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Assessment on allelopathic activity and potential allelochemicals of *Turnera subulata* Sm.

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Turnera subulata Sm. has potential in controlling insect pest population in the rice field as well as have medicinal properties. However, there is limited study has been conducted related to the allelopathic activity of *T. subulata*. Therefore, this study was carried out to assess the allelopathic activity and to identify potential allelochemicals from *T. subulata* leaf on the development of environmentally friendly weed management approach. The allelopathic assessment was conducted using two methods: sandwich and dish-pack. Four different types of receiver plants were used; *Lactuca sativa* (lettuce), *Brassica chinensis* (mustard), *Oryza sativa* var sativa (weedy rice) and *Triticum aestivum* (wheat). The sandwich method was carried out using 5 mg, 10 mg, and 50 mg of *T. subulata* leaves. Meanwhile, the dish-pack method was carried out using four different distances (41, 58, 82, and 92 mm) away from the well donor source. HPTLC analysis was developed to identify potential allelochemicals of *T. subulata* leaves. Result shows that the radicle elongation of weedy rice had the highest growth inhibition (79.0 % compared to control) at 50 mg of *T. subulata* leaves in the sandwich method. Whilst, at the distance of 41 mm from the donor source well (*T. subulata* leaves), the radicle of lettuce was the highest inhibited (72.4 % compared to control) by using dish pack. HPTLC analysis revealed two potential compounds which were rutin and naringin that identified from the methanolic extract of *T. subulata* leaves. Overall, these results show that *T. subulata* leaves have the significant allelopathic inhibitory properties and had the potential to be used as the weed suppressing agent.

Keywords: *Turnera subulata*, sandwich method, dish pack method, HPTLC.

INTRODUCTION

Allelopathy is an ecological interaction in which a plant can affect the growth and development of other plant via the release of secondary metabolites or allelochemicals into its environment (Farooq et al. 2011). These chemical compounds are released into the environment from plant parts through leaching, root exudation, volatilization, residue decomposition and other processes in both natural and agricultural systems (Albuquerque et al. 2011). Allelochemicals are easily degraded (Duke et al. 2002), cheaper and environmental friendly, thus provide better option compared to herbicide (Mushtaq et al. 2020). The use of allelochemicals meets the emerging demand of global effort in modern agriculture in minimizing the use of herbicides for pest management (Jabran et al. 2017; Nornasuha and

Ismail, 2017).

Turnera subulata Sm. is a herbaceous subshrub species, mostly distributed in subtropical and hot tropical zone of America (Solis Neffa and Fernandez, 2000), Indonesia, Malaysia, Florida and several Pasific Islands (Saravanan et al. 2018). This species is a perennial herb growing with dense taproot, woody cylindrical stem with simple leaves (de Brito Filho et al. 2014). Species of *Turnera* is recognized for their medicinal properties. In galenic medicine, this plant is used to treat bronchitis, coughs, gastrointestinal complaints, tumours, pain relief, pulmonary and respiratory ailments (Agra et al. 2007; Szewczyk and Zidorn, 2014). Moreover, *T. subulata* that planted in the rice field could help in controlling insect pest population as this plant provides refuge for the natural enemies of insect rice pest (Badrulhadza et al. 2018).

From the previous study, it has been reported that *T. subulata* aqueous leaves extract contained high amount of antioxidant compounds compared to other plant part (Chai and Wong, 2012). Moreover, the essential oil that present in aerial part of *T. subulata* exhibited effective antibacterial activity against various strains of *Staphylococcus aureus* with the MIC values between 25 µg/mL and 1600 µg/mL. Several major compounds had been identified from aerial part of *T. subulata* essential oil such as trans-caryophyllene, citronellol, sphatulenol, α-cadinol, n-tricosano, geraniol, trans-geranilacetone, n-pentacosano, globulol, caryophyllene oxide (Fernandes et al. 2014). Saravanan et al. (2018) reported the crude extracts of *T. subulata* in chloroform, ethyl acetate and ethanol when tested using LC/MS-MS method have been detected with the present of flavonols, dihydroflavonols, flavonones, flavones, anthocyanins, isoflavonoids, phenolic acids, and hydroxybenzaldehyde.

The active ingredients of potentially allelopathic plant species have benefit to medicinal properties activities. (Mirmostafaee et al. 2020). Since *T. subulata* leaves have the medicinal properties and had the ability to form dense and dominant population (Silvestre et al. 2013), this study was carried out to evaluate the allelopathic potential of *T. subulata* leaves and to identify the potential allelochemicals of this plant.

MATERIALS AND METHODS

Plant Materials

Turnera subulata leaves which is the donor plant species were collected from MADA (Muda Agricultural Development Authority) Nursery centre, at municipality of Jitra (6°16'23.0"N, 100°24'37.3"E), in the state of Kedah, Malaysia. This species have two colours of petals (i.e; white and yellow) with stained at the base (MyBIS, 2020). In this study, yellow petal of flower is used and this yellow flower is also known as synonym to *Turnera trioniflora* Sims. The leaves samples were collected from January 2019 until March 2019.

Collected fresh leaves were washed with tap water to remove dust, dirt and other undesired materials prior to dry at 60 ºC for 24 hours. The dried leaves were stored in at room temperature (±28 ºC) for further use. Seeds of *Lactuca sativa*, *Brassica chinensis*, *Oryza sativa* var *sativa* and *Triticum aestivum* were used as the receiver plant species. *Lactuca sativa* seeds act as common bioassay species used in allelopathy, *B. chinensis* seeds act as dicot tested bioassay species, *T. aestivum* seeds act as monocot tested bioassay species and *O. sativa* var sativa (weedy rice) seeds act as weed bioassay species.

Sandwich Method

The allelopathic potential of *Turnera subulata* leaves was determined using sandwich method as this method simulates the release of allelochemicals from leaves litters in natural condition (Appiah et al. 2015). In this study, allelopathic effects of leaf litter leachate of *T. subulata* leaves were determined by using sandwich method under controlled conditions. Three different amounts of leaves were used in this study, which were 5, 10 and 50 mg, and then placed in multi-dishes (Nalge Nunc Intl., Roskilde, Denmark). The experiments were performed in triplicate. For media preparation, agar powder (Nacalai Tesque, Kyoto, Japan) were prepared in 0.75% w/v and autoclaved at 121°C for 15 minutes. First layer of agar (5 mL) were added by using the micropipette into each well of the six-well multidish. The agar medium was allowed to solidify at room temperature. Then, 5 mL (second layer) of agar medium were added to each well of multi-dish and allowed to solidify (Fujii et al. 2004 and Appiah et al. 2015). In each well , five seeds of receiver bioassay species were placed vertically above agar surface at equal distances, covered with plastic tape and labelled appropriately for incubation in the dark for four days at 21 ºC (for *L. sativa*) and 28 ºC (for *B. chinensis*, *O. sativa var sativa* and *T. aestivum*)

respectively. The hypocotyl and radicle length (mm) of receiver plants were recorded after four days (Fujii et al. 2004; Nornasuha et al. 2019).

Dish pack method

The allelopathic potential was determined by using dish-pack method to test the effect of emitted leaves volatilization on the receiver species. The diffusion speed and activity intensity of volatile compounds from *T. subulata* leaves were estimated based on the relationship of the receiver species distances from *T. subulata* leaves and the inhibition on the receiver species growth (Fujii et al. 2005). In this study, the allelopathic potential of leaves volatilization from *T. subulata* was determined by using dish-pack method under controlled conditions. This method is widely used because it can determine the effect of volatile allelochemicals on the receiver plants very quickly (Shinwari et al. 2013). By using this method, the allelopathic activities of *T .subulata* were screened for possible volatile substances that can affect the growth of receiver plants. Multi-well plastic dishes with six wells (36 mm x 18 mm each) were used in this experiment.

Approximately 200 mg of the dried leaves sample were placed in the well at the corner six well multi-dishes. Four different distances from the sample (41 mm, 58 mm, 82 mm and 92 mm) were tested for allelopathic activity from leaves volatilization. Whatman (Grade: 1 Size: 33mm) filter paper was placed in each other five wells with seven lettuce seeds and moistened with 0.7 mL of distilled water. Multi-dishes were covered, and sealed with adhesive tape to prevent compounds volatilization from volatized. In the control dish, sample well was left blank while other processes were repeated similar of each sample dish. Aluminium foils were wrapped around the multi-dishes to prevent interference of light. All multi-dishes were incubated for four days at 21 ºC for *L. sativa* and 28 ºC for *B. chinensis*, *O. sativa* var sativa and *T. aestivum* respectively. The radicle and hypocotyl length were measured and recorded thus compared to with control sample.

Identification of allelochemicals compound by HPTLC

Preparation of standard solutions

The standard solution (concentration 1mg/ml) was prepared as 1 mg rutin and naringin were dissolved respectively in 0.5 ml HPLC by using standard methanol grade. The solution was sonicated for 20 minutes and then centrifuged at 1000 rpm for 10 minutes. The standard for working purpose was prepared freshly before use then the stock was stored in the chiller (± 4 °C) for further uses.

Preparation of extract for HPTLC Analysis

Approximately 50 g of dried *T. subulata* leaves were extracted with methanol and shaken in an orbital shaker for 72 hours at room temperature (28 °C ± 2). The supernatant was filtered through Whatman No. 42 filter paper. To prevent the growth of microorganisms, the solution was filtered again through a 0.2 µm Nalgene filter (Becton Dickinson Labware, NJ, USA). The methanol extract was evaporated to dryness under vacuum at 40°C using a rotary evaporator. The samples extract then centrifuged at 3000rpm for 5 minutes. This solution was used as test solution for HPTLC analysis.

HPTLC analysis

Some modifications were conducted by using HPTLC analysis (Rejila et al., 2012). Approximately 2 µl of methanolic extracts were loaded as 8 mm band length on the 20 x 10 Silica gel 60F TLC plate using Hamilton syringe and TLC sampler ATS 4 CAMAG LINOMAT 5 instrument. The loaded plate was kept in TLC twin trough developing chamber (after saturation with solvent vapour) with respective mobile phase (flavonoid compound) and the plate was developed in the respective mobile phase (ethyl acetate-ethanol-formic acid- water 28:2.4:4.8:4.8) up to 85 mm. The developed plate was dried using hot air to evaporate remaining solvents and sprayed with aluminium chloride reagent. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and image of each plate was capture at UV366 nm. Finally, the plate was fixed in scanner stage and scanned at 254nm.

Methanolic extracts were spotted in the form of bands with Camag microlitre syringe on a pre-coated silica gel plates 60F 254 [10 cm X 10 cm with 0.2 mm thickness, E.Merck] using Camag linomat IV applicator. Automatic sample spotter of band at 2 mm width. The plates were developed in a solvent system in CAMAG glass twin through chamber previously saturated with the solvent for 30 min and the distance was 8 cm subsequent to the scanning, TLC plates were air dried and scanning was performed on a TLC Scanner. The TLC Scanner 3 is controlled by win CATS

software 1.4.6.2002 (CAMAG, Switzerland) (Shah et al. 2008)

Statistical Analysis

All experiments were conducted using Completely Randomized Design (CRD) with three replications. The results presented are the mean of the results of these three replicates. The experimental data were subjected to one-way analysis of variance (ANOVA). Means were compared using the Duncan Multiple range test (DMRT) at the 5% level of significance. The statistical analysis was done using the SPSS software (version 21). The degree of inhibition of hypocotyl or radicle length was calculated for each treatment by comparing to the control with the following formula:

The calculation of inhibition percentage (%) =

$$\frac{(\text{Average Control Length} - \text{Average Treatment Length})}{\text{Average Control Length}} \times 100$$

RESULTS

Determination of allelopathic potential using sandwich method

Table 1 shows the hypocotyl and radicle length parameters for the control seedlings and seedling tested using different amount of leaves of *T. subulata*. In the present study, *T. subulata* leaf litter leachates were found to have significant allelopathic effects on *Lactuca sativa*, *Brassica chinensis, Oryza sativa* and *Triticum aestivum*. Leaf litter leachate of *T. subulata* at the amount of 10 mg and 50 mg caused the significant inhibitory effect on the radicle and hypocotyl length of all receiver species (Table 1).

The hypocotyl length of *L. sativa*, *B. chinensis, O. sativa* and *T. aestivum* were significantly lower than among the control seedlings at 50 mg amount of *T. subulata* leaves with the inhibitory effect on the hypocotyl length of *T. aestivum* was the least. At similar amount of leaves, the highest inhibitory effect of hypocotyl elongation was shown at *B. chinensis* by 60.31% compared to the control. Whereas, 5 mg of leaf litter leachate of *T. subulata* showed significant stimulatory effect on the hypocotyl length of *T. aestivum* compared to control.

In this study, the amount of 10 mg and 50 mg of *T. subulata* leaves, showed significant inhibitory effect on the radicle length of all tested receiver species. The radicle length of *O. sativa* was found being the highest inhibited (by 79.05% compared to control) at the amount of 50 mg of *T. subulata* leaves. The allelopathic effect of *T. subulata* leaves was strongly exhibited on the radicle length of *O. sativa* compared to other receiver species as the inhibition percentage was 49.95% at the amount of 5 mg (which is the lowest amount of *T. subulata* leaves applied).

Determination of allelopathic potential using dish pack method

From Table 2, it was observed that the hypocotyl length of all tested receiver species were significantly inhibited at the distance of 41 mm compared to control. The hypocotyl length of *Lactuca sativa* was found to be the highest inhibited by 56.42 % compared to control at this distance. At the beyond of 41 mm distance well, all receiver species except *T. aestivum* exhibited the significant inhibitory effect on the hypocotyl length compared to control. In addition, the radicle length of *L. sativa, B. chinensis,* and *O. sativa* were significantly inhibited at the distance of 41 mm compared to control. However, there was stimulation on radicle length of *T. aestivum* at this distance by 15.04% of control and at other distances (i.e; 58 mm, 82 mm and 92 mm) as well.

Screening for identification of the potential allelochemicals from *T. subulata* by HPTLC

HPTLC profile of methanol extract of *T. subulata* leaf is presented in Table 3. Blue and yellow color zone that were detected in UV after derivatization in the chromatogram suggested the presence of flavonoids (Fig. 1, 2, 3 and 4). The Rf value of the leaf methanolic extract of *T. subulata* was found to be at 0.15, 0.20, 0.29, 0.33, 2.48, 0.56, 0.64, 0.76 and 0.86 for peak 1, 2, 3, 4, 5, 6, 7, 8,9 and 10 respectively (Table 3). Among them, peaks 5 and 6 were identified as rutin and naringin. The peak height of the detected peaks is given in the Table 3. Peak 5, identified as rutin had Rf of 0.38, peak area of 13291.1 and peak area percentage of 15.78%. Peak 6, identified as naringin had Rf value of 0.48, peak area of 4347.9, and peak area percentage of 5.16%.

Table 1: Allelopathic effects of different amount of *Turnera subulata* leaf litter leachate on the receiver plants as determined by Sandwich method (mean ± standard error)

Bioassay species	Treatments	Length							
		Hypocotyl (mm)				Radicle (mm)			
Lactuca sativa	Control	24.14	±	0.82	a	25.92	±	1.187	a
	5mg	24.54	±	1.06	a	17.82	±	0.986	b
	10mg	20.60	±	1.02	b	14.32	±	0.869	c
	50mg	13.92	±	0.63	c	7.78	±	0.408	d
Brassica chinensis	Control	21.32	±	0.58	a	23.46	±	0.884	a
	5mg	17.38	±	0.44	b	12.48	±	0.349	b
	10mg	16.06	±	0.52	b	9.48	±	0.507	c
	50mg	8.46	±	0.38	c	4.94	±	0.27	d
Oryza sativa var *sativa*	Control	71.08	±	1.05	a	56.22	±	2.364	a
	5mg	73.30	±	1.02	a	28.14	±	0.935	b
	10mg	53.12	±	1.55	b	21.72	±	0.42	c
	50mg	32.10	±	0.60	c	11.78	±	0.555	d
Triticum aestivum	Control	54.94	±	1.57	bc	64.4	±	3.307	a
	5mg	60.94	±	1.55	a	62.74	±	1.515	a
	10mg	58.64	±	1.85	ab	54.92	±	1.082	b
	50mg	51.70	±	1.69	c	33.9	±	0.93	c

Note: Means within the column in each bioassay species followed by the same alphabet were not significantly different ($p<0.05$) according to DMRT.

Table 2: Allelopathic effects of different distance of *Turnera subulata* leaves on the receiver plants as determined by Dish-pack method (mean ± standard error)

Bioassay species	Treatments	Length							
		Hypocotyl (mm)				Radicle (mm)			
Lactuca sativa	Control	25.70	±	0.65	a	37.70	±	1.17	a
	41 mm	11.20	±	1.25	c	10.40	±	1.40	c
	58 mm	17.30	±	1.52	b	18.30	±	1.99	b
	82 mm	14.70	±	1.61	bc	16.30	±	2.40	b
	92 mm	16.40	±	1.47	b	17.80	±	1.66	b
Brassica chinensis	Control	16.80	±	0.55	a	29.70	±	2.02	a
	41 mm	8.60	±	0.27	d	23.90	±	1.87	bc
	58 mm	11.30	±	0.62	c	18.20	±	1.28	d
	82 mm	10.90	±	0.49	c	20.10	±	2.35	cd
	92 mm	13.30	±	1.18	b	25.80	±	1.72	ab
Oryza sativa var *sativa*	Control	62.90	±	1.24	a	49.90	±	0.92	a
	41 mm	36.60	±	1.66	d	30.20	±	2.39	c
	58 mm	55.50	±	1.78	b	46.70	±	2.50	a
	82 mm	57.30	±	1.81	b	42.80	±	3.32	ab
	92 mm	47.30	±	2.04	c	38.70	±	2.46	b
Triticum aestivum	Control	71.30	±	2.62	ab	45.20	±	1.94	d
	41 mm	47.80	±	2.02	c	52.00	±	2.12	c
	58 mm	68.10	±	2.08	ab	77.60	±	1.48	b
	82 mm	65.60	±	1.78	b	77.00	±	2.23	b
	92 mm	72.70	±	1.48	a	89.40	±	3.55	a

Note: Means within the column in each bioassay species followed by the same alphabet were not significantly different ($p<0.05$) according to DMRT

Table 3: The peak table of flavonoid compounds and unknown compounds of *Turnera subulata* methanolic leaves extract

Peak	Start Rf	Start Height	Max Rf	Max Height	End Rf	End Height	Area	Area %	Assigned substance
1	0.15	9.7	0.18	49.0	0.20	27.2	1218.4	1.45	AutoGenerated8
2	0.20	27.5	0.25	480.0	0.29	182.6	18129.4	21.52	AutoGenerated2
3	0.29	187.6	0.31	421.2	0.33	381.2	12856.0	15.26	AutoGenerated4
4	0.33	382.7	0.35	431.6	0.38	104.5	12173.6	14.45	AutoGenerated3
5	0.38	106.8	0.42	389.5	0.46	52.1	13291.1	15.78	AutoGenerated1
6	0.48	53.8	0.51	119.8	0.55	20.7	4347.9	5.16	AutoGenerated6
7	0.56	22.3	0.59	43.3	0.61	29.2	1389.1	1.65	AutoGenerated10
8	0.64	20.2	0.66	44.1	0.71	0.4	1413.5	1.68	AutoGenerated9
9	0.76	2.5	0.81	135.8	0.85	2.9	4223.2	5.01	AutoGenerated7
10	0.86	0.1	0.93	371.8	0.96	174.9	15192.4	18.04	AutoGenerated5

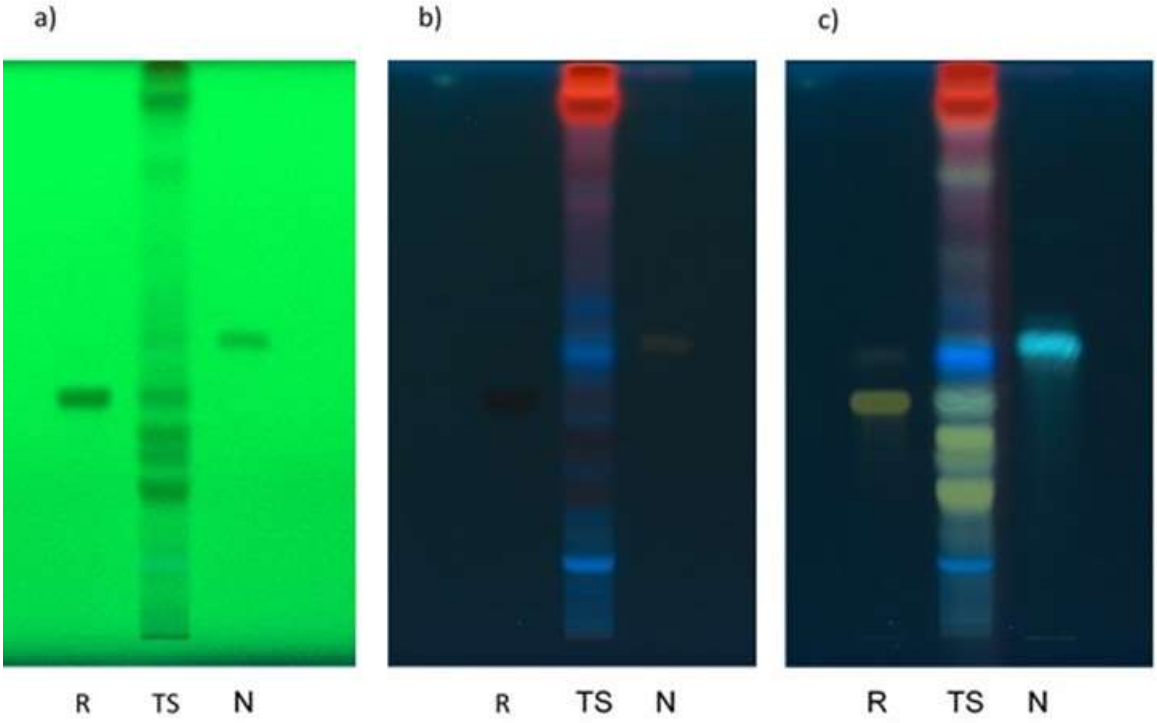

Figure 1: Chromatogram under a) 254 nm, b) 366 nm before derivatization and c) 366 nm after derivatization. R: Rutin, TS: *Turnera subulata* leaves methanolic extract and N: Naringin.

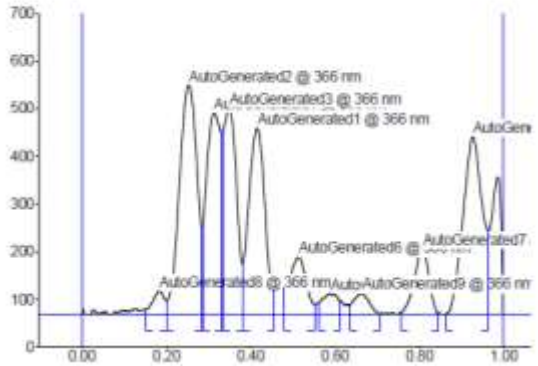

Figure 2: HPTLC Chromatogram of *Turnera subulata* leaves extract showed Peak densitogram display (Scanned at 366nm)

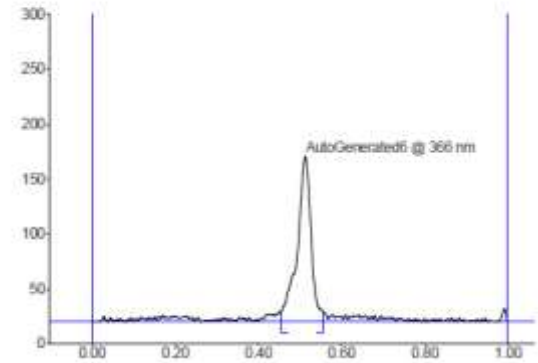

Figure 3;HPTLC Chromatogram showed peak densitogram display Rutin (Scanned at 366nm)

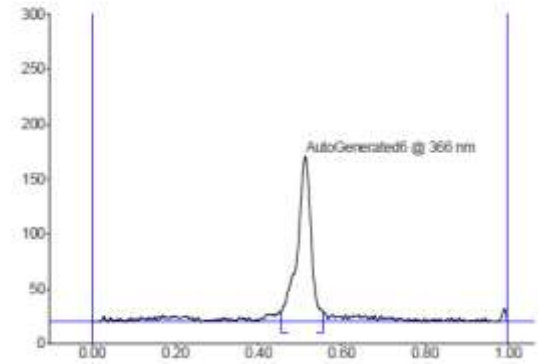

Figure 4: HPTLC Chromatogram shows peak densitogram display Naringin (Scanned at 366nm)

DISCUSSION

Allelopathic activities from *T. subulata* leaves show different degree of radicle and hypocotyl inhibition of selected bioassay species. From the experiment, the degree of radicle and hypocotyl length inhibition shows significantly increased as compared to the control as the amount of the tested *T. subulata* leaves increased from 5 mg to 50 mg. This is consistent with Silvestre et al. (2018), which the decrement value of main root length and hypocotyl length were directly proportional to the concentration of *T. ulmifolia* hexane leaves and branches extract. This concentration dependent activity were also found in the previous research where it was reported that high concentration showed the highest inhibitory activity (Ghnaya et al., 2016; Islam et al., 2018; Suwitchayanon et al., 2017). It had been stated by Hossain and Alam (2010) that allelochemicals exhibited inhibitory effect at high concentration and stimulatory effect at low concentration on germination and growth of receiver plants. Furthermore, allelopathy of other species of *Turnera* (which were *T. ulmifolia* and *T. diffusa*) were reported had inhibitory effect on the development of cucumber seedling (Silvestre et al., 2018).

In this study, the hypocotyl length of all receiver species showed lower inhibitory percentage compared to the radicle length at 50 mg of *T. subulata* leaves. This happened due to the radicle is the first organ that has contact with the agar of tested leaves. Besides, the root tissue has higher permeability compared to the shoot tissue (Islam & Kato-Noguchi 2013). Franco et al. (2015) reported that the root development of receiver species was inhibited due to the ability of allelochemicals that can affect the genes which is responsible for cellular characterization of ground tissue and endoderm.

In addition, this study shows that the effect of leaf volatilization from *T. subulata* on the hypocotyl and radicle length was depending on the types of receiver species. Table 2 shows that the length of hypocotyl and radicle of *L. sativa, B. chinensis, O. sativa* significantly decreased as the wells were further distant from the source well. This indicated high volatility of the volatile compounds from *T. subulata* leaf. The inhibitory activities on the growth of *L. sativa* from leaf volatilization of other plants were also previously reported (Appiah et al., 2015; Kang et al., 2019).

Rutin and naringin are regarded as flavonoids, which are widely distributed in plants. These flavonoids compounds may contributed to allelopathic effects of *T. subulata* as the flavonoids are also known for their phytotoxic activity (Einhellig, 2004). It had been reported by Ghimere et al. (2019), rutin had been found significantly inhibited the seed germination of alfalfa. In addition, rutin also had been observed can reduce the protein content in Arabidopsis cells (Hussain and Reigosa., 2016). This is due to ability of rutin in impairing respiration and ATP levels in embryogenic cells (Takahashi et al., 1998).

CONCLUSION

This present study revealed that *T. subulata* leaves have allelopathic effects on the growth of selected bioassay species which are *L. sativa, B. chinensis, O. sativa* and *T. aestivum*. The leaf litter leachate of *T. subulata* gives the highest inhibitory effect on the growth of weedy rice. However, the leaf volatilization of *T. subulata* gives the highest inhibitory effect on the lettuce growth. The screening test of leaf litter leachate and leaves volatilization in these experiments provided the clear clue for the presence of allelochemicals in the *T. subulata*. HPTLC chromatogram of methanolic extract shows the presence of flavonoids in the sample extract. The possible allelochemicals identified by HPTLC elucidated from methanolic extract were rutin and naringin. Further studies should be carried out on the effect of *T. subulata* leaves in controlling weedy rice in the greenhouse and field. Exploring the potential of *T. subulata* allelopathy could be promising in achieving better weed management for sustainable agriculture.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NY and NHY designed the experiments, performed data analysis and wrote the manuscript. AA performed the experiments and data analysis. KAA, KM, and KCL read and approved the final version.

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