incubator (NTS Model MI-25S) at 22°C for 72 hours (3 days). The lengths of the radicle and hypocotyl were measured using a graphing paper. These data were used to calculate the elongation percentage compared with the control (Eq. 1).

$$Elongation\ (\%) = \frac{average\ \ length\ of\ radicle/hypocotyl}{average\ length\ of\ control\ radicle/hypocotyl} \times 100 \quad \text{(Eq. 1)}$$

Preparation of test solution

Five milligrams of *S. macrophylla* dried leaf litter was soaked in 50 mL of methanol 90% and incubated for 24 hours. Crude extract of *S. macrophylla* leaf litter was filtered using filter paper (125 mm ø, Toyo Roshi Kaisha, Ltd., Tokyo). *S. macrophylla* leaf litter extraction was repeated twice, collected in an Erlenmeyer flask (Pyrex), and used as test solution. The final concentration ranged from 1 to 9 mg dry weight equivalent mL^{-1} (mg D.W. eq. mL).

Growth-inhibitory activity bioassay

The growth-inhibitory activity bioassay was adopted from Takemura *et al.*(2013). Filter paper (27 mm ø, Toyo Roshi Kaisha, Ltd., Tokyo) was placed into a glass Petri dish (27 mm ø). The test solution (700 µL) was added into the filter paper in the Petri dish and dried completely in vacuo (Eyela NVC-2200 and Eyela DPE-1130). After distilled water was added (700 µL), 5 pre-germinated (18 h at 22°C in the dark) seedlings of lettuce (*Lactuca sativa* L. var. Legacy; Takii Company, Kyoto, Japan) were placed on the filter paper. All of the Petri dishes were placed in the same chamber and wrapped with aluminum foil and incubated in an incubator (NTS Model MI-25S) at 22°C for 48 hours. After incubation, the lengths of the radicle and hypocotyl were measured using a graphing paper. These data were used to calculate the elongation percentage compared with the control (Eq. 1).

Statistical analysis

The experiment was conducted using a completely randomized design with three independent replications. Either the radicle or hypocotyl elongation percentage was calculated (Eq. 1) and confirmed with a normal distribution curve. Categorization of strength inhibitory activity was calculated by "Standard Deviation Variance" (SDV) based on radicle elongation of 50 mg of dried leaf litter. In the statistical analysis, evaluation of the mean (M), standard deviation (SD), and SD variance (SDV) were calculated using Microsoft Excel ver. 2013®. The effective concentration required to induce half-maximal inhibition of growth (EC_{50}) was calculated by linear regression analysis using Microsoft Excel ver. 2013®.

Results and Discussion

Allelopathic potential of S. macrophylla leaf litter on lettuce growth

The allelopathic potential categorization was utilized to observe allelopathic activity of *S. macrophylla* compared with the other leaf litter species. The number of comparator leaf litter species were as well as more than 50 leaf litter species. However, *S. macrophylla* only compared with 46 leaf litter species from 22 families because several leaf litter species did not have suitable sample weight for assessment. The most abundant species belonged to Cupressaceae (9 species), Pinaceae (6 species), Podocarpaceae (6 species), Acanthaceae (2 species), Verbenaceae (2 species), followed by one species from each of the other families. Meanwhile, the lettuce seeds, a small seeded species were used as test plant material in the sandwich bioassay method because of their rapid germination, homogeneity and high sensitivity to the phytotoxic compound. It had similar character with lettuce (*L. sativa* 'Great Lakes 366') seeds

used in the previous sandwich bioassay method (Fujii *et al.*, 2007).

The allelopathic potential of the leaf litter samples was determined by "Standard Deviation Variance" (SDV) method based on radicle elongation in 50 mg D.W. and then compared with other leaf litter species. According to Table 1, *S. macrophylla* leaf litter exhibited strong allelopathic activity when compared with 46 other leaf litter species, and included in the top ten of allelopathic leaf litter species. This could be related to previous allelopathy research showing that *S. macrophylla* leaf litter inhibited the

Table 1. Allelopathic activity of 47 leaf litter species using sandwich method.

Plant families	Collection site†	Scientific name	Dry leaf content (10 ml agar^{-1})				Criteria (*)
			10 mg		50 mg		
			R%	H%	R%	H%	
Verbenaceae	YMBG	*Duranta repens* L.	29	54	9	28	****
Plumbaginaceae	YMBG	*Plumbago auriculata* Lam.	54	112	15	74	***
Meliaceae	**INDO**	***Swietenia macrophylla* King**	**59**	**102**	**19**	**72**	***
Solanaceae	YMBG	*Brunfelsia australis* Benth.	30	57	20	69	***
Moraceae	YMBG	*Ficus superba* Miq.	54	84	24	50	**
Berberidaceae	TKBG	*Nandina domestica* Thunb.	34	69	25	72	**
Myrtaceae	YMBG	*Feijoa sellowiana*(O. Berg.) O. Berg.	73	112	33	84	**
Pinaceae	TKBG	*Pinus densiflora* Siebold & Zucc.	97	84	36	65	**
Theaceae	KSBG	*Camellia japonica* L. F. leunctha Makino	54	66	37	67	**
Pinaceae	TKBG	*Tsuga sieboldii* Carrière	76	84	38	78	*
Cupressaceae	KSBG	*Taxodium distichum* (L.) Rich.	87	91	39	64	*
Celastraceae	KSBG	*Euonymus chibae* Makino	88	103	42	78	*
Acanthaceae	YMBG	*Strobilanthes anisophyllus* (G. Lodd.) T. Anders.	81	98	46	119	*
Linaceae	YMBG	*Reinwardtia indica* Dumort.	78	79	48	92	*
Podocarpaceae	TKBG	*Saxegothaea conspicua* Lindl.	82	97	50	87	*
Hamamelidaceae	KSBG	*Hamamelis mollis* Oliv.	108	98	53	92	
Verbenaceae	YMBG	*Lantana camara* L.	88	89	54	108	
Pinaceae	KSBG	*Pinus koraiensis* Siebold & Zucc.	104	87	57	65	
Fagaceae	TKBG	*Quercus sessiliflolia* Salisb.	87	98	58	106	
Pinaceae	JDBG	*Abies sachalinensis* Mast.	100	92	63	79	
Acanthaceae	YMBG	*Eranthemum pulchellum* Andrews	103	99	64	107	
Cupressaceae	JDBG	*Cyrptomeria japonica* (Thunb. ex L.f.) D. Don	84	74	65	71	
Pinaceae	JDBG	*Pinus ellioti* 'Elliotti' Engelm	89	86	68	86	
Cupressaceae	JDBG	*Thujopsis dolabrata* (Thunb. ex L.f.) Siebold & Zucc.	94	116	70	122	
Cupressaceae	JDBG	*Chamaecyparis pisifera* (Siebold & Zucc.) Endl. 'Filifera'	103	101	70	91	
Pinaceae	KSBG	*Cedrus deodara* (Roxb.) G. Donn f.	93	86	70	86	
Ericaceae	KSBG	*Rhododendron macrosepalum* Maxim	127	115	71	129	
Cupressaceae	KSBG	*Juniperus luthuensis* Koidz.	83	95	74	100	
Caprifoliaceae	YMBG	*Lonicera periclymenum* L.	111	112	75	110	
Araliaceae	TKBG	*Dendropanax trifidus* (Thunb.) Makino ex Hara	93	104	75	124	
Cannabaceae	TKBG	*Celtis sinensis* Pers.	117	109	76	100	

Table 1. Allelopathic activity of 47 leaf litter species using sandwich method (cont.).

Plant families	Collection site	Scientific name	Dry leaf content (10 ml agar^{-1})				Criteria
			10 mg		50 mg		
			R%	H%	R%	H%	
Podocarpaceae	TKBG	*Podocarpus nivalis* Hook.	100	107	81	123	
Symplocaceae	TKBG	*Symplocos sawafutagi* H. Nagamasu	123	106	82	106	
Ericaceae	TKBG	*Rhododendron hyperythrum* Hayata	109	112	83	120	
Ericaceae	KSBG	*Rhododendron transiens* Nakai cv Hatrusimo	104	112	84	114	
Cupressaceae	KSBG	*Chamaecyparis obtusa* (Siebold & Zucc.) Siebold & Zucc. Ex Endl.	105	106	85	128	
Cupressaceae	JDBG	*Cunninghamia lanceolata* (Lamb.) Hook.	106	99	86	105	
Proteaceae	YMBG	*Banksia ericifolia* L.f.	103	93	91	108	
Cupressaceae	TKBG	*Sequoia sempervirens* (Lamb. Ex D. Don) Endl.	109	90	91	94	
Ericaceae	KSBG	*Rhododendron socibrum* D. Dm.	114	108	92	130	
Ericaceae	KSBG	*Rhododendron pulchrum* Sweet cv. Sen-e-ohmurasaki	121	116	92	130	
Ericaceae	KSBG	*Rhododendron hybrida* Has	125	122	96	134	
Ericaceae	KSBG	*Rhododendron transiens* Nakai cv Asukagawa	116	108	98	140	
Cupressaceae	TKBG	*Metasequoia glyptostroboides* Hu & W.C. Cheng	100	100	104	132	
Podocarpaceae	JDBG	*Podocarpus macrophyllus* (Thunb.) Sweet	116	99	112	104	
Aquifoliaceae	TUAT	*Ilex integra* Thunb.	125	98	115	136	
Podocarpaceae	TKBG	*Podocarpus totara* G. Bennet ex D. Donn.	127	104	120	107	
Mean (M)			92.8	96.5	65.0	97.6	
Standard Deviation (σ)			25.1	15.2	27.8	25.7	
M -0.5(σ)	*				51.1	84.7	
M -1(σ)	**				37.2	71.9	
M -1.5(σ)	***				23.3	59.0	
M -2(σ)	****				9.4	46.1	

R%: percentage of radicle length compared with the control; H%: percentage of hypocotyl length compared with the control; Criteria (*) were categorized by "Standard Deviation Variance" (SDV) based on radicle elongation at 50 mg of leaf litter; Asterisks indicate the exhibited strength of inhibitory activity; ****: strongest inhibitory activity, ***: strong inhibitory activity, **: medium inhibitory activity and *: low inhibitory activity; †: INDO: Central Java, Indonesia; JDBG: Jindai Botanical Garden; KSBG: Koishikawa Botanical Garden; TKBG: Tsukuba Botanical Garden; TUAT: Tokyo University of Agriculture and Technology, Fuchu campus and YMBG: Yumenoshima Botanical Garden.

germination of sunflower seeds (Muhartini, 1987). In previous studies, both Duranta repens leaves and *Nandina domestica* leaves exhibited allelopathic activity on lettuce (Appiah *et al*., 2015; Takemura *et al*., 2013). In comparison, *S. macrophylla* leaf litter has been observed and showed allelopathic potential higher than *Nandina domestica* but lower than *Duranta repens*. The results of this research suggest that leaf litter contributed to inhibition of plant growth under *S. macrophylla* trees.

The radicle and hypocotyl elongation percentages of the lettuce seedlings varied depending on the milligram dry weight (D.W.) of leaf litter species (Table 1). When treated with 10 mg D.W. and 50 mg D.W. samples,

the radicle elongation percentages ranged 29–127% and 9–120%, respectively, compared with the control. Meanwhile, hypocotyl ranged 54–122% and 28–140%, respectively. Inhibition of radicle lettuce seedlings ~50% was exhibited in 3 species at 10 mg D.W. and 15 species at 50 mg D.W., respectively. The results indicate that increasing leaf litter dry weight enhanced the inhibition of radicle growth in lettuce seedlings.

Direct interaction between leaf litter and lettuce seeds is prevented by the presence of a bilayer agar. Movement of allelochemicals from leaf litter is facilitated by the principle of diffusion. As Table 1 shows, the radicle elongation in lettuce seedlings was less than that of hypocotyls with either 10 mg or 50 mg of dried leaves. This might be related to the absorption and concentration of phytotoxins in the root tissue by direct contact with the allelochemicals (Morikawa *et al*., 2012).

Growth-inhibitory activity of S. macrophylla leaf litter

The inhibitory activity on lettuce seedling growth can be represented as effective concentration (EC_{50}) required to induce half-maximal inhibition of growth (Takemura *et al*., 2013). Determination of concentration intervals was started by evaluating a wide range of concentrations—e.g. 0.1, 1, 3, 10, 30, 100 mg dry weight (D.W.) equivalent mL^{-1} (mg D.W. eq. mL^{-1}) (data not shown). The second concentration interval was determined in the specific range of concentration, which included the half-maximal inhibition of lettuce seedling growth. The range of specific concentration interval was 1, 3, 5, 7, 9 mg dry weight (D.W.) equivalent mL^{-1} (mg D.W. eq. mL^{-1}). The allelopathic potential of *S. macrophylla* leaf litter is illustrated in Figure 1.

It showed that increasing *S. macrophylla* leaf litter concentration is concomitant with inhibition of radicle lettuce seedling growth compared with the control. Twenty species that have strong growth-inhibitory activity had an EC_{50} ranging from 0.28 to 6.90 mg F.W. eq. mL^{-1} (Takemura *et al*., 2013; NIEAS, 2016). Based on the linear regression analysis, the EC_{50} of *S. macrophylla* was estimated to be 3.25 mg D.W. eq. mL^{-1} and was considered to have strong growth-inhibitory activity on lettuce radicle elongation. Based on the EC_{50} value, *S. macrophylla* has stronger growth-inhibitory activity than *Nandina domestica* (Berberidaceae; estimated EC_{50} : 6.21 mg F.W. eq. mL^{-1}) and related to the previous study using sandwich method bioassay. The interaction between *S. macrophylla* leaf litter and lettuce seedlings investigated in this study is novel because previous research has only explored plant–microbe and plant–insect interactions.

S. macrophylla leaves contain limonoid, essential oils, and polyphenols (quercetin, catechin, and kaemferol) (El Zalabani *et al*.,

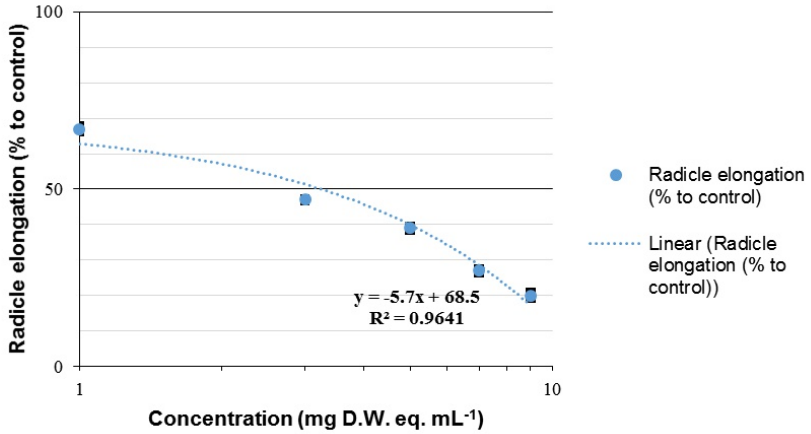

Figure 1. Effect of mahogany (*Swietenia macrophylla* King) leaf litter on the growth of lettuce seedlings based on a growth-inhibitory effects bioassay. Each value represented mean ± S.D. from three independent experiments. Range of concentration is shown in logarithmic scale and exhibited unit of mg *S.macrophylla* leaf litter dry weight (D.W.) equivalent mL^{-1}. The effective concentration of 50 percent (EC_{50}) could be calculated based on linear regression analysis.

2012; Moghadamtousi *et al*., 2013; Paritala *et al*., 2015; Roy and Saraf, 2006). Limonoid is insoluble in the water but soluble in the alcohol. It might be extracted by methanol 90% but solvent evaporation caused binding of limonoid to the filter paper. When distilled water added to the filter paper, limonoid might not be soluble in the distilled water. Therefore, it might not contribute to induce inhibition of radicle elongation in pre-germinated lettuce seedlings. Meanwhile, the lone pair electron in the hydroxyl group (-OH) of polyphenol might bind with hydrogen atom of methanol 90%. Furthermore, *S. macrophylla* leaf litter contained lignin and tannin included in the polyphenol group. Both of them might be extracted by methanol 90%. Therefore, the possible allelochemicals were polyphenol, lignin and tannin. The possible allelochemicals will interact on the radicle of lettuce seedling and might inhibit radicle and hypocotyl elongation. Meanwhile, total activity method could be used to investigate induction of allelopathic activity, either by a single compound or multiple compounds.

Further research should investigate the allelopathic potential of *S. macrophylla* leaf litter towards weeds or crops using a specific activity (EC_{50}) bioassay, determine the chemical constituents in *S. macrophylla* leaf litter using the total activity method, and observe polyphenols as allelochemicals using an isolation method and total activity.

Conclusions

S. macrophylla exhibits strong allelopathic potential on lettuce using the sandwich method. The effective concentration (EC_{50}) of *S. macrophylla* leaf litter was estimated to be 3.25 mg D.W. eq. mL^{-1} and was considered to have strong specific activity. The results of the present study are new findings that suggest a possibility of allelopathic potential of leaf litter in plant–plant interaction under *S. macrophylla* trees.

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