Scientific Note

Contribution of phenolic acids and dimethyl sulfone to the allelopathic effect of invasive *Tridax procumbens*[1]

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ABSTRACT

Tridax procumbens is an invasive weed with a strong allelopathic activity. In this study, the contribution of phenolic acids and dimethyl sulfone to the allelopathic effect of *T. procumbens* was evaluated against *Raphanus sativus*. Phenolic acids (benzoic, ellagic and ferulic), vanillin and dimethyl sulfone were identified and quantified from the strongest fraction of *T. procumbens*, in an allelopathic assay by high performance liquid chromatography and gas chromatography - mass spectrometry. The contribution of phenolic acids and dimethyl sulfone to the allelopathic effect of *T. procumbens*, expressed as a total activity, was evaluated by comparing the IC_{50} value to the concentration of each allelochemical, in a completely randomized design. The benzoic acid presented the strongest inhibitory effect (115 mg kg^{-1}) and the highest contribution (0.483) to the allelopathic effect of *T. procumbens*, followed by vanillin, dimethyl sulfone and ferulic acid.

KEYWORDS: Invasive weed, benzoic acid, ferulic acid, vanillin.

RESUMO

Contribuição de ácidos fenólicos e dimetilsulfona para o efeito alelopático de *Tridax procumbens* invasivo

Tridax procumbens é uma erva daninha invasiva com forte atividade alelopática. Objetivou-se avaliar a contribuição de ácidos fenólicos e dimetilsulfona para a alelopatia de *T. procumbens* contra *Raphanus sativus*. Ácidos fenólicos (benzoico, elágico e ferúlico), vanilina e dimetilsulfona foram identificados e quantificados a partir da fração mais forte de *T. procumbens*, em ensaio alelopático por cromatografia líquida de alta eficiência e cromatografia gasosa - espectrometria de massa. A contribuição dos ácidos fenólicos e dimetilsulfona para a alelopatia de *T. procumbens*, expressa como atividade total, foi avaliada comparando-se o valor de IC_{50} com a concentração de cada aleloquímico, em delineamento completamente randomizado. O ácido benzoico apresentou o maior efeito inibitório (115 mg kg^{-1}) e a maior contribuição (0,483) para o efeito alelopático de *T. procumbens*, seguido por vanilina, dimetilsulfona e ácido ferúlico.

PALAVRAS-CHAVE: Erva daninha invasiva, ácido benzoico, ácido ferúlico, vanilina.

Tridax procumbens is a weed present in more than 80 countries. It is known as "tridax daisy", "coat buttons" or "gletang" (in Indonesia) and is reported to decrease the yield of more than 30 crops (Holm et al. 1997). This plant shows a strong allelopathic activity against *Lactuva sativa*, *Alium cepa* and *Raphanus sativus* (Mecina et al. 2016, Nurul et al. 2016, Andriana et al. 2018). The major allelochemicals identified in this plant were phenolic compounds (Andriana et al. 2018).

Phenolic compounds are a group of the most essential and natural allelochemicals of plants in the ecosystem, which consists of a hydroxyl group (-OH) bonded directly to a benzene ring (Li et al. 2010). Phenolic compounds have been identified in weed species and reported to act as allelochemicals. For example, the p-coumaric, gallic, ferulic, p-hydroxybenzoic and anisic acids were detected in *Ageratum conyzoides* and reported to have a phytotoxic action (Batish et al. 2009). Vanillin and p-hydroxybenzoic, protocatechuic, p-coumaric, ferulic and caffeic acids were detected in *Bidens pilosa* and acted as allelochemicals (Deba et al. 2007). Additionally, some phenolic compounds, such as nopinene, eucalyptol, D-limonene, as well as triterpenoids, Lantadenes A and B, have been

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detected from *Lantana camara* and reported to have a strong allelopathic activity (Gindri et al. 2020)

On the other hand, dimethyl sulfone, a naturally-derived sulfur compound, was detected in *T. procumbens* by gas chromatography - mass spectrometry (GC-MS) analysis (Andriana et al. 2018). Dimethyl sulfone, known as $DMSO_2$, MSM, methyl sulfone or methylsulfonylmethane, with the chemical formula $(CH3)_2SO_2$, was also detected in many food sources and plants. It is used for wound healing in humans and animals, as well as a dietary supplement in the United States (Sousa-Lima et al. 2016). However, information on the allelopathic action of dimethyl sulfone is still limited.

Previously, by column chromatography, the separation of allelochemicals from *T. procumbens* using a gradient solvent system technique was conducted. It was found that the F1 fraction, separated from the ethyl acetate extract, showed the strongest allelopathic activity against *R. sativus*. This fraction reduced the chlorophylls contents of *R. sativus*, but stimulated a lipid peroxidation formation as a response to oxidative stresses (Andriana et al. 2018). Phenolic acids (benzoic, ferulic and ellagic) and vanillin were identified in the F1 fraction of *T. procumbens* and, among them, the benzoic acid showed the strongest allelopathic activity (Andriana et al. 2019). However, the contribution of each phenolic acid and dimethyl sulfone to the allelopathic activity of *T. procumbens* remains unknown. In order to continue the research about *T. procumbens* allelopathy, this study was carried out to evaluate the contribution of phenolic acids and dimethyl sulfone to the allelopathic activity of *T. procumbens*.

The plant material used in this study was *Tridax procumbens* collected in Subang, Indonesia (6º33'56.0"S and 107º44'54.9"E), and was authenticated by the Herbarium Bogoriense, Botany Division, Research Center for Biology, Indonesian Institute of Sciences, Indonesia. The target plant used in this study was *Raphanus sativus*. The seeds of the target plant were obtained from the Sakata Seed Corporation (Yokohama, Japan). The evaluated sample of phenolic acids and dimethyl sulfone contents was the most active fraction in the preliminary allelopathic research obtained by ethyl acetate extract (1.99 g) separation, using the gradient elution technique in a column chromatography (Andriana et al. 2018). The identification and quantification of phenolic acids and dimethyl sulfone contents were performed by high performance liquid chromatography (HPLC) and GC-MS, respectively.

An HPLC system (LC-Net II/ADC, UV-2075 Plus and PU-2089 Plus, Jasco, Tokyo, Japan) with a UV detector at 254 nm was employed to detect and quantify the phenolic acids contents in *T. procumbens* (Tuyen et al. 2017). It was used the RP C18 column (Jasco, Tokyo, Japan), with 250.0 mm in length, 4.6 mm of internal diameter and 5.0 µm in thickness. The mobile phase was methanol 99.8 % (A) and 0.1 % acetic acid (v/v) (B), at a flow rate of 1 mL min^{-1}. The gradient elution was performed as it follows: 0-5 min (5 % A); 5-10 min (20 % A); 10-20 min (50 % A); 20-30 min (80 % A); 30-40 min (100 % A); 40-50 min (100 % A); 50-60 min (5 % A). Phenolic standards and samples at the concentration of 1 mg mL^{-1} were injected to the HPLC column of 5 µL. The phenolic compositions were identified based on the retention times, and their concentrations were calculated by comparing the peak areas of the samples with those of the standards.

To identify and quantify the dimethyl sulfone, a GC-MS system (JMS-T100 GCV, JEOL Ltd., Tokyo, Japan) was used. A volume of 1 µL of the F1 fraction or $DMSO_2$ standard dissolved in methanol was injected into a GC-MS system (Minh et al. 2019). The column used in the GC-MS system was the DB-5MS (Agilent Technologies, J & W Scientific Products, Folsom, CA, USA), with 30 m in length, 0.25 mm of internal diameter and 0.25 µm in thickness. Helium was chosen as a carrier gas, and the split ratio was 5.0/1.0. The operating condition of GC oven temperature was maintained as it follows: the initial temperature was set at 50 ºC with no hold time and, then, it was increased at a rate of 10 ºC min^{-1} up to a final temperature of 300 ºC (hold for 20 min). The injector and detector temperatures were set at 300 ºC and 320 ºC, respectively, and the mass range scanned from 29 to 800 amu. The obtained peak was analyzed using the JEOL's GC-MS Mass Center System version 2.65a. To determine the concentration of dimethyl sulfone in the sample, several concentrations of standards were injected into the GC-MS system and plotted in a regression line. Then, the obtained peak of the sample was calculated by a linear equation.

Phenolic acids from *T. procumbens* were separated from an amount of 1.2 g ethyl acetate

extract. The extract was then diluted by chloroform in a column chromatography to obtain a 0.22 mg fraction. This fraction was then tested for phenolic acids and dimethyl sulfone contents, as well as allelopathic acitity against *R. sativus*. The *R. sativus* seeds were sterilized with sodium hypochlorite (5 %) for 10 min and rinsed three times with distilled water. Then, a volume of 300 µL test solution containing different concentrations of phenolic acids and dimethyl sulfone dissolved in methanol was added to each well of a 12-well plate (22.1 mm in diameter × 35 mm in height) that lined with filter paper. After that, the methanol in the wells was allowed to evaporate within 6 hours at ambient conditions, and an aliquot of 300 µL (2.22-fold of filter paper weight) distilled water was added to each well, a process repeated each day until the fifth day, totaling 1,500 µL, as recommended by the International Seed Testing Association (Hampton & Tekrony 1995). A total of 10 seeds of *R. sativus* were sowed in each well of the 12-well plate and placed in a growth chamber (Biotron NC system, Nippon Medical & Chemical Instrument, Co. Ltd, Osaka, Japan). The photoperiod was set for day/night, 12/12 h, with 25/23 ºC. Methanol without tested allelochmeicals, applied using a protocol similar to the one aforementioned, was used as control. After five days, germination, root length and shoot elongation were observed. The data were expressed as percentage of inhibition over the controls, and the IC_{50} value of each sample was also calculated (Andriana et al. 2018).

The contribution of the phenolic acids and dimethyl sulfone to the allelopathic effect of *T. procumbens* was expressed in terms of specific and total inhibitory activities for germination and growth of *R. sativus* (Golisz et al. 2007). The specific activity is presented by the IC_{50}, meaning the effective concentration of the compound to inhibit half of the maximum inhibition that was calculated by plotting several concentrations against the inhibition percentage. The equations for calculating the specific activity (IC_{50}) were: vanillin (root: y = 46.902x + 11.623; shoot: y = 37.118x + 11.913); ferulic acid (root: y = 60.701x + 13.37; shoot: y = 27.467x + 6.0119); benzoic acid (germination: y = 128.57x - 31.667; root: y = 46.939x + 51.623; shoot: y = 65.59x + 36.087). The total activity was calculated following the equation by Hiradate (2006), representing a function of total concentration of examined compound per specific activity in the organism: Total activity = (1/specific activity) × concentration.

In the present study, all data were presented as means and standard deviations, and analyzed by one-way Anova, using the Minitab 16.2.3 software (Minitab Inc., Philadelphia, USA). A completely randomized design with a single experimental factor was employed. The categorical factor, type of allelochemical, consisted of vanillin, benzoic, ferulic and ellagic acids, and dimethyl sulfone with three concentration levels (0.25, 0.5 and 1.0 mg mL^{-1}) was implemented in the experimental design, while the response parameters were inhibition of germination, root and shoot height, expressed as inhibition percentage or IC_{50} values (germination, root and shoot heights). The mean differences were determined by the Tukey test ($p < 0.05$), and the study conducted in triplicate.

The chemicals and reagents used in this study, such as phenolic standards including vanillin and caffeic, benzoic, cinnamic, catechol, chlorogenic, ferulic, ellagic, protocatechuic, gallic, p-hydroxybenzoic, p-coumaric, sinapic, vanillic and syringic acids, were obtained from Kanto Chemical Inc. (Tokyo, Japan), while the dimethyl sulfone standard, methanol and acetic acid for the HPLC analysis were purchased from Sigma-Aldrich (Tokyo, Japan).

The results showed that phenolic acids (ferulic, benzoic and ellagic) and vanillin were detected in the fraction of *T. procumbens* separated from the ethyl acetate extract by chloroform dilution in an HPLC system. The retention time for the vanillin and ferulic, benzoic and ellagic acids were 21.9, 23.6, 25.4 and 26.7 min, respectively (Figure 1), while dimethyl sulfone was detected by GC-MS in the retention time of 7.99 min (Figure 2).

The concentrations of the phenolic acids and dimethyl sulfone (mg kg^{-1} of dry weight) in *T. procumbens* are shown in Table 1. Vanillin showed the maximum concentration, followed by the benzoic acid, dimethyl sulfone, ellagic acid and ferulic acid.

Similarly to a previous study conducted by Andriana et al. (2018), the phenolic compounds were the major allelochemicals detected in *T. procumbens*. In the present study, phenolic acids (benzoic, ferulic and ellagic) and vanillin were detected as allelochemicals in *T. procumbens*, with vanillin being the major component. As a phytotoxic compound, vanillin was also found in many other plants, such as *Oryza sativa* (Khang et al. 2016), *Bidens pilosa*

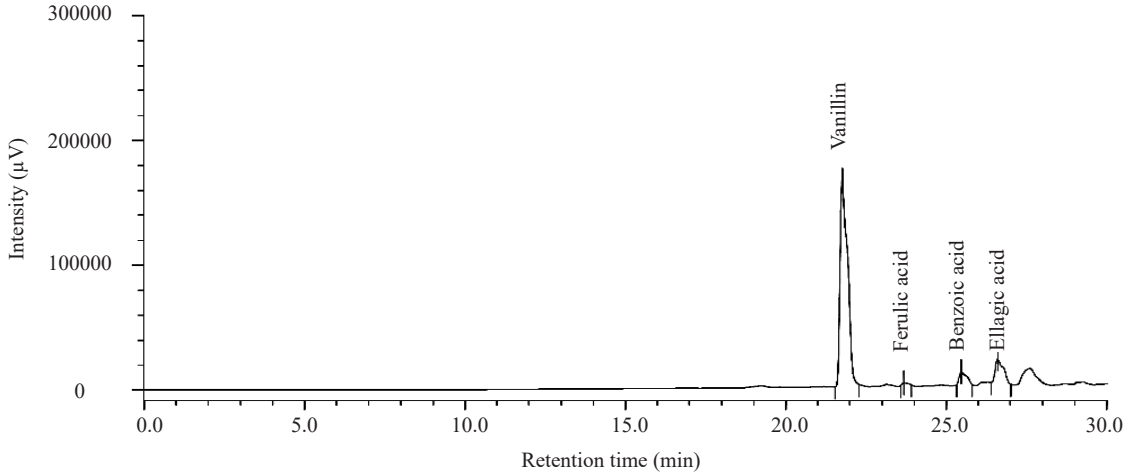

Figure 1. Phenolic acids detected in *Tridax procumbens* by high performance liquid chromatography.

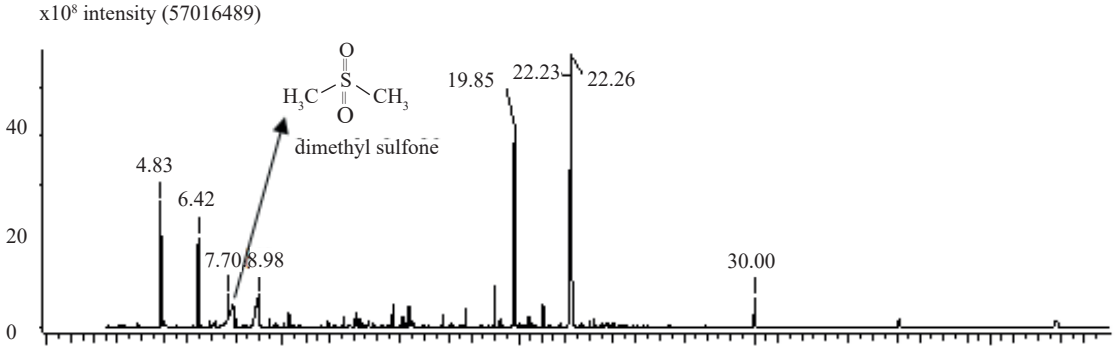

Figure 2. Dimethyl sulfone detected in *Tridax procumbens* by gas chromatography - mass spectrometry.

(Deba et al. 2007) and *Imperata cylindrica* (Xuan et al. 2009).

By analyzing the germination and growth assays, the benzoic acid had a higher inhibitory effect on radish seed germination, followed by vanillin, ferulic acid, dimethyl sulfone and ellagic acid (Table 2). Vanillin, ferulic acid and dimethyl sulfone suppressed the radish root and shoot elongation, while the ellagic acid stimulated the radish growth (17.39-54.36 % of root and shoot elongation).

Table 1. Concentration of phenolic acids and dimethyl sulfone in *Tridax procumbens*.

Compound	Concentration (mg kg^{-1} of dry weight)
Vanillin	291.7
Ferulic acid	2.8
Benzoic acid	55.9
Ellagic acid	14.1
Dimethyl sulfone	23.2

Dimethyl sulfone caused more inhibition in shoot elongation than in root growth.

The contribution of each phenolic acid and the dimethyl sulfone to the allelopathic effect of *T. procumbens* against *R. sativus* was illustrated in Table 3. Benzoic acid was the compound that most contributed to the allelopathy of *T. produmbens*, with total activity values around 0.09, 0.48 and 0.26 for inhibition of germination, root elongation and shoot growth of *R. sativus*, respectively. Benzoic acid is also found in several plants, such as *Azadirachta indica* (Xuan et al. 2004a), *Cucumis sativus* (Yu & Matsui 1997) and *Ageratum conyzoides* (Xuan et al. 2004b). This compound has been known to affect plant physiological processes such as nutrient uptake, stomatal conductance and net photosynthetic rate, resulting in growth inhibition (Quy et al. 2019). For the other phenolic acids detected, ferulic and ellagic acids, both were identified in three species, namely *Lupinus albus* (Stalikas 2007), *Avena fatua*

Table 2. Effect of phenolic acids and dimethyl sulfone of *Tridax procumbens* on germination and growth of *Raphanus sativus*.

Treatment	Concentration (mg mL^{-1})	Inhibition (%)		
		Germination	Root length	Shoot height
Vanillin	1.00	26.67 ± 11.55 b	50.32 ± 24.47 bc	43.04 ± 9.79 b
	0.50	3.33 ± 5.77 d	48.05 ± 17.21 bc	36.52 ± 3.98 bc
	0.25	0.00 ± 0.00 d	30.19 ± 10.41 c	33.04 ± 11.09 bc
Ferulic acid	1.00	0.00 ± 0.00 d	68.51 ± 6.26 b	30.38 ± 32.93 bc
	0.50	0.00 ± 0.00 d	47.05 ± 16.59 bc	23.04 ± 10.35 bc
	0.25	0.00 ± 0.00 d	44.16 ± 7.3 bc	18.69 ± 1.99 bc
Benzoic acid	1.00	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a
	0.50	23.33 ± 32.15 bc	70.78 ± 12.20 b	43.04 ± 32.01 b
	0.25	6.67 ± 5.77 cd	66.23 ± 7.38 b	38.69 ± 13.80 bc
Ellagic acid	1.00	0.00 ± 0.00 d	(+) 43.18 ± 8.66 e	13.07 ± 9.50 c
	0.50	0.00 ± 0.00 d	(+) 54.36 ± 29.74 e	(+) 26.18 ± 15.23 c
	0.25	0.00 ± 0.00 d	(+) 43.50 ± 24.47 e	(+) 17.39 ± 9.13 c
Dimethyl sulfone	1.00	0.00 ± 0.00 d	17.70 ± 0.35 d	32.20 ± 2.54 bc
	0.50	0.00 ± 0.00 d	12.28 ± 1.33 d	10.34 ± 4.36 c
	0.25	0.00 ± 0.00 d	10.51 ± 0.30 d	18.36 ± 5.17 b
Control	0.00	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 d

Data expressed by means and standard deviations (±). Means within a column followed by a similar letter are not significantly different by the Tukey test ($p < 0.05$). (+) = promote.

Table 3. Contribution of phenolic acids and dimethyl sulfone to germination and growth inhibition.

Compound	Concentration (mg kg^{-1})	Specific activity (IC_{50}; mg kg^{-1})			Total activity (C/IC_{50})		
		Germination	Root	Shoot	Germination	Root	Shoot
Vanillin	291.7	-	818	1,226	-	0.3620	0.2820
Ferulic acid	2.8	-	603	1,601	-	0.0054	0.0027
Benzoic acid	55.9	629	115	212	0.09	0.4830	0.2630
Ellagic acid	14.1	-	-	-	-	-	-
Dimethyl sulfone	23.2	-	2,258	1,827	-	0.0130	0.0120

"-" = no negative effect on the radish germination and growth. Linear equation for IC_{50} calculation: vanillin (root: y = 46.902x + 11.623; shoot: y = 37.118x + 11.913); ferulic acid (root: y = 60.701x + 13.37; shoot: y = 27.467x + 6.0119); benzoic acid (germination: y = 128.57x - 31.667; root: y = 46.939x + 51.623; shoot: y = 65.59x + 36.087).

and *Xanthium strumarium* (Qasem & Foy 2001). Ellagic acid has been reported to reduce the effect of salinity tolerance and enhance the plant growth (Khan et al. 2017).

In this study, the benzoic acid gave a higher contribution to the allelopathy of *Tridax procumbens* against *Raphanus sativus*, when compared to vanillin, ferulic acid and dimethyl sulfone. If compared to the other tested compounds, the ellagic acid was the only one that stimulated the *R. sativus* growth. The benzoic acid might have a role in the allellopahic effect of *T. procumbens*, indicated by presenting a lower inhibition on *R. sativus* germination than the root and shoot elongation. Thus, this study suggests that the benzoic acid showed to be the most essential allelochemical, and might be responsible for the allelopathy of *T. procumbens*.

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