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Chemical Characterization and Phytotoxicity of Foliar Volatiles and Essential Oil of *Callistemon viminalis*

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Abstract: We investigated the chemical composition and phytotoxicity of foliar volatiles (directly released from the macerated leaves) and essential oil extracted from the leaves of *Callistemon viminalis* against four weed species. Essential oil (EO) and foliar volatiles caused reduction in germination, seedling growth and dry matter accumulation in *Bidens pilosa*, *Cassia occidentalis*, *Echinochloa crus-galli* and *Phalaris minor*. *Bidens pilosa* was found to be the most sensitive towards foliar volatiles and EO, whereas *C. occidentalis* was the least sensitive. The chemical analyses of foliar volatiles and EO revealed the presence of 1,8-cineole and α-pinene as the main monoterpenes. The study concludes that volatile components of *C. viminalis* possess phytotoxicity against weeds and thus may hold promise for the management of weeds under sustainable agriculture.

Key words: Foliar volatiles, Monoterpenoids, Cineole, Biological activity, Weed control.

Introduction

Plants emit a variety of volatile organic compounds that are involved in multiple ecological functions such as defense against herbivores and pathogens, as attractants of pollinators and seed dispersers, and plant-plant interactions including allelopathy [19,32]. The volatile organic compounds especially emanated from the leaves (hereafter foliar volatiles) are receiving much attention by scientists for their role in allelopathy that can be further exploited for weed management owing to their environmentally benign properties [6,7,36]. The foliar volatiles remain stored within the plants in the special histological structures like secretory cavities, resin ducts, laticifers or glandular trichomes [17,37]. These are easily released into the environment at ambient temperature by crossing over the membranes freely either through diffusion or other biological processes [25,38]. Environmental conditions like temperature, light and injury influence the quantity of volatile organic compounds emitted from the foliar tissues [14]. Upon release, these may be involved in allelopathic interactions bringing significant alterations in the structure and composition of the plant communities that leads to vegetation patterning [21]. Muller and Muller [22] reported the formation of bare zones (devoid of grasses) around the aromatic bushes of *Salvia* species owing to the release of certain volatile terpenes (mainly cineole, camphor, α- and β-pinene) from these bushes. It forms a characteristic pattern of vegetation around *Salvia* which was referred to as "*Salvia* phenomenon" [27]. Similarly, the exclusion of herbs around aromatic shrubs

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of *Artemisia californica* Less. was also attributed to the release of volatile toxins under natural conditions [16]. Later, the phytotoxic nature of the foliar volatiles, particularly against weeds, was also reported in *Nepeta* × *faassenii* Bergmans ex Stearn [11]; *Anisomeles indica* (L.) Kuntze [6]; *Calamintha nepeta* (L.) Kuntze [3] and *Tinospora tuberculata* Beumee [4]. These studies indicate that foliar volatiles can serve as potential candidates for weed management. Since there is difficulty in trapping these volatiles, their pool available as essential oils within the leaf tissues, may be exploited for this purpose. It is thus important to isolate and compare these with foliar volatiles and determine their chemical profiles and phytotoxicity.

The genus *Callistemon* R. Br. (Family Myrtaceae) is represented by species that are mostly shrubs or small trees. These plants bear evergreen persistent foliage and brush-like flowering spikes that provide them the common name of bottlebrushes [33]. Though a native of Australia, *Callistemon* species are cultivated worldwide because of ornamental and medicinal value and their edible fruits. *C. viminalis* (Gaertn.) G. Don., commonly known as weeping bottlebrush, is one of the very common species of *Callistemon*, extensively cultivated in the gardens, parks and road sides in different parts of the world including India [2]. It has pendulous evergreen foliage with crimson red flowers and has aromatic leaves and inflorescence [24]. Being a rich source of chemical compounds with fungicidal, antimicrobial, antioxidant and insecticidal properties, *C. viminalis* is widely used as environmental bioindicators [28,29,39]. de Oliveira *et al*. [8] reported that *C. viminalis* possesses allelopathic properties owing to presence of essential oil in flowers and its constituent monoterpenes. We observed scarce vegetation growing beneath *C. viminalis* trees. We, therefore, hypothesized that this tree may inhibit the growth of under-storey or nearby plants due to the release of volatile compounds from its foliage. Further, studies on foliar volatiles and essential oil of *C. viminalis* in plant-plant interactions have not been investigated. In order to accomplish this, the present study was conducted to determine the chemical composition and phytotoxic effect of foliar volatiles (directly released from the macerated leaves) and essential oils (extracted from the leaves) against some weeds.

Materials and Methods

Materials

Mature leaves (~ 5 kg) were collected from trees of *C. viminalis* growing in Panjab University campus (30°45' 29.15"N, 76°46' 06.34"E; 350 m a.s.l.), Chandigarh, India, in November, 2013. The voucher specimen with PAN No. 20314 has been deposited in Herbarium, Department of Botany, Panjab University, Chandigarh, India. Seeds of *Cassia occidentalis* L., *Bidens pilosa* L., *Echinochloa crus-galli* [L.] Beauv. and *Phalaris minor* Retz. were collected from the outskirts of Chandigarh. The seeds of *C. occidentalis* were treated with concentrated sulphuric acid for 1 min for scarification and then washed for 5 min in running tap water followed by distilled water before use. All chemicals were purchased from Sisco Research Laboratory, Mumbai, India; Loba-Chemie Pvt. Ltd., India and Sigma- Aldrich, St. Louis, USA.

Extraction of essential oil

The essential oil was extracted from mature leaves of *C. viminalis* using Clevenger's apparatus. The mature leaves (approximately 1 kg) were cleaned and placed in round bottom flask, boiled for 5 h and the oil obtained from the nozzle was dried over anhydrous sodium sulphate (yield=5.70 ± 0.05 ml kg^{-1} dry weight). It was further referred as *Callistemon* EO (essential oil). The extraction of essential oil was repeated thrice. It was stored at 4°C for further analysis by GC-MS and bioassay.

Germination and growth studies

Phytotoxicity of foliar volatiles

The inhibitory effect of foliar volatiles of *C. viminalis* was determined as per Batish *et al*. [6] with some modifications. Mature leaves were collected from the tree and used immediately for the experiment. Leaves weighing (1, 2, 4, and 6 g fresh weight) were macerated into small pieces, wrapped in cheese cloth and placed in glass jar. Before use *C. occidentalis* seeds were scarified with concentrated sulphuric acid. Pre-imbibed (for

16 h in distilled water) seeds of test weeds (25 of *P. minor*, 20 of *E. crus-galli*, 15 of *B. pilosa* and 10 of *C. occidentalis*) were placed on moistened (with approximately 15 ml) filter paper in each glass jar. Seeds placed in similar manner but without leaves served as control. All the jars were sealed with Cello tape to prevent loss of volatiles. After 6 days, percent germination, root and shoot/ coleoptile length, and seedling dry weight (after oven drying at 80°C for 48 h) were measured.

Phytotoxicity of *Callistemon* EO

Pre-imbibed seeds (10 of *C. occidentalis*, 15 of *B. pilosa,* 20 of *E. crus-galli* and 25 of *P. minor* per Petri dish) were placed in 15 cm diameter Petri dishes lined with Whatman #1 filter circle wetted with 10 ml of distilled water. The filter paper was treated with essential oil or distilled water (to serve as control) to have a concentration ranging from 0.1 mg/ml to 1 mg/ml. Petri dishes were sealed with a Cello tape to avoid volatilization of essential oil. After 7 days, the number of seeds that germinated was counted, and root and coleoptile / shoot length and seedling dry weight were measured.

GC-MS analysis

Foliar volatiles were analyzed by Headspace-GC. For this, the mature leaves of *C. viminalis* were plucked and directly put into a glass vial that was immediately sealed to retain the volatiles present in the foliage. After 30 min, a needle (temperature controlled needle with syringe temperature of 100°C) was used to withdraw 1 µL of headspace from the vial, which was then injected to Thermo GC (Trace 1300 Gas chromatograph) equipped with TG 5 column [30 m × 0.25 mm (inner diameter), and 0.25 µm film thickness] and FID (flame ionization detector). GC-MS was done using TSQ 8000 triple Quadrapole mass spectrophotometer equipped with TG 5MS column. Helium was used as a carrier gas at a split ratio of 1:150 and the column flow rate was 1 ml/min. The oven temperature was programmed from 60°C (held isothermally for 2 min) to 250°C with ramp of 3°C per min and held at 250°C for 5 min. Injector temperature and ion source temperature were set at 250°C and 230°C, respectively. For *Callistemon* EO, 1 µL of oil (in hexane solution) was injected, whereas all other conditions were the same as for the analysis of foliar volatiles. The mass spectra were scanned in the range of *m/z* 41-600 (for foliar volatile) and 30-400 (for *Callistemon* EO).

Different components were identified on the basis of (i) comparison of their retention indices (RI) relative to homologous series of n-alkanes (C_7-$C_{30,}$ Sigma Aldrich), (ii) computer matching of mass spectra with the NIST library, and (iii) consulting libraries of NIST 98 [34], Pherobase and compilation by Adams [1]. The co-GC was done on the basis of retention times of authentic reference compounds.

Statistical analyses

The germination and growth studies were conducted in a randomized design with five replicates for each treatment, including the control. The data on percent germination was analyzed by Dunnett's test at $P \leq 0.05$ and $P \leq 0.01$. The statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL). The data on root and shoot/ coleoptile length, and dry weight were analyzed by linear regression models.

Results

Foliar volatiles and Callistemon EO are phytotoxic

The foliar volatiles from *C. viminalis* inhibited the germination, root and shoot/ coleoptile length and dry weight in all the test weeds. Foliar tissue of *C. viminalis* (1-6 g) caused a significant ($P \leq 0.01$) reduction in germination in test weeds, except in *B. pilosa* and *C. occidentalis* in response to 1 g leaf tissue. Germination declined ($P \leq 0.01$) by 70 %, 67 %, 63 % and 53 % in *B. pilosa*, *P. minor*, *E. crus-galli* and *C. occidentalis* upon exposure to 6 g leaf tissue (Table 1). Root and shoot/coleoptile length and seedling dry weight of test weeds declined ($P \leq 0.05$) in a dose-dependent manner upon exposure to leaf tissue (Fig. 1a, 1b, 1c). Root length declined ($P \leq 0.05$) by 81 % in *E. crus-galli* and 74 % in *P. minor* on exposure to 6 g leaves of *C. viminalis*, over that in the control. Coleoptile/shoot length declined ($P \leq 0.05$) by ~69 % in *B. pilosa*, 52 % in *P. mi-*

Table 1. Percent decline in germination (over the control) of test plants in response to the foliar volatiles of *C. viminalis*

	Target plant			
Amount of leaves (g)	*B. pilosa*	*C. occidentalis*	*E. crus-galli*	*P. minor*
1	14.63	10.00	15.68*	20.31**
2	41.47**	26.67*	23.53**	42.19**
4	60.97**	46.67**	50.98**	54.68**
6	70.73**	53.33**	62.74**	67.19**

*, ** within a column for a particular test plant represent significance at $P \leq 0.05$ and $P \leq 0.01$, respectively, applying Dunnett's test

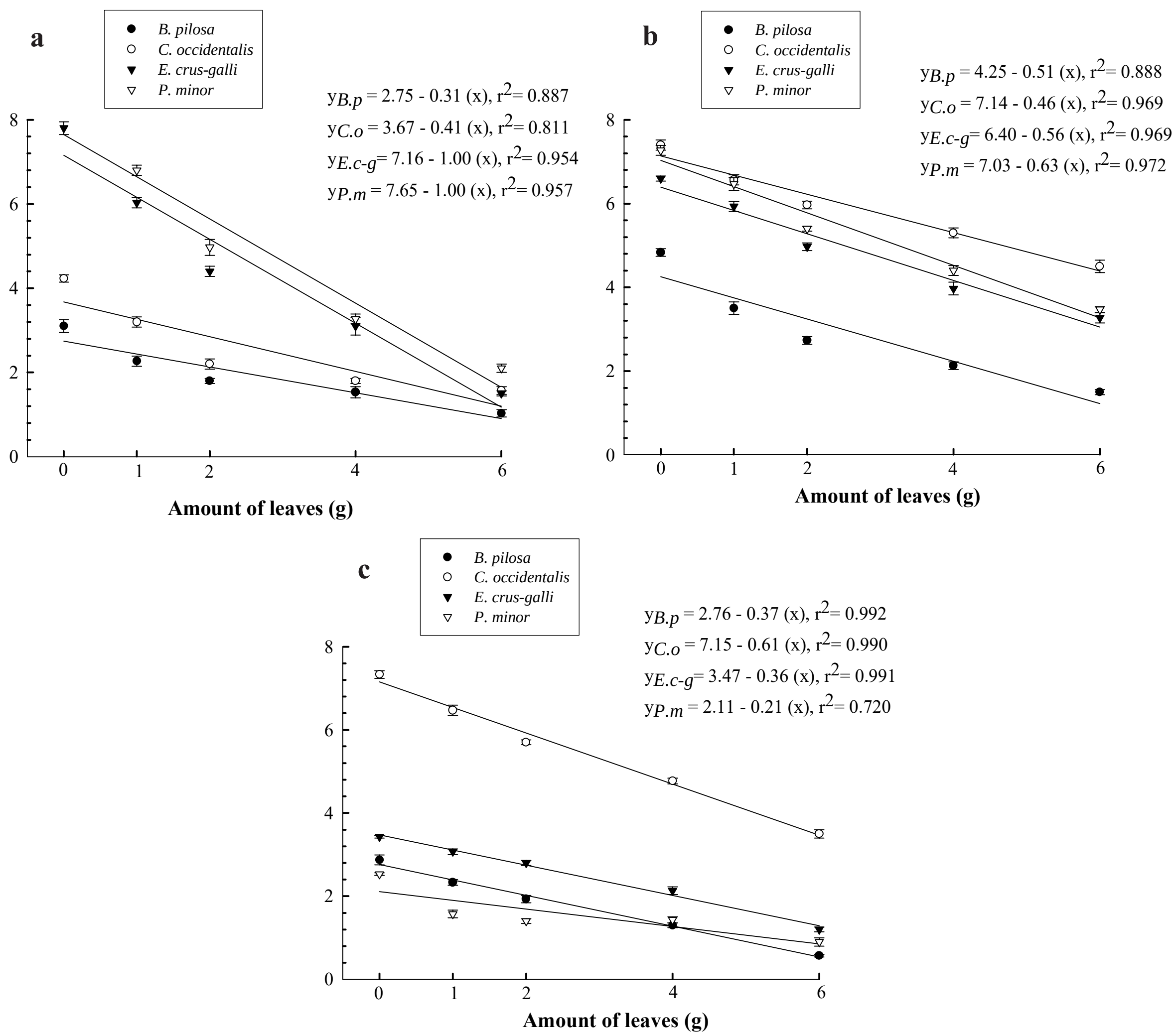

Figure 1. Effect of foliar volatiles of *C. viminalis* on **a**) root length, **b**) shoot length, and **c**) dry weight of test plants. Vertical bars along each data point represent the standard error of the mean. Data were analysed by linear regression. Black lines represent regression lines, and r^2 represents coefficient of determination. All the regressions were significant at $P \leq 0.05$, except for dry weight of *P. minor*

nor, 51 % in *E. crus-galli* and 39 % in *C. occidentalis* over their controls (Fig. 1b). The seedling weight was found to be the least in *C. occidentalis.* Volatiles released from 6 g leaf tissue decreased seedling dry weight by 80 %, 65 % and 64 % in *B. pilosa*, *E. crus-galli* and *P. minor* (Fig. 1c).

Callistemon EO (0.1-1 mg/ml) reduced ($P \leq 0.05$) seed germination, root and shoot/coleoptile length, and dry matter accumulation in test weed species. The percent reduction in seed germination was significant ($P \leq 0.01$) at $\geq$0.1 mg/ml *Callistemon* EO, except in *B. pilosa* ($P \leq 0.01$ at 0.25 mg/ml) and *C. occidentalis* ($P \leq 0.01$ at 0.50 mg/ml) (Table 2). None of the seed of *P. minor* and *B. pilosa* germinated at 1 mg/ml and 0.75 mg/ml *Callistemon* EO, respectively (Table 2). Root length declined by 16-100 %, 16-78 % 21-100 %, and 4-69 % in *B. pilosa*, *E. crus-galli* and *P. minor* and *C. occidentalis* in response to 0.1 to 1 mg/ml of *Callistemon* EO (Fig 2a). The inhibitory effect of *Callistemon* EO was the most pronounced in *B. pilosa* ($P \leq 0.05$ at 0.50 mg/ml) (Fig. 2b). The dry weight was affected the most in *B. pilosa* followed by *P. minor, E. crus-galli* and *C. occidentalis* (Fig. 2c).

Chemical profiles of foliar volatiles and *Callistemon* EO

A total of 5 compounds were identified in foliar

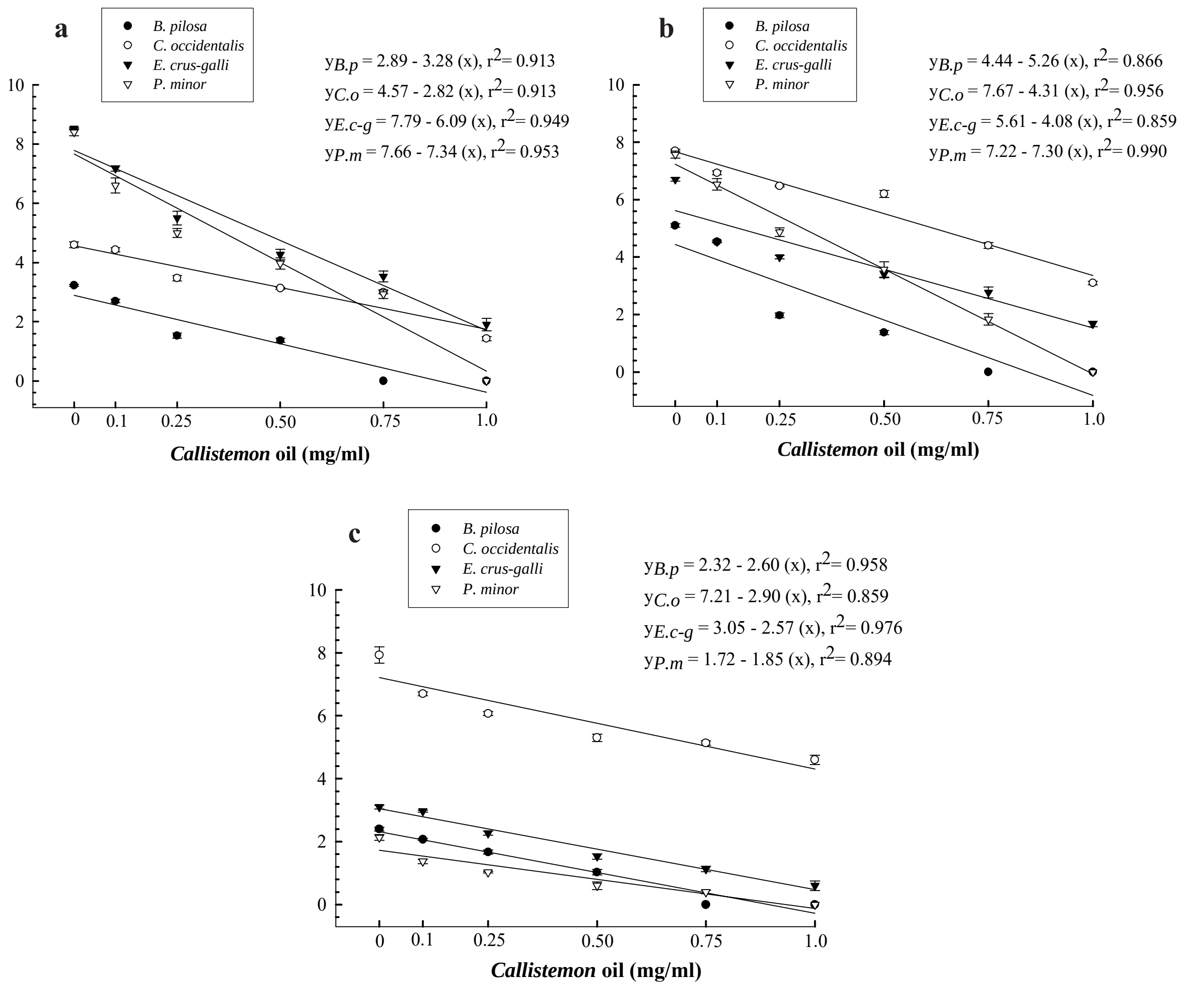

Figure 2. Effect of *Callistemon* EO on **a**) root length, **b**) shoot length, and **c**) dry weight of test plants. Vertical bars along each data point represent the standard error of the mean. Data were analyzed by linear regression. Black lines represent regression lines, and r^2 represents coefficient of determination. All the regressions were significant at $P \leq 0.05$, except for shoot length of *B. pilosa*

Table 2. Percent decline in germination (over the control) in test weeds in response to *Callistemon* EO

	Target plant			
Conc. (mg/ml)	***B. pilosa***	***C. occidentalis***	***E. crus-galli***	***P. minor***
0.1	13.51	6.67	18.37*	26.23**
0.25	45.95**	13.33	26.53**	42.62**
0.50	86.49**	36.67**	46.95**	55.74**
0.75	100.00**	40.00**	57.14**	83.61**
1.0	100.00**	46.67**	81.63**	100.00**

*, ** within a column for a particular test plant represent significance at $P \leq 0.05$ and $P \leq 0.01$, respectively, applying Dunnett's test

Table 3. Chemical characterization of foliar volatiles emitted from mature leaves of *C. viminalis* as revealed through Headspace GC and its composition by chemical class

No.	RT	Component[a]	Percent [b]	Identification method [c]
1	5.28	α-Pinene	31.20	MS, co-GC
2	7.99	D-Limonene	15.21	MS, co-GC
3	8.10	1,8-Cineole	41.89	MS, co-GC
4	24.30	Z-Cinerolone	3.81	MS*
5	24.80	9-Ethoxy-10-oxatricyclo [7.2.1.0(1,6)]dodecan-11- one	7.89	MS*
		Chemical Class	**Percent (%)**	
		Total monoterpenoids (1,2,3)	88.30	
		Monoterpenoid hydrocarbons (1,2)	46.41	
		Oxygenated monoterpenoid (3)	41.89	
		Total sesquiterpenes	-	
		Sesquiterpene hydrocarbons	-	
		Oxygenated sesquiterpenes	-	
		Others (4,5)	11.70	
		Total identified	100.00	

[a]Compounds present in order of elution from the TG-5MS capillary column
[b]Percentage based on FID peak area normalization ($n = 3$)
[c]Methods: co-GC; identification based on retention times of authentic reference compounds
*MS, tentatively identified on the basis of computer matching of mass spectra of peaks only

volatiles emitted from macerated leaves of *C. viminalis* (Table 3). It included 46 % monoterpene hydrocarbon, 42 % oxygenated monoterpenes and 12 % miscellaneous compounds (Table 3). 1,8-cineole (~42 %) and α-pinene (~31 %) were the major components followed by D-limonene (~15 %), 9-ethoxy-10-oxatricyclo[7.2.1.0 (1,6)]dodecan-11-one (~8 %) and *Z*-cinerolone (~4 %) (Table 3). GC-MS analysis of light brown coloured *Callistemon* EO revealed the presence of 22 compounds, majority of which were monoterpenes (97.6 %) (Table 4). In general, the

Table 4. Chemical characterization of *Callistemon* EO through GC-MS and its composition by chemical class

No.	RT[a]	RI[b]	Component[c]	Percent[d]	Identification method[e]
1	5.76	913	Propanoic acid, 2- methyl-, 2-methyl propyl ester	0.14	MS,RI
2	6.38	933	α-Pinene	16.85	MS,RI, co-GC
3	6.80	947	Camphene	0.05	MS,RI, co-GC
4	7.70	976	β-Pinene	0.38	MS,RI, co-GC
5	8.17	991	β-Myrcene	0.09	MS,RI, co-GC
6	8.66	1006	α-Phellendrene	0.43	MS,RI, co-GC
7	8.94	1013	Propanoic acid, 2-methyl-, 3- methylbutyl ester	0.16	MS
8	9.73	1033	1,8-Cineole	63.82	MS,RI, co-GC
9	10.31	1048	β-(Z)-Ocimene	0.11	MS,RI, co-GC
10	10.73	1058	γ-Terpinene	0.23	MS,RI
11	12.44	1102	Linalool	0.45	MS,RI, co-GC
12	13.43	1125	3-Octanol, acetate	0.07	MS,RI
13	14.06	1140	L-Pinocarveol	0.28	MS,RI
14	15.30	1168	Santolina alcohol	0.24	MS
15	15.74	1179	Terpinen-4-ol	0.32	MS,RI, co-GC
16	16.37	1193	α-Terpineol	9.88	MS,RI, co-GC
17	19.10	1256	Piperitone oxide	3.64	MS,RI
18	19.85	1273	n-Amyl ether	0.58	MS
19	21.04	1301	*trans*-Ascaridole	0.50	MS,RI
20	26.01	1419	Caryophyllene	0.22	MS,RI, co-GC
21	28.54	1481	Germacrene D	0.11	MS,RI
22	32.37	1579	(+)-Spathulenol	0.18	MS,RI

Chemical Class	Percent (%)
Total monoterpenoids (2-6,8-11,13,15-19)[f]	97.61
Monoterpenoid hydrocarbons (2-6,9,10)	18.14
Oxygenated monoterpenoid (8,11,13,15-19)	79.47
Total sesquiterpenes (20,21,22)	0.51
Sesquiterpene hydrocarbons (21,22)	0.33
Oxygenated sesquiterpenes (22)	0.18
Others (1,7,12,14)	0.61
Total identified	98.73

[a] Retention Time of compounds on TG-5MS capillary column
[b] Retention index relative to *n*-alkanes (C_{7-30}) on the TG -5MS capillary column
[c] Compounds present in order of elution from the TG-5 capillary column
[d] Percentage based on FID peak area normalization (*n*=3)
[e] Methods: MS, tentatively identified on the basis of computer matching of mass spectra of peaks with NIST 98 and pherobase libraries
RI, tentatively identified on the basis of matching of the retention index with published literature
co-GC; identification based on retention times of authentic reference compounds

Callistemon EO was monoterpenoid in nature with ~79 % oxygenated and 18 % hydrocarbon monoterpenes (Table 4). 1,8-Cineole (~64 %) was the major component followed by α-pinene (~17%), and α-terpineol (~10 %) (Table 4).

Discussion

We observed inhibitory effects of *Callistemon* foliar volatiles and *Callistemon* EO on germination and seedling growth of the test plants. In general, compared to the foliar volatiles, *Callistemon* EO was more phytotoxic. This may be attributed to immediate volatilization of foliar volatiles into the atmosphere in the form of vapors that are difficult to trap due to injury / maceration of leaves. *B. pilosa* was the most sensitive to both the foliar volatiles and *Callistemon* EO, whereas *C. occidentalis* was the least sensitive. These observations are in accordance with earlier studies indicating the phytotoxic nature of foliar volatiles and essential oils of aromatic plants towards growth of other plants [3,5,6,18]. Eom *et al.* [11] observed that fresh leaves (0, 5, 10, and 20 g) of *Nepeta× faassenii* inhibited seedling growth of *Lepidium sativum* L. by 44 % and it was attributed to the presence of foliar volatiles. Batish *et al.* [6] demonstrated reduction in seedling growth and dry matter content in *B. pilosa*, *C. occidentalis*, *Amaranthus viridis* L. and *E. crus-galli* in response to foliar volatiles of *Anisomeles indica*. Aslani *et al.* [4] reported inhibition in seed germination, radicle and hypocotyl length in *E. crus-galli* in response to volatiles of *Tinospora tuberculata*. In fact, *in-vitro* volatile bioassays have been considered a potential means to assess the impact of leaf detachment from the living plants [11]. Upon detachment, production of abscisic acid and ethylene increases due to alterations in water status or its availability [35]. It is well-known that these hormones induce metabolic changes in plant system, which further changes production of volatiles [12,20]. Volatile oils of *Eucalyptus citriodora* reduced seed germination of *Triticum aestivum* L., *Zea mays* L., *Raphanus sativus* (L.) Domin, *C. occidentalis*, *A. viridis* and *E. crus-galli* [5]. Kaur *et al.*[18] reported a decrease in seed emergence and seedling growth of weeds, *Achyranthes aspera* L., *C. occidentalis*, *Parthenium hystero-phorus* L., *E. crus-galli* and *Ageratum conyzoides* L. in response to *Artemisia scoparia* Waldst. & Kit. oil. *C. viminalis* leaves synthesize and emit a significant amount of various volatiles through surface glands as these have been observed in abundant on its adaxial surface.

GC-MS analyses of both *Callistemon* foliar volatiles and EO revealed 1,8-cineole and α-pinene as the major components. These observations are in agreement with previous studies of Silva *et al.* [29] and de Oliveira *et al.* [8] who reported that leaf oil of *C. viminalis* from Brazil contained 65-67 % of 1,8-cineole and 12-16 % of α-pinene. It is well known that various factors such as age and development stage of the plant, organ and climate have a great influence on total amount, nature and proportion of metabolites [15]. Interestingly, the component *D*-limonene, 9-ethoxy-10-oxatricyclo[7.2.1.0(1,6)]dodecan-11-one and *Z*-cinerolone present in the foliar volatiles were completely absent in the oil. The presence of new components in the foliar volatiles may be due to *de novo* synthesis that may be induced upon injury / maceration [13] or their conversion into other compounds / monoterpenes for storage as essential oil [9].

Aromatic plants exhibit their phytotoxic effect through the release of foliar volatiles and their constituent monoterpenes. The major monoterpenes identified in present study are well known growth inhibitors [26,40]. Singh *et al.* [31] reported inhibitory effect of two monoterpenes, cineole and citronellol, on germination and early growth of *Ageratum conyzoides* and reported that cineole was more phytotoxic. Nishida *et al.* [23] observed a significant reduction in root length of *Brassica campestris* L. upon treatment with α-pinene, β-pinene, camphor and 1,8-cineole. Singh *et al.* [30] revealed that monoterpene, α-pinene inhibited radicle length of *C. occidentalis* by inducing oxidative damage due to enhanced generation of reactive oxygen species. The observed growth inhibitory effects of leaves of *C. viminalis* may be attributed to the synergistic effect of their major constituents which were found to be same in both the chemical profiles of foliar volatiles and essential oil.

Conclusions

The present study concludes that foliar volatiles and EO of *C. viminalis* were monoterpenoid in nature and 1,8-cineole and α-pinene were identified as the predominant monoterpenes. These possess phytotoxic properties towards the test weed species and thus can be utilized for the development of a bioherbicide.

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