Variation in the phytotoxic activity of *Tinospora tuberculata* extracts as influenced by solvent type and chemical profile

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A study was conducted to evaluate the role of secondary metabolites on the allelopathic activity of methanol and water extracts obtained from aerial parts of *Tinospora tuberculata* on seed germination and the radicle and hypocotyl lengths of barnyardgrass. The higher suppressive effects were observed on germination and seedling growth of barnyardgrass, when the methanol extracts of *Tinospora tuberculata* stem or leaf were applied in comparison to the water extracts. Ultra-fast liquid chromatography analysis confirmed that methanol extracts and leaf extracts contained higher number and amount of chemical compounds than did those of the water extracts and stem extracts respectively. Moreover, the concentrations of 11 identified compounds of the extracts and an equimolar mixture of the chemicals required for 50% growth inhibition on barnyardgrass germination, radicle and hypocotyle were determined. Trans-cinnamic acid and benzoic acid had the highest allelopathic activity, while chlorogenic acid and orientin had the lowest on the basis of the rank values. Benzoic acid was found in the highest concentration in the methanol leaf extract, while this compound was not identified in the water leaf extract. On the other hand, the predominant compound was orientin for stem extracts. These results suggest that these compounds may be involved in the allelopathy activity of *Tinospora tuberculata* depending on their number, concentration, combination and inhibitory activity. *Tinospora tuberculata* could be a potential source of natural inhibitor compounds employable for eco-friendly agriculture.

Keywords: Allelopathy, cluster analysis, phytotoxic components, *Tinospora tuberculata*.

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ALLELOPATHY is a sophisticated mechanism of plant defence. It has been defined as any direct or indirect effect of one plant on the survival, growth and reproduction of another[1]. A number of plant species have shown allelopathic activity on the growth of other plants[2]. These plants are capable of activating defence mechanisms against other plants by producing hundreds of secondary metabolites

These substances have received special attention because of their agricultural potential as either natural herbicides or templates for new synthetic herbicides[3]. Thus, the identification of natural plant components could contribute to the discovery of allelopathic agents. Recently, many research reports have been published on the screening of allelopathic plants, and identification, confirmation, bioactivity test and structural analysis of allelochemicals[4,5].

The extraction of plant tissues is the first step in the isolation of bioactive compounds. In the extraction process, compounds contained in a raw material of a plant are transferred into a solvent[6]. Solvent type, solvent ratio and efficiency of mass transfer are the main parameters that effect the quantity of extraction[7]. The appropriate selection of solvent increases the chemical solubility and rate of the mass transfer[6]. Li and Jin[8] observed high extraction efficiencies when polar solvents (methanol, ethanol, acetone, acetonitrile) were used.

Malaysia has been classified as one of the top rich countries in terms of biodiversity. There are 15,000 plant species, of which about 10% is known to be medicinal[9]. Nevertheless, there is little information available regarding Malaysian allelopathic plants.

Tinospora tuberculata (*Tinospora rumphii* Boerl or *Tinospora crispa*; Malay name Batawali), belongs to the family Menispermaceae, and is a traditional medicinal plant[10]. It is a wild plant that grows in the primary rainforests all over Malaysia, Thailand and Indonesia.

This study was performed to evaluate the role of phytotoxic compounds on the allelopathic activity of methanol and water extracts obtained from aerial parts of *Tinospora tuberculata* on the germination and early growth of barnyardgrass (*Echinochloa crus-galli*).

Tinospora tuberculata plants growing in the Herbal Garden of Universiti Putra Malaysia (02°59′N, 101°43′E and 64 m amsl), Selangor, Malaysia were used in this study. Plants (excluding root) were harvested, cleaned several times with tap water and air-dried for 3 weeks. The plants were separated and bulked into the two major parts – leaf and stem. Both bulked plant parts were then ground into a fine powder in a laboratory blender and sieved through a 40-mesh sieve. Seeds of barnyardgrass (*E. crus-galli*) were purchased from Herbiseed (Wokingham, UK).

Syringic acid (98%), trans-ferulic acid (99 + %), trans-cinnamic acid (99 + %), *p*-anisic acid (99 + %), chlorogenic acid (98%), vanillic acid (99%), coumarin (98%), gallic acid (99%), caffeic acid (99%) and benzoic acid (99.5%) were obtained from Chemtron Biotechnology

Sdn. Bhd (Kuala Lumpur (KL), Malaysia). Vitexin, isovitexin (98 + %), orientin (97+%) and isoorientin (98 + %) were obtained from Sigma-Aldrich (KL, Malaysia). Methanol and acetic acid were obtained from Friendemann (KL, Malaysia). Water was twice distilled and all chemicals used were HPLC grade.

The phytotoxic activity of *Tinospora tuberculata* on germination and initial growth of barnyardgrass was studied using water and methanol extracts. Leaves and stems of *Tinospora tuberculata* were soaked in distilled water and methanol–distilled water solution/mixture (80 : 20, v/v) separately to obtain water and methanol extract respectively. They were shaken in an orbital shaker at room temperature (24–26°C) for 48 h. The solution was filtered through four layers of cheese cloth to remove any debris and then centrifuged at 3000 rpm for 1 h. The supernatant was filtered through one layer of Whatman No. 42 filter paper. To prevent the growth of microorganisms, the solution was filtered again through a 0.2 mm Nalgene filter (Becton Dickinson Labware, NJ, USA). The methanol extracts were evaporated to dryness under vacuum at 40°C using a rotary evaporator[11]. The resulting solid was the desired methanol extract, which was stored at –20°C until use in the bioassays[12].

Each stock extract of the stem and leaf of *Tinospora tuberculata* was diluted appropriately with distilled water to obtain extract concentrations of 3.12, 6.25, 12.5, 25, 50 and 100 g l^{-1} for bioassay.

Thirty seeds of barnyardgrass were placed on two layers of filter paper in a 90 × 15 mm petri dish. Then 5 ml of each extract (water or methanol) at the different concentrations (3.12, 6.25, 12.5, 25, 50 and 100 g l^{-1}) or distilled water as the control were applied to the petri dishes.

All the petri dishes were placed in a growth chamber at a 12 h day/12 h night photoperiod with temperatures of 25°C and 20°C respectively. Germination percentage, and radicle and hypocotyl lengths were measured on all seedlings in each petri dish and sealed flask at 7 days after seeding. The bioassay tests were arranged in a completely randomized design with four replications and the experiment was repeated twice.

Ultra-fast liquid chromatography (UFLC) analysis was performed using a UFLC system (Shimadzu, Kyoto, Japan) equipped with a SPD-20A detector and LC-20AD pump, which provide unmatched gradient speed and precision, a SIL-20A autosampler and a DGU-20-A5 degasser. Sample solutions were separated on a C_{18} reversed-phase column (Luna® 5 µm C18 (2) 100 Å, LC column 250 × 4.6 mm i.d. Phenomenex) which was maintained at 30°C. The detector was set between 190 and 400 nm. The solvent flow rate was 1 ml min^{-1} and 10 µl of sample solution was injected in each run.

Chromatographic separation was achieved using a linear gradient elution in 48 min at a flow rate of 1 ml min^{-1}, consisting of mobile phase A: distilled water–acetic acid (97 : 3), and mobile phase B: methanol (100%). The gradient was optimized as: 100% solvent A for 1 min followed by a linear increase of solvent B to 95% in 48 min. Solvents were filtered through a 0.20 um, 47 mm nylon filter membrane (Phenex). All solvents and distilled water were degassed before use.

The reference compounds were chromatographed alone and in a mixture. Retention times of the standard compounds and major peaks in the extract were recorded. The phenolic and flavonoid compounds were analysed by their retention times with authentic standards, according to the methods of Sodaeizadeh *et al.*[13].

The commercial standards were dissolved in a mixture of H_2O : MeOH (20/80 v/v). The concentration of the stock standard solutions was 1 mg ml^{-1}. Solutions were filtered through 0.2 µm, 15 mm syringe filters (Phenex-NY, Non-Sterile, Luer/Slip). The concentrations of compounds were quantified from peak areas of the studied samples and the corresponding standards using calibration curves. Calibration curves were prepared by UFLC injection of external standard solutions by diluting the stock solutions at the level of 1000, 500, 250, 125 and 62.2 ppm. The concentrations of the compounds in *Tinospora tuberculata* leaves and stems are expressed in mg kg^{-1}.

The inhibitory effects of the individual and mixture of pure compounds were tested by interfering in the germination and initial growth of barnydgrass. The compounds were dissolved in distilled water to achieve six different concentrations (0, 100, 200, 400, 800 and 1600 µM), and then placed in an ultrasonic bath and sonicated at 60 kHz for 1 h at 30°C. Thirty seeds of barnydgrass were placed on a sheet of filter paper in petri dishes of 9 cm diameter. The filter papers were moistened with 5 ml of pure compound solution at different concentrations. Controls were treated with 5 ml distilled water in the same manner. Germination percentage, and radicle and hypocotyl lengths were measured on all seedlings in each petri dish at 7 days after seeding in a growth chamber. The bioassay tests were arranged in a completely randomized design with four replications and the experiment was repeated twice.

In order to determine any significant differences among treatments, a one-way ANOVA was carried out using the PROC GLM procedure in SAS software (SAS 9.3, SAS Institute Inc., NC, USA). Separation of treatment means from the control at 0.05 probability level was conducted using the LSD test, as outlined in the SAS procedure (version 9.3). Effective doses capable of inhibiting 50% of tested parameters were calculated as EC_{50}. The EC_{50} values were calculated by probit analysis based on percentage of parameter inhibition. Ranking for each of the most effective compounds was determined as an index using the following equation for each plant tested

$$R_e = EC_{g50}\ (\text{germination}) + EC_{r50}\ (\text{radicle}) + EC_{h50}\ (\text{hypocotyle}),$$

where R_e is the rank of each compound and the EC_{g50}, EC_{r50}, and EC_{h50} are the concentrations of treatments (compounds) that inhibit 50% of germination, radicle growth, and hypocotyl growth of barnyardgrass respectively. Compounds with the lowest Re values are the most phytotoxic. To further clarify the allelopathic activity of compounds against barnyardgrass, affinity propagation clustering was performed using R packages[14].

The potential allelopathic contribution of methanol extracts of the aerial parts of *Tinospora tuberculata* has been studied[15–17]. In addition, our previous work revealed that the extent of inhibition of leaf extract was more than the stem extract[16,18]. This study was conducted to evaluate the correlation between phytotoxic activity and chemical profile in the methanol and water extracts of *Tinospora tuberculata* aerial parts.

Table 1 shows that water and methanol extracts of *Tinospora tuberculata* exhibited a significant reduction in the germination and early growth of barnyardgrass compared with the non-treated control. The methanol extracts of both leaf and stem have a greater effect in reducing germination, radicle and hypocotyl growth of barnyardgrass. For instance, at highest concentrations of leaf methanol extract, the radicle growth was completely suppressed. While leaf water extract reduced 81% of radicle development of barnyardgrass. In addition, in the presence of the stem water extract at the dose of 100 g l^{-1}, germination, radicle and hypocotyle growth revealed significant inhibition of 5%, 71% and 33% respectively, as against 15%, 76% and 40% for stem methanol extract respectively. Therefore, the magnitude of the effect was dependent on the type of solvent (water and methanol). In line with our finding, Ali *et al.*[19] observed the methanol extract of root, stem and leaf of *Thymus numidicus* had the highest allelopathic effects against *Medicago sativa* and *Triticum aestivum* followed by ethyl acetate than water extract.

The variation of allelopathic activity of extracts suggested that they contain different amounts and types of substances. Therefore, the bioactive extracts of *Tinospora tuberculata* aerial parts were evaluated by UFLC analysis

Table 1. Germination and early growth of barnyardgrass treated with water and methanol extracts of *Tinospora tuberculata* aerial parts

		Leaf water extract			Leaf methanol extract		
Tested plants	Concentration (g l^{-1})	Germination (%)	Radicle length (mm)	Hypocotyl length (mm)	Germination (%)	Radicle length (mm)	Hypocotyl length (mm)
Barnyardgrass	0.00	90.0 ± 1.1 a (0)	54.5 ± 4.3 a (0)	37.0 ± 0.0 a (0)	90 ± 1.2 a (0)	54.5 ± 4.1 a (0)	37.0 ± 0.9 a (0)
	3.15	86.6 ± 4.0 ab (3.7)	51.3 ± 2.9 a (5.8)	37.8 ± 0.8 a (-2.16)	87.7 ± 2.2 ab (2.4)	40.3 ± 7.0 b (26)	36.3 ± 2.4 a (1.9)
	6.25	86.6 ± 6.7 ab (3.7)	51.4 ± 0.54 a (5.6)	34.5 ± 0.7 b (6.7)	87.7 ± 1.1 ab (2.47)	44.1 ± 0.1 ab (19)	33.3 ± 2.1 ab (9.9)
	12.50	85.5 ± 1.1 abc (5)	49.0 ± 3.2 a (10.0)	31.9 ± 0.5 c (13.7)	81.1 ± 6.7 b (9.87)	19.5 ± 4.0 c (64.22)	30.0 ± 2.8 b (18.8)
	25.00	76.6 ± 4.8 bc (14.8)	30.3 ± 2.7 b (44.4)	15.7 ± 0.1 d (57.5)	69 ± 2.0 c (23.3)	11.4 ± 0.8 cd (79.0)	21.0 ± 0.5 c (43.3)
	50.00	73.3 ± 4.0 c (18.5)	24.7 ± 2.3 b (54.6)	15.3 ± 1.0 d (58.6)	67.7 ± 8.0 c (24.7)	1.0 ± 0.5 d (98.0)	9.50 ± 0.22 d (74.36)
	100.00	56.6 ± 2.2 d (37.1)	10.15 ± 1.9 c (81.3)	12.9 ± 0.4 e (65.1)	46.6 ± 1.6 d (48.1)	0 ± 0 d (100)	5.4 ± 0.6 e (85.4)
		Stem water extract			Stem methanol extract		
Barnyardgrass	0.00	83.3 ± 1.9 a (0)	60.3 ± 4.5 a (0)	45.4 ± 1.0 a (0)	84.4 ± 1.1 a (0)	60.3 ± 2.4 a (0)	45.4 ± 1.1 a (0)
	3.15	83.33 ± 3.8 a (0)	59.7 ± 2.2 a (1)	44.2 ± 1.7 ab (2.6)	84.3 ± 0.01 a (0.1)	50.4 ± 2.4 a (16.4)	39.4 ± 1.6 ab (13.24)
	6.25	80 ± 1.9 a (3.9)	56.3 ± 1.6 ab (6.6)	43.7 ± 3.6 ab (3.7)	83.3 ± 0 a (1.3)	49.4 ± 4.2 a (18.1)	33.6 ± 1.4 bc (26.1)
	12.50	80 ± 3.8 a (3.9)	48.9 ± 3.1 b (18.9)	41.6 ± 2.0 ab (8.3)	83.3 ± 5.0 a (1.3)	50 ± 1.6 a (17.1)	33.0 ± 0.6 bc (27.3)
	25.00	76.66 ± 5.0 a (7.9)	50.3 ± 3.0 b (16.5)	38.9 ± 0.8 b (14.3)	78.3 ± 1.6 b (7.2)	42.2 ± 4.8 b (29.9)	30.8 ± 0.2 c (32.1)
	50.00	78.88 ± 1.1 a (5.3)	38.0 ± 1.6 c (36.9)	38.6 ± 1.6 b (14.9)	73.3 ± 0 c (13.1)	28.8 ± 3.4 c (52.1)	29.4 ± 0.8 c (35.2)
	100.00	78.88 ± 4.0 a (5.3)	16.9 ± 1.6 d (71.9)	30.3 ± 1.4 c (33.2)	71.0 ± 0.02 d (15.9)	14.2 ± 0.8 d (76.3)	27.2 ± 0.4 c (40.13)

Data are expressed as means ± SE. Mean with the same letters in the column is not significantly different at $P < 0.05$. Values in parentheses are percentage over control.

Table 2. Major peaks detected in the tested extracts of *Tinospora tuberculata*

		Peak area/mg kg^{-1} DW			
Retention time	Detected compounds	Leaf methanol extract	Leaf water extract	Stem methanol extract	Stem water extract
8.6	Unknown	1,062,860/-	1,381,765/-	–	–
9.0	Unknown	318,661/-	440,246/-	162,699/-	92,881/-
10.1	Unknown	524,362/-	1,057,653/-	120,220/-	86,055/-
11.0	Unknown	139,589/-	768,707/-	–	–
12.7	Unknown	193,109/-	201,128/-	–	–
14.5	Unknown	129,553/-	0	–	60,007/-
20.7	Unknown	278,742/-	310,034/-	–	106,090/-
21.1	Unknown	506,050/-	629,812/-	–	–
22.0	Unknown	151,346/-	102,698/-	64,502/-	–
22.4	Unknown	–	–	65,168/-	–
22.9	Chlorogenic acid	296,467/427.8	3,322/174.4	420,344/534.9	8,687/179
23.4	Unknown	13,964/-	–	–	–
24.4	Unknown	293,366/-	234,997/-	57,180/-	–
24.9	Unknown	228,913/-	52,532	–	180,484/-
26.4	Caffeic acid	4,086,476/2,148.4	3,891,165/2,048.6	755,091/445.4	486,200/308
26.6	Unknown	–	–	178,094/-	15,0961/-
27.0	Syringic acid	616,852/363	57,510/21.7	138,735/71.3	61,771/20.6
27.8	Unknown	2,436,595/-	2,189,888/-	–	727,620/-
28.2	Unknown	1,820,658/-	1,744,264/-	1,414,215/-	979,856/-
28.9	Orientin	1,588,288/1,022.5	1,278,317/858.1	1,259,838/848.3	768,646/587.8
29.4	Isoorientin	4,280,765/1,847.4	930,463/273.9	146,825/-	–
30.7	Vitexin	3,262,457/1,783.7	2,399,952/1335.1	617,503/408	914,849/562.7
31.4	Trans-ferulic acid	565,064/389.6	311,284/237.6	112,692/118.8	0.00
31.9	Isovitexin	9,022,654/4,817	1,741,994/974	343,128/239.4	46,439/80.2
32.1	Unknown	568,654/-	–	–	–
33.4	Unknown	200,231/-	65,972/-	–	131,432/-
33.8	Unknown	751,735/-	–	76,268/-	–
34.6	Unknown	225,044/-	–	–	–
35.0	Unknown	319,847/-	–	–	–
35.6	Benzoic cid	1,125,753/4,129	20,313/46.70	29,826/78.4	36,971/104.8
36.6	*p*-Anisic acid	534,587/115.5	18,591/17.6	1,299/14.3	27,593/19.3
39.9	Trans-cinnamic acid	618,176/141.6	36,208/35.8	10,045/31	2,603/29.6
41.1	Unknown	–	–	90,685/-	–
42.5	Unknown	0	–	82,513/-	–
43.2	Unknown	78,708/-	–	87,058/-	–
45.5	Unknown	182,424/-	–	–	–
Peak area of major peaks		36,421,950/17,185.5	19,868,815/6023.5	6,087,103/2789.8	4,801,978/1,872.7
Number of major peaks		32	24	22	18

to determine their profile of secondary metabolites associated with the observed growth inhibition.

The leaf extracts from methanol and water had 25 and 22 peaks in the UFLC profile respectively. The methanol stem extract had 22 peaks, while 21 peaks were observed in the water stem extract (Table 2). Twenty-three known allelochemicals were subjected to UFLC to identify some substances from the tested extracts according to the method of Sodaeizadeh *et al.*[13]. The UFLC analysis revealed only 11 of these compounds, including benzoic acid, caffeic acid, chlorogenic acid, isoorientin, isovitexin, orientin, *p*-anisic acid, syringic acid, trans-cinnamic acid, trans-ferulic acid, and vitexin had the same retention time with the peaks of the tested extracts. However, benzoic acid was not found in water leaf extract and isoorientin, trans-ferulic and *p*-anisic acid were not present in water stem extract.

Table 2 shows large differences among the UFLC profiles of extracts in terms of the peak areas. The amount of compounds present was the highest in leaf methanol extract (peak area = 36,421,950), followed by leaf water (peak area = 19,868,815), methanol stem extract (peak area = 6,087,103) and water stem extract (peak area = 4,801,978). In terms of quantification, all the identified compounds were detected at higher amounts in leaf extracts and methanol extracts than those of the stem extracts and water extracts respectively (Table 2). Taken together, leaf methanol extract (36,421,950/17,185.5 peak area/mg kg^{-1} DW and number = 32) had the highest number and amount of identified and unknown compounds followed by leaf water extract (19,868,815/6023.5 peak area/mg kg^{-1} DW and number = 23), stem methanol extract (6,087,103/2789.8 peak area/mg kg^{-1} DW and number = 22) and stem water extract (4,801,978/1872.7 peak

Table 3. Allelopathic activity of phytotoxic compounds and sensitivity of examined initial growth parameters

Allelopathic compound	Values in μM (lower-upper)			Rank
	EC_{g50}	EC_{r50}	EC_{h50}	
Benzoic acid	1,488.9 (1421.4–1589.4)	650.2 (580.8–772.1)	1,299.8 (1183.3–1446)	3,439.04
Caffeic acid	2,503.6 (2446.6–2585.8)	959.3 (819–1045.6)	2,372.8 (2302.5–2480.8)	5,835.84
Chlorogenic acid	6,674.5 (6588.3–6802.8)	1,778.2 (1674–1878.08)	4,710.4 (4460.3–4816.9)	1,3163.2
Isovitexin	2,016.9 (1872.3–2147)	1,018.5 (943.3–1258.8)	2,133.4 (2013.7–2385.1)	5,168.96
Isoorientin	2,953.6 (2775.1–3118.4)	502.7 (442.6–619.5)	2,500.1 (2311.6–2746.5)	5,956.48
Mixture	2,839.3 (2591–3096.5)	402.2 (224.9–601.1)	1,813.7 (1547.8–2049.6)	5,055.36
Orientin	5,585.2 (5180.4–59213.1)	829.1 (642.2–1004.4)	2,402.8 (2188.4–2797.1)	8,817.28
p-Anisic acid	1,786.8 (1482.2–2098.7)	509.7 (380.4–685.2)	2,316.4 (2054.4–2576.9)	4,613.12
Syringic acid	2,355.8 (2017.6–2581.9)	720.6 (477.4–979.3)	2,756.8 (2615.2–2919.1)	5,833.28
Trans-cinnamic acid	1,301.7 (1039.2–1539.6)	175 (105.6–201.2)	1,429.4 (1255.5–1799.2)	2,906.24
Trans-ferulic acid	4,649.6 (4328.5–4985.1)	920.6 (642.5–1304.1)	2,042.8 (1726.7–2491)	7,613.12
Vitexin	2,078.0 (1767.6–2404.4)	236.4 (172.6–363.7)	2,041.9 (1686.8–2567.2)	4,356.48
Rank	36,234.56	8703.04	27,820.8	72,758.4

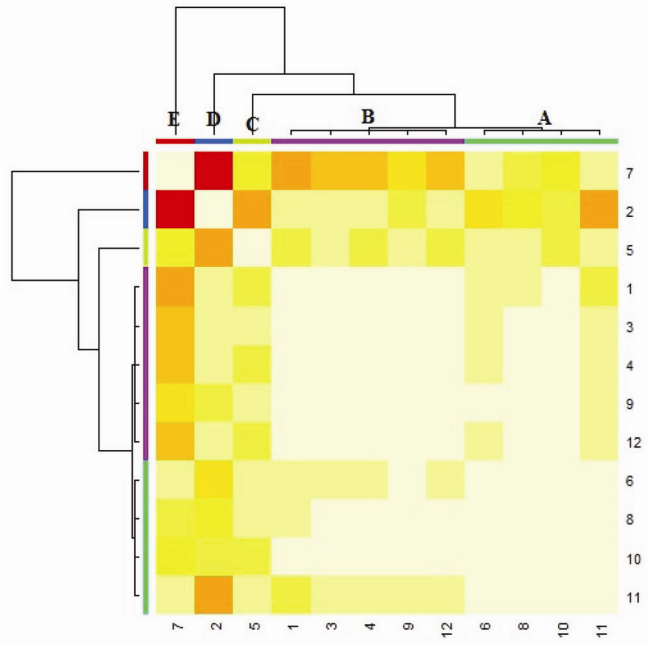

Figure 1. Cluster analysis of the reduction values of seed germination, radicle and hypocotyl elongations of barnyardgrass treated with the detected allelochemicals showing the similarity of phytotoxic activity among allelopathic compounds. 1, Caffeic acid; 2, Chlorogenic acid; 3, Syringic acid; 4, Trans-ferulic acid; 5, Benzoic acid; 6, *p*-Anisic acid; 7, Trans-cinnamic acid; 8, Mixture, 9, Isovitexin; 10, isoorientin; 11, Vitexin; 12, Orientin.

area/mg kg^{-1} DW and number = 18). Verdeguer *et al.*[20] suggested that the suppressive effect of the extracts is dependent on the chemical composition.

The results show that the degree of phytotoxicity found in the filter paper bioassay closely coincides with the amount and number of components in the tested extracts. This suggests that methanol can enhance the chemical solubility and efficiency of the mass transfer relative to water[6]. Omezzine and Haouala[21] observed that methanol extracts had the highest amount of the total phenolics, flavonoids, flavonols and flavones, and precipitable alkaloids among other organic extracts (water, hexane and ethyl acetate).

Phytotoxicity of 11 identified compounds from the aerial parts of *Tinospora tuberculata* was evaluated by the concentration–response bioassay against the seeds of barnyardgrass to assess their possible contribution in the allelopahic activity of *Tinospora tuberculata* extracts.

The concentrations required for 50% growth inhibition (defined as EC_{50}) of germination (EC_{g50}), and radicle (EC_{r50}) and hypocotyle (EC_{h50}) growth of barnyardgrass were calculated. Table 3 shows the examined compounds and their combinations exhibiting various degrees of growth inhibitory effects on the early growth of barnyardgrass. The differences are evident from the rank values of the compounds.

Trans-cinnamic acid (R_e = 2906.24) and benzoic acid (R_e = 3439.04) show higher inhibitory power on germination and initial growth of barnyardgrass. While chlorogenic acid (R_e = 13163.2) and orientin (R_e = 8817.28) show the weakest allelopathic activities compared to the others. The ranking for the other compounds according to R_e value is as follows: trans-ferulic acid > isoorientin > caffeic acid > syringic acid > mixture > *p*-anisic acid > vitexin > isovitexin.

Cluster analysis was also done to integrate parameters measured in previous sections to identify discrete groups of allelochemicals with similar phytotoxicity. Parameters included germination, and radicle and hypocotyl elongation of barnyardgrass 7 days after exposure. Clustering was performed using a centroid hierarchical approach based on a minimum variance linking method with Euclidean distance as the similarity measure.

The dendrogram in Figure 1 shows that the allelopathic activities of the tested components and their combinations in bioassay can be clustered into five interpretable groups (branches A–E; Figure 1).

Clusters C and E consist of benzoic acid and trans-cinnamic acid respectively, characterized by high inhibitory effects and low rank values. Chlorogenic acid clustered into branch D which has the lowest allelopathic activity. Barnyardgrass root growth is suppressed only 3–44% by chlorogenic acid.

Cluster B is comprised of caffeic acid, syringic acid, trans-ferulic acid, orientin and isovitexin (Figure 1). These compounds exert a relatively weak inhibitory effect in comparison with clusters A, C and E.

Finally, cluster A consists of *p*-anisic acid, mixture, isoorientin and vitexin. This branch has moderate allelopathic components, reducing barnyardgrass root growth by 10–95% at all tested rates. The main differences evidenced in the cluster were due to the effects on barnyardgrass.

Benzoic acid was found in the methanol leaf extract at the highest concentration, while this compound was not identified in the water leaf extract. On the other hand, the predominant compound was orientin for methanol and water extracts of the stem. Therefore, the higher inhibitory activity of the methanol leaf extract compared to the water leaf extract, and higher activity of leaf extracts compared to stem extracts can be explained by the level of benzoic acid as most effective compound and orientin as one of the weakest allelopathic compounds. Therefore, based on the concentration and growth inhibitory effect of each tested compound, it seems that benzoic acid, among the other tested compounds, is the most effective in *Tinospora tuberculata* allelopathic activity.

The mixture of all tested compounds showed moderate inhibitory effect when compared with the allelopathic activity of individual compounds (Table 3). This may indicate that interactions of concentration and combination of compounds determine the allelopathic activity of a mixture. Therefore, it might be the allelopathy activity of *Tinospora tuberculata* depending on the concentration, combination and inhibitory activity of its bioactive compounds. Phenolic and flavonoid compounds have been found in a wide range of plants and often exert allelopathic responses[22].

The present study indicates that *Tinospora tuberculata* aerial part extracts show phytotoxic activity on barnyardgrass. The degree of inhibition is largely dependent on the plant part and solvent type. UFLC analysis also shows larger number and amount of chemical compounds being present in the most suppressive extracts. Bioassays of pure compounds indicate that trans-cinnamic acid and benzoic acid are the most active and the mixture of compounds has moderate allelopathic activity. In addition, maximum amount of benzoic acid is present in the most active extracts, although the interaction of other organic compounds cannot be omitted. Therefore, allelopathy activity in each extract varies depending on the number, concentration, combination and phytotoxic activity of each compound. Chemical profile of *Tinospora tuberculata* can be employed in developing new types of herbicides as well as for biorational management tools in organic agriculture. However, their effects on natural enemies and the environment have not been fully studied. It could be profitable to (i) characterize, isolate and determinate the allelopathic activity of the unknown chemical components in *Tinospora tuberculata*; (ii) evaluate the persistence of *Tinospora tuberculata* phytotoxic compounds in the soil, and (iii) assess the mechanism of action of allelopathic compounds on weed species.

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Reproductive biology of *Elaeocarpus blascoi* Weibel, an endemic and endangered tree species of Palni Hills, Western Ghats, India

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***Elaeocarpus blascoi* Weibel is a lesser known endemic tree species growing in the Vattakanal shola forest of Palni Hills, Western Ghats, India. A study on reproductive biology of the species was conducted in the natural habitat to study its phenology, floral biology, pollen biology, fruit set and seed germination. Flowers are bisexual, anther dehisces 2–3 hours after anthesis and stigma becomes receptive on the day of anthesis and extends up to 6 days. Breeding experiments confirmed that the species permits autogamy and geitonogamy. Six different pollinators were observed during peak flowering period and *Apis dorsata* (honey bee) was found to be an effective pollinator and it takes 55 ± 15 sec per flower. Percentage of fruit set observed in the natural habitat was 78 and seed germination rate was found to be less than 5 in the natural habitat. Tests showed that more than 70% of seeds lost their viability after a year and most of the seeds were infested with *Fusarium* sp., *Lasiodiplodia* sp. and *Penicillium* sp. Further, the natural habitat of the species is altered by commercial plantations, tourism and urbanization in the Palni Hills, which leads to their reduction.**

Keywords: *Elaeocarpus blascoi*, endemic species, reproductive biology, shola forest.

IN Asia, the genus *Elaeocarpus* consists of 120 species, of which 25 have been reported from India[1]. Most of the species of *Elaeocarpus* are confined to the North East and southern India, and a few species to Andaman and Nicobar Islands. Eleven species were reported from the Western Ghats of Tamil Nadu (TN), including the recently reported *Elaeocarpus aristatus*[2]. The species of *Elaeocarpus* generally prefer a warm humid climate and usually occurs between 500 and 2000 m amsl (ref. 3). Usually, the genus consists of large trees with buttressed root along the perennial streams of tropical forests, mostly in the evergreen and shola forests. Though it is widely distributed, the species is never found in abundance in any particular locality. The fruits of *Elaeocarpus* species are endowed with a hard and highly ornamental stony endocarp. In nature, the rate of seed germination of most species of *Elaeocarpus* is low and erratic, since the nuts are unable to imbibe water[4]. Poor or no germination

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