Journal of Applied Botany and Food Quality 86, 33 - 36 (2013), DOI:10.5073/JABFQ.2013.086.005

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Plant growth inhibitory terpenes from *Eupatorium adenophorum* leaves

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(Received October 15, 2012)

Summary

Eupatorium adenophorum is commonly known as Crofton weed, growing widely throughout the northern hilly terrains. Bioactive potential of *E. adenophorum* leaves was investigated through phytochemical study and *in vitro* plant growth inhibitory. Essential oil was hydrodistilled from leaves and analyzed by GCMS. Leaf material was extracted using cold extraction process followed by evaporation of solvent under reduced pressure. Five cadinene sesquiterpenes and one sterol were isolated from hexane and EtOAc concentrates and their structures were established spectroscopically. Plant growth inhibitory activity of extractives and isolated terpenes were studied against different seeds of weed and crops. Essential oil was moderate seedling growth inhibitor. Among various extracts, EtOAc extract was found to be most inhibitory to *Phalaris minor* seeds (EC_{50} 117 µg mL^{-1}). Among the sesquiterpenes, 5,6-dihydroxycadinan-3-ene-2,7-dione was found to be most active and inhibited both shoot and root growth of *Phalaris minor* seed (EC_{50} 97 µg mL^{-1}) and *Polygonum plebejum* (EC_{50} 117 µg mL^{-1}).

Introduction

Eupatorium adenophorum Spreng (syn. *Ageratina adenophora* King and Robinson) is fast growing perennial herbaceous plant, belonging to the family Compositae (Duan, 2003). The plant flourishes abundantly forested and cultivated lands, and is widely distributed from tropical to temperate region such as America, Europe, Australia, South Africa, India, Thailand and China (Auld, 1966). The plant is introduced in India in 19th century since then widely proliferating in the hilly terrain of northern, north eastern region and other lower hilly regions of southern India (Borthakur, 1977). Due to significant essential oil content in its aerial parts (Weyerstahl et al., 1998; Pala-Paul et al., 2002), it is considered as a valuable raw material for perfumery industry (Sharma et al., 1998; Adhikari and Kraus, 1994). The plant is reported to contain an array of bioactive constituents like monoterpenes, sesquiterpenes, flavonoids, phenyl propanoids and their derivatives (Proksch et al., 1983). Sesquiterpenoids are the major constituent of leaves and flowers (Ding et al., 1999; Zinghui and Jingkai, 1999). Cadinene sesquiterpenes were also identified in leaves (Lan et al., 2006). The plant is also well known for its insect repellent activities (Sood et al., 2000; Li et al., 2001; Mehta et al., 2002; Wang, 2002; Rajmohan and Ramaswamy, 2007). Potential molluscicidal activity of aqueous extract of the plant was reported against *Oncomelania hupensis* (Zou et al., 2009). Besides, growth inhibitory activity of *E. adenophorum* litter was also studied on survival and growth of *Lantana camara* (Kaul and Bansal, 2002). However, except some sporadic attempts on plant growth inhibitory activity (Zheng and Feng, 2005), detailed growth inhibition assay of cadinenes from *E. adenophorum* has not been studied. As far as our literature survey could ascertain, there is no report on antifungal activity of the cadinene derivatives isolated from leaves of *E. adenophorum*. The present study was therefore aimed to isolate and evaluate major bioactive constituents of *E. adenophorum* leaves for plant growth inhibitory activity against seeds of three weeds and two crops.

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Materials and methods

Reagents and instruments

All solvents and reagents were of the highest purity needed for each application. Solvents and chemicals were purchased from Sigma® (USA) and Merck® India Ltd. and used without further purification. Materials used for column chromatography was silica gel (100-200 mesh; Merk Specialities Private Ltd. Mumbai, India). $HSGF_{254}$ silica gel TLC plates (Merck Specialities Private Ltd. Mumbai, India) were used for analytical TLC and spots were detected under UV light or by heating after spraying with 98% H_2SO_4. Preparative TLC (0.4-0.5 mm) was performed on glass plates precoated with silica gel GF_{254}. UV and IR spectra were recorded respectively with a Jasco V-650 spectrophotometer and an FTS-40 infrared spectrometer with KBr pellets. NMR spectra were recorded on a Brucker 400 AC (400 and 75.5 MHz) NMR spectrometer with TMS as an internal standard. ESI-MS/MS were measured on a Thermo LC/MSD Trap XCT mass spectrometer (Finnigan MAT Incos 50).

Plant material

Leaves of *E. adenophorum* were collected during 2009 from forest area of Kangra, Himachal Pradesh, India. After identification of specimen, a voucher sample (EA-EAA-05-09) was deposited in the herbarium of Department of Botany, Himachal Pradesh Agricultural University, Himachal Pradesh, India.

Essential oil extraction and GC-MS analysis

Shade dried leaf powder (5 kg) was subjected to hydrodistillation for 4 hours using a Clevenger apparatus to obtain essential oil. Essential oil was collected and dried over anhydrous sodium sulfate and preserved in a sealed vial at 4 °C until analysis. GC-MS analysis was carried out on a Thermo Fischer capillary gas chromatograph equipped with flame ionization detector (FID) for FI-detection, which is further directly coupled to the mass spectrometer system and HP-5MS capillary column (30m × 0.25 mm i.d. × 0.25 μm film thickness). Temperature programming was done 60-125 °C at 2 °C/min hold time 2 min., 125-160 °C at 0.5 °C /min hold time 5 min. and 160-240 °C at 5 °C/min. Injector and detector temperatures were maintained at 220 °C and 290 °C, respectively. Helium was used as carrier gas with a flow rate of 1 mL/min. Ion source temperature was 270 °C and mass transfer line 250 °C with split ratio 1:20. Identification of the constituents of essential oil was performed by comparison of their retention times, retention indices and comparison of mass spectral fragmentation pattern.

Cold extraction of leaves

Shade dried leaves (5 kg) of *E. adenophorum* was extracted separately with MeOH (10 L). The solvent was filtered and evaporated

in vacuuo at 42 °C to yield methanolic concentrates. Chlorophyll was removed from methanolic concentrate (920 g) by precipitation with lead acetate (5 %). Methanolic concentrate (10 g) was dissolved in aqueous ethanol (70 %), lead acetate solution (5 %) was added and kept undisturbed for 10 minutes. Chlorophyll lead acetate complex precipitated was filtered to remove undesired precipitates and the filtrate was partitioned sequentially with hexane, EtOAc and *n*-BuOH to obtain respective concentrates.

Isolation of terpenes

EtOAc concentrate (70 g) was subjected to silica gel column chromatography (100-200 mess particle size, pre-activated at 110 °C) using eluent mixtures of EtOAc in hexane (20-100 %, v/v) to obtain 50 fractions. Fractions 15-23 showing four major spots on TLC plates were combined and re-chromatographed using hexane-EtOAc (10 %) followed by preparative-TLC to give EA-1 (75 mg) and EA-2 (55 mg). Fractions 37-46 showing similar spots on TLC were combined and re-chromatographed using a gradient of hexane-EtOAc (50-80 %) as eluent to provide 16 fractions (Fr. I-Fr. XVI). Fraction IX-XII were combined and subjected to preparative TLC to obtain comparatively less polar EA-3 (45 mg) and more polar EA-4 (55 mg). On the other hand, hexane concentrate (50 g) was silica gel column chromatographed with the gradient of increasing hexane in EtOAc (0-50 %). Total number of 32 fractions was collected and finally 5 sub-fractions obtained on combining the eluates based on their similar behavior on TLC. Sub-fraction 1 was subjected to preparative-TLC with hexane-EtOAc (80 %) to obtain EA-5 (60 mg) and EA-6 (32 mg).

Plant growth inhibition assay

Seeds of three weeds *Phalaris minor*, *Polygonum plebejum*, *Chenopodium album* and two field crops wheat (*Triticum aestivum* L.) cv. HD 2329 and chickpea (*Cicer arietinum* L.) cv. BGD72 were collected from Seed Technology Centre, Division of Seed Science and Technology, IARI, New Delhi. Test solutions of essential oil, extracts and terpenes were prepared using DMSO (0.1 %, v/v) as the initial solvent carrier followed by diluting with distilled water to a final concentration of 25 μg mL^{-1}. Other test solutions (i.e., 50, 100, 250, 500 μg mL^{-1}) were prepared by dilution with an aqueous solution of DMSO (5 %). Seeds were washed with ethanol (70 % v/v) for 2 min and surface sterilized using sodium hypochlorite (0.5 % v/v) for 2 min, followed by three washes with sterile distilled water. After sterilization seeds were stored in a refrigerator at 4 °C for 3 days before use. Three layers of filter papers were put in 6 cm diameter glass Petri plates, and the filter papers were impregnated with test solutions. To avoid toxic effect of organic solvent, filter paper treated with DMSO solution was placed in a fume hood for 1 h to allow complete solvent evaporation. Fifty seeds of each test crop were evenly placed on the moist filter paper in each Petri plates. Two controls (filter paper treated with 3 mL of DMSO and filter paper without any treatment) were set. Each treatment had three duplicates. Seeds were allowed to germinate under 12 hrs light at 25 °C. The root and shoot length was measured in both treated (T) and control (C) plates (after 7 days) until all the seeds in the control Petri plates were fully germinated. Percentage inhibition of growth (I %) and ED_{50} (μg mL^{-1}) were determined.

Statistical analysis

Experimental data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) to determine significant differences among treatment means. Differences were considered significant at p levels of 0.05 and 0.01.

Results and Discussion

Chemical constituents

Light yellowish essential oil (0.90 % v/w, dry weight basis) with strong aromatic odour was hydrodistilled from leaves. GC-MS spectrum indicated total twenty six volatile compounds, representing 83.5 % of total composition. About 71.9 % sesquiterpenes and 11.3 % monoterpenic constituents were identified in essential oil (Tab. 1). γ-Cadinene (13.56 %) and germacrene-D (10.03 %) were the major constituents. Two cadinene sesquiterpenes were isolated and purified from hexane concentrate. Similarly, EtOAc fraction was further investigated for its phytochemical compositions. Four cadinene derivatives were isolated and their structures were determined by NMR and mass fragmentation pattern. Further identification of these cadinene derivatives were also done by comparison with the literature data. Cadinene derivatives were iden-tified as 7-hydroxycadinan-3-ene-2-one (EA-1) (Bohlmann and Gupta, 1981), 5,6-dihydroxycadinan-3-ene-2,7-dione (EA-2) (Zhao et al., 2009), 2-acetyl-cadinan-3,6-diene-7-one (EA-3) (Baruah et al., 1994), stigmasterol (EA-4), cadinan-3-ene-2,7-dione (EA-5) (Lan et al., 2008) and cadinan-3,6-diene-2,7-dione (EA-6) (Lan et al., 2008) (Fig. 1). Copies of the original spectra are obtainable from author.

Plant growth inhibition activity

A number of studies have been reported in literature which showed that plant allelochemicals interfere with the growth and establish-

Tab. 1: Essential oil constituents of *E. adenophorum* leaves.

Compounds	RT	RI	Content (%)
Cymene	9.10	1026	0.3±1.2
Camphor	14.95	1100	1.4±1.1
Borneol	16.20	1155	1.8±0.3
Camphene	17.66	1162	2.2±0.6
Verbenol	18.19	1171	0.3±0.9
Bornyl acetate	23.36	1268	1.7±1.3
Geranial	27.17	1294	2.2±2.6
Limonene	31.35	1352	1.5±2.5
Aromadendrene	32.53	1408	3.1±0.7
β-Cedrene	32.94	1418	1.3±1.4
α-Bergamotene	33.32	1433	0.7±2.6
β-Farnesene	34.20	1445	6.7±1.0
Elemene	35.56	1449	9.8±3.6
Caryophyllene	35.87	1457	4.6±1.2
α-Humulene	36.52	1462	2.6±1.8
Germacrene-D	38.03	1464	10.0±1.1
α-Curcumene	38.27	1479	0.7±0.6
Bicyclogermacrene	39.66	1489	3.0±3.7
Cadina-1,4-diene	40.72	1496	2.3±4.1
β-Bisabolene	40.98	1503	1.5±0.4
Sesquiphellandrene	42.19	1508	0.7±1.5
Spathulenol	47.44	1519	1.6±1.0
γ-Cadinene	50.60	1529	13.6±1.2
Cadinol	53.29	1653	0.7±1.8
Farnesol	58.17	1656	5.4±1.5
Nerolidol	59.40	1677	3.8±0.7

RT=Retention time (min)
RI = Relative index to n-alkanes (C_9-C_{21}) on HP-5 MS column

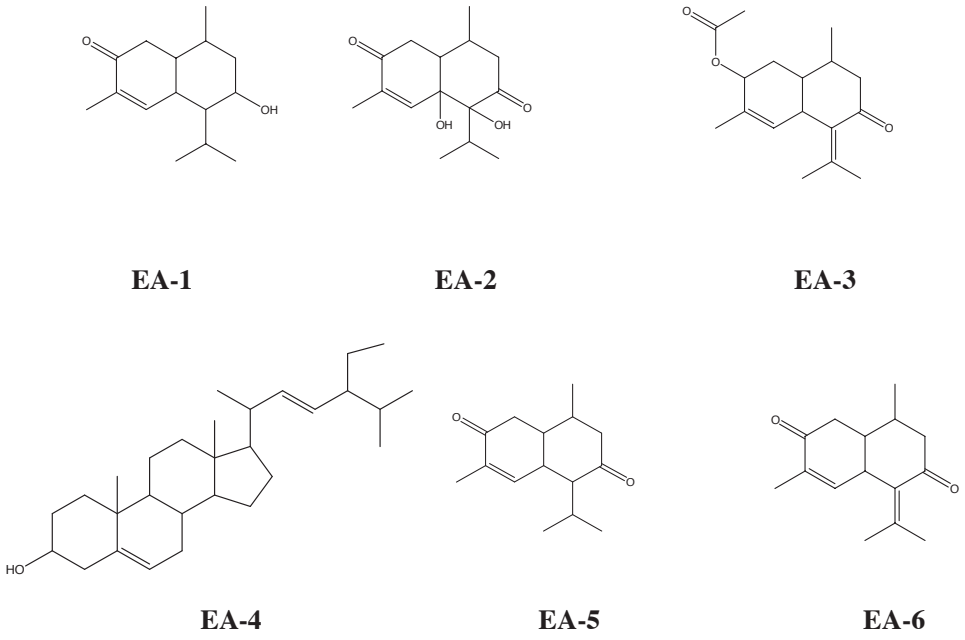

Fig. 1: Structures of terpenes isolated from *E. adenophorum* leaves.
(**EA-1**), 7-hydroxycadinan-3-ene-2-one; (**EA-2**), 5,6-dihydroxycadinan-3-ene-2,7-dione; **(EA-3)**, stigmasterol; (**EA-4**), 2-acetyl-cadinan-3,6-diene-7-one; (**EA-5**), cadinan-3-ene-2,7-dione; (**EA-6**), cadinan-3,6-diene-2,7-dione

ment of other crops or weeds (WHITTAKER, 1971; ENS et al., 2009). Several allelopathic compounds such as catechin, juglone, apigenin, gallic acid derivatives have been discovered and investigated against various weed/crop species.

Our study indicated that both hexane and EtOAc concentrate was effective on *P. minor* and *P. plebejum*. Essential oil was moderately active on shoot and root growth inhibition of weed seeds. However, seedling growth of crop seeds was not hampered. Growth inhibition percentage was dose dependant and detailed lethal median concentration was depicted in Tab. 2. Shoot length of *T. aestivum* seed was also found to be inhibited by the extracts. MeOH concentrate was active against *P. minor* and *C. album*. There was no growth inhibition effect observed with *n*-BuOH extract. Root and shoot growth inhibition of *C. arietinum* was significantly less. Since, solvent concentrates were found to be active; it was investigated in details to isolate bioactive principle. Among the cadinene constituents, 5,6-dihydroxycadinan-3-ene-2,7-dione exhibited highest shoot and root growth inhibition against *P. minor* (Tab. 2). Similar trend was observed in growth inhibition of *P. plebejum*. However, *C. album* and *C. arietinum* growth was not much hampered at the same concentration. As evident from the data, growth inhibition of *C. arietinum* was below 20 %. 5,6-Dihydroxycadinan-3-ene-2,7-dione was most effective in inhibiting coleoptile growth of *P. minor* (EC_{50} 97 μg mL^{-1}) and *P. plebejum* (EC_{50} 117 μg mL^{-1}) than *C. album* (EC_{50} 516 μg mL^{-1}). It was also highly inhibitory of root growth of *P. minor* (EC_{50} 190 μg mL^{-1}). 7-Hydroxycadinan-3-ene-2-one was found to be active in reducing the shoot length of *P. plebejum* (EC_{50} 147 μg mL^{-1}) and *P. minor* (EC_{50} 169 μg mL^{-1}) than *C. album* (EC_{50} 601 μg mL^{-1}). Root growth of these weeds was not much affected by 7-hydroxycadinan-3-ene-

Tab. 2: Plant growth inhibitory activity of extracts and cadinene sesquiterpenes.

Compounds / Extracts	EC_{50} (μg mL^{-1}) please round the values									
	P. minor		*P. plebejum*		*C. album*		*T. aestivum*		*C. arietinum*	
	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Essential oil	375	252	468	265	636	412	841	740	755	617
Hexane extract	216	161	427	186	456	517	346	226	678	521
MeOH extract	346	257	527	397	338	277	457	307	735	616
EtOAc extract	270	117	312	190	534	435	345	155	789	630
n-BuOH extract	982	790	858	690	975	757	>1000	992	>1000	876
EA-1	279	169	327	147	639	501	334	198	637	519
EA-2	190	97	200	117	435	516	291	111	601	576
EA-3	287	144	300	162	574	413	421	169	620	525
EA-4	590	437	802	679	945	583	622	480	>1000	947
EA-5	432	279	489	311	767	570	410	301	790	657
EA-6	468	333	429	353	672	520	424	361	724	580

2-one. On the other hand, stigmasterol showed lowest shoot and root growth inhibition against all test species. 2-Acetyl-cadinan-3,6-diene-7-one was also exhibited shoot length inhibition of *P. minor* (EC_{50} 144 μg mL^{-1}). In general, shoot growth inhibition was more as compared to root growth inhibition. Cadinan-3-ene-2,7-dione and cadinan-3,6-diene-2,7-dione exhibited moderate growth inhibition of test seeds.

Our study indicated that 5,6-dihydroxycadinan-3-ene-2,7-dione was most potential plant growth inhibitor against *P. minor* seeds. Higher plant growth inhibitory activity of 5,6-dihydroxycadinan-3-ene-2,7-dione was attributed to the fact that this compound is relatively more polar than the other isolated compounds. In general crude extracts were also active towards the weed seeds. Further, the relative effect on coleoptile growth was greater than that of root growth. These results are in agreement with the previous reports (TRIPATHI et al., 1981). Furthermore, coleoptile and root growth of the test species exhibited different responses towards cadinene derivatives, confirming the contention of WHITTAKER, 1971 that plant growth inhibitory activity depends on the nature of test species and concentration of the compounds. Experimental results suggested that the cadinenes are the major constituents of *E. adenophorum* leaves. Chemical constituents and possible growth inhibitory activity of leaves of *E. adenophorum* are most important examined features. In view of botanical origin of active ingredient and huge availability of the plant material makes it potential source of naturally occurring bioactive constituents.

Acknowledgements

Authors are thankful to Head, Division of Agricultural Chemicals, Indian Agricultural Research Institute, India for providing necessary facilities for carrying out the research work.

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