AENSI Journals

Australian Journal of Basic and Applied Sciences

ISSN:1991-8178

Journal home page: www.ajbasweb.com

Phytotoxic Properties of Monoterpenes on *Silybum marianum* (L.) Gaertn. and Their Inhibitory Effect on Carbonic Anhydrase Activity

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ARTICLE INFO

Article history:
Received 20 August 2014
Received in revised form
19 September 2014
Accepted 23 October 2014
Available online 16 November 2014

Keywords:
Monoterpenes, Herbicidal activity,Silybummarianum, Carbonic anhydrase,Mode of action

ABSTRACT

Background: Monoterpenes are common essential oil constituents that provide the essence and the odor of a plant.The natural pesticidal properties of monoterpenes make them useful as potential alternative pest control agents as well as good lead compounds for the development ofsafe, effective, and fully biodegradable pesticides. Monoterpeneshave been reported to possessallelopathic and herbicidal properties.**Objective:**This study has been under taken to investigate the effect of tenmonoterpenes, namely camphene, (*R*)-camphor, (*R*)-carvone, 1,8-cineole, (*S*)-fenchone, geraniol, (*S*)-limonene, (*R*)-linalool, (*1R,2S,5R*)-menthol and thymol, on seed germination and seedling growth of *Silybummarianum*and toevaluate theirinhibitory effects on carbonic anhydrase activity. **Results:**All monoterpenes reduced seed germination and inhibited seedling growth but to varying extents. Among the tested compounds, thymol was the most potent seed germination inhibitor at the tested concentrations of 0.5, 1 and 2mM.Four compounds, thymol, (*1R,2S,5R*)-menthol, (*R*)-camphor and (*R*)-carvone, caused complete inhibition of seed germinationat concentration of 4 mM.Furthermore, the monoterpenes strongly inhibited root growth of the weed with thymol and camphene being the most potent compounds as EC_{50} value for both compounds was 0.40 mM, while(*R*)-linalool, geraniol and 1,8-cineole showed the weakest root growth inhibition activity. On the other hand, the results of shoot growth inhibition revealed that thymolcaused the highest growth inhibition, followed by (*1R,2S,5R*)-menthol and (*R*)-camphor with EC_{50} values were 0.40, 0.43 and 0.50 mM, respectively. Six of tested monoterpenes were tested for theireffect on carbonic anhydrase (CA) activity. The monoterpenes strongly inhibited the enzyme with (*S*)-fenchone (I_{50} = 0.08 mM) being the most potent, followed by (*R*)-camphor (I_{50} = 0.11mM) and 1,8-cineole (I_{50} = 0.12 mM). This is the first report on the inhibitory effects of monoterpenes oncarbonic anhydrase.The structure-activity relationship of the tested compounds was disclosed. **Conclusion:**The results of this study suggest that the monoterpenes,thymol, (*1R,2S,5R*)-menthol, (*R*)-camphor, camphene and (*R*)-carvone possess strong phytotoxic potential and can thus serve as lead compounds for the development of new bioherbicides.

To Cite This Article: Samir A.M. Abdelgaleil, Neama A. Gouda and Mona M.G. Saad., Phytotoxic Properties of Monoterpenes on Silybum marianum (L.) Gaertn. and Their Inhibitory Effect on Carbonic Anhydrase Activity. ***Aust. J. Basic & Appl. Sci.,*** *8(16): 356-363, 2014*

INTRODUCTION

Weeds are mainly controlled by synthetic herbicides, but these may result in environmental and human health problems, such as groundwater contamination, residual toxicity in foods, and evolution of resistance to herbicides. Therefore, an effort is warranted to find alternatives to currently used herbicides. One of the strategies to reduce dependence on synthetic herbicides is to use plants with phytotoxic properties for weed control (Duke *et al.* 2002; Copping and Duke 2007). Several higher plants and their metabolites have been observed to possess allelopathicand herbicidal properties (Turk and Tawaha 2002;Chon *et al.* 2005;Amoo*et al.* 2008).

Monoterpenes are the main constituents in the majority of plant essential oils and give plants their unique odoriferous properties because of their low boiling points. Several hundred naturally occurring monoterpenes are known. They are biosynthesized from geranyl pyrophosphate, the ubiquitous acyclic C10 intermediate of the isoprenoid pathway (Windholz *et al.* 1983). Monoterpenes can be classified into two major groups: monoterpene hydrocarbons that include acyclic aliphatic, monocyclic aliphatic, and dicyclic aliphatic and

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oxygenated monoterpenes that include acyclic monoterpenoids, monocyclic monoterpenoids, and dicyclicmonoterpenoids. The latter group includes many alcohols, aldehydes, ketones, ethers, esters and acids (Templeton 1969).Monoterpenes exert a wide spectrum of biologicalactions that are important in food chemistry, chemicalecology, and the pharmaceutical industry.It has been also demonstrated that monoterpenes are involved in multiple ecological functions in plants, such as protection against herbivores and microbial diseases, attraction of pollinators, and in allelopathy. The natural pesticidal properties of some monoterpenes make them useful as potential alternative pest control agents as well as good lead compounds for the development of safe, effective, and fully biodegradable pesticides (Kohli*et al.* 1998;Isman 2000; Romagni*et al.* 2000).

Silybummarianum(L.)Gaertn. (milk thistle, family Asteraceae) is a serious weed in many areas of North and South America, Africa, Australia, and the Middle East (Holm *et al.* 1997). In Egypt, it is considered very dangerous invasive weed which spread through both the new reclaimed lands and desert areasespecially, in wheat fields. Milk thistle is grown commercially as a medicinal plant in Europe, Egypt, China, and Argentina.

Previous studies have demonstrated that monoterpenes are able to inhibit seed germination and growth of several plant species. For example, Reynolds (1987) has reported comparative phytotoxic effects of a series of open chain, cyclic, and bicyclic monoterpenes for their activity against the indicator species lettuce. Similarly,Vokou*et al.* (2003) compared the bioactivity of 47 different monoterpenoids on the seed germination and seedling growth of *Lactuca sativa*.Furthermore, Singh *et al.* (2002) described theinhibitory effect of four monoterpenes, citronellol, citronellal, cineole and linalool, on the germination, seedling length and biomass of *Cassia occidentalis* L. Vaughn and Spencer (1993) explained the inhibitory effect of 18 monoterpenes on germination and growth of nine different plant species. The authors found the phytotoxic effect of the tested monoterpenes was species dependent. This remarkable phytotoxic effect of monoterpenoids coupled with their biodegradable nature and low toxicity against mammals and other non-target species makes these compounds of interest and potential for agricultural industry (Duke *et al.* 2000; Isman, 2000). Some studies have already reported their potential use for weed and pest management insustainable agriculture (Isman 2000; Romagni*et al.* 2000; Singh *et al.* 2002).The commercial herbicide cinmethylinis a2-benzyl ether-substituted analogue of the monoterpene 1,4-cineole used for monocot weed control (Duke *et al*. 2000).

Carbonic anhydrase (CA) catalyses the hydration of CO_2 and dehydration of HCO_3 (Wilkinson*et al.* 2007).Carbonic anhydrase (CA) is important in many physiological functions that involve carboxylation or decarboxylation reactions, including both photosynthesis and respiration. In addition, it is clear that CA also participates in the transport of inorganic carbon to actively photosynthesizing cells or away from actively respiring cells (Henry 1996). The inhibition of CA reduced availability of CO_2 within the cells and could also divert photosynthetic electrons from CO_2 fixation to O_2, leading to the formation of reactive oxygen species (ROS). In fact, some monoterpenes were reported to cause their phytotoxic effects through inhibition of respiration and photosynthesis (Pauly*et al.* 1981; Kohli*et al.* 1998; Macias *et al.* 2007) and enhancing generation of ROS (Singh *et al.* 2006). These processes may be attributed to the inhibition of carbonic anhydrase.

There are no reported studies on phytotoxic and inhibitory effects of monoterpeneson *S. marianum*and carbonic anhydrase enzyme. Therefore the aim of the present study was to evaluate the effect of 10 monoterpenes belonging to several chemical classes on seed germination and seedling growth of this weed and to examine their effects on carbonic anhydrase activity.

MATERIALS AND METHODS

Chemicals:

Tenmonoterpenes, camphene (95%), (*R*)-camphor (98%), (*R*)-carvone (98%), 1,8-cineole (99%), (*S*)-fenchone (98%), geraniol (98%), (*S*)-limonene (96%), (*R*)-linalool (95%), (*1R,2S,5R*)-menthol (98%) and thymol (98%) were purchased from Sigma–Aldrich Chemical Co., Steinheim, Germany. Chemical structures of these monoterpenes are shown in Fig. 1. Tribenuron-methyl (95%,Granstar®) was supplied by DuPont de Nemours and Company, Inc., (Wilmington, Delaware, USA). All chemicals were of highest grade commercially available.

Test seeds:

Milk thistle, *Silybummarianum*(L.)Gaertn. (Asteraceae), field biotype seeds were collected from Alexandria Desert Research Station Farm, Alexandria, Egypt. All undersized or damaged seeds were discarded, and the seeds of uniform size were selected. Germination tests were carried out before experiments and the germination percent was 60%.

Phytotoxic bioassay:

Phytotoxic effects of the tested monoterpenes were evaluated on milk thistle(*Silybummarianum*germination and subsequent seedling growth. The solutions of tested monoterpnes were first prepared in dimethyl sulfoxide

(DMSO). Serial dilutions of these solutions were prepared with distilled water containing 0.02% of an emulsifying agent (Tween 80) to give the concentrations of 0.5, 1, 2, 4, and 6 mM. An aqueous solution of DMSO (0.5% v/v) and Tween 80 was used as control treatment. Three replicates, each of 20 seeds, were prepared for each treatment using glass Petri dishes (9 cm) lined with Whatman No. 2 filter paper. Six milliliters of each test solution were added to the Petri dish. Afterward, Petri dishes were sealed tightly with Parafilm kept on a germination cabinet at 26±2 °C with a 12-h photoperiod. Tribenuron-methyl was used as reference herbicide. After 9 days of sowing, the percentages of seed germination and the length of roots and shoots were determined. The growth inhibition percentages of length roots and shoots were calculated from the following equation: I (%) = [1 - T/C] × 100; T is the length of roots or shoots of treatment (cm) and C is the length of roots or shoots of control (cm). The concentrations causing 50% inhibition in root or/and shoot growth were calculated from a probit analysis (Finney 1971).

Carbonic anhydrase activity assay:

Carbonic anhydrase was extracted from *S.marianum* and measured colorimetrically according to the procedures described by the method of Barman (1974) In this method, plants biotype were allowed to growto reach four leaves growth stages. The enzyme was extracted from the green and fresh leaves collected from healthy plants. Plant leaves were immediately placed in a beaker with ice. Theleaves were homogenized in a cold extraction buffer (10 ml per 0.2 g of tissue) using a polytron homogenizer for 30 s. The extraction buffer contained 50 mMTris (hydroxyl methyl) amino methane at pH 8.2. The homogenate was filtered through four layers of cold cheesecloth to remove extracellular material while keeping temperature at (4°C). The filtrate was centrifuged at 3,000 g for 5 min at 4°C. The supernatant was used as source ofcarbonic anhydrase. The reaction mixture contained enzyme extract (1 ml) was mixed with 2 ml of freshly prepared reaction buffer. The reaction buffer contained 50 mMTris (hydroxyl methyl) amino methane at pH 8.2, 0.1 ml of bromothymol blue 0.01% and 2 ml of 50mM sodium bicarbonate.Monoterpenes were prepared dimethyl sulfoxide (DMSO) and added to the reaction mixtures togive final concentrations of 0, 0.3, 0.6, 0.8 and 1 mM.Three replicates of each treatment, control (without monoterpene) and blank (without enzyme) were prepared.The time required for change indicator color from blue to yellow was measured. The enzyme unit (E.U) was calculated from the equation: $EU= 2(T_0-T_c)/T_c$, whereT_0 is the time in seconds required to change indicator color from blue to yellow in the blank andT_c is the time in seconds required to change indicator color from blue to yellow in the control and treatments. Specific activity of carbonic anhydrase was calculated as follow: Specific activity (SA) = EU/mg protein. The protein content was quantified spectrophotometrically at 595 nm according to Bradford's method (Bradford, 1976) for all samples using bovine serum albumin as standard. I_{50} (concentration of monoterpenes required to cause a 50% inhibition of enzyme activity) values were determined by a linear regression method (Finney, 1971).

Statistical analysis:

Germination percentages root and shoot lengths were subjected to one-way analysis of variance followed by Student–Newman–Keuls test (Cohort software Inc. 1985) to determine significant differences among mean values at the probability level of 0.05. The concentration–response data were subjected to probit analysis (Finney, 1971)to obtain the EC_{50} and I_{50} values using the SPSS 12.0 software program (Statistical Package for Social Sciences, USA).

RESULTS AND DISCUSSION

Effect of monoterpenes on germination ofSilybummarianum:

Germination percentages of *S. marianum* seeds treated with different concentrations of the ten tested monoterpenes and a reference herbicide,tribenuron-methyl, are shown in Table 1. All of the tested monoterpenes significantly reduced the seed germination in a concentration-dependent manner. The results showed thatthymol and (*1R,2S,5R*)-menthol were the most potent seed germination inhibitors at the concentration of 0.5 mM. The germination percent for both compounds was 30%, and both compounds were more potent than the herbicidetribenuron-methyl (germination percent = 33.3%) at this concentration. Camphene and (*S*)-limonene were not active at this concentration and showed similar germination percent (56.7%) as control. Similarly, thymol (germination percent = 23.3%) displayed the highest reduction in seed germination at the concentration of 1mM, followed by menthol, geraniol and (*R*)-camphor. Thymol revealed higher germination inhibition thantribenuron-methyl.Likewise,Thymol (germination percent = 3.3%) was the most potent inhibitor for seed germination at 2 mM, followed by (*R*)-camphor and camphene, while (*S*)-limonene and (*R*)-linalool were the least effective compounds. At concentration of 4 mM, four compounds, thymol, (*1R,2S,5R*)-menthol, (*R*)-camphor and (*R*)-carvone (98%), caused complete inhibition for seed germination. Complete failure of seed germination was observed by all monoterpenes except camphene, 1,8-cineole and(*S*)-limonene at concentration of 4 mM. In general, thymol, (*R*)-camphor, (*1R,2S,5R*)-menthol and (*R*)-carvone revealed the highest seed germination reduction at the tested concentrations.

In the literature, there were no previous studies on germination inhibition of the tested monoterpenes against *S. marianum*. However, inhibitory effects of some tested monoterpenes, such as camphene camphor, carvone, linalool, 1,8-cineole, geraniol, limonene and thymol on germination of other plants and weed species were described(Singh *et al.* 2002; Vokou*et al.* 2003; He *et al.* 2008; Martino *et al.* 2010). In agreement with our results, previous studies showed that essential oils isolated from various plant species and their major constituents, monoterpenes, exert potent phytotoxic effects on weed germination of other species (Angelini*et al.* 2003; Nishida *et al.* 2005, Kordali*et al.* 2007; Salamci*et al.* 2007; Areco*et al.* 2014).

Table 1: Effect of monoterpenes on *Silybummarianum*seed germination 9 d after sowing[a].

Conc mM	Germination % ± SE			
	Camphene	(*R*)-Camphor	(*R*)-Carvone	1,8-Cineole
0	56.7±3.34a	56.7±3.34a	56.7±3.34a	56.7±3.34a
0.5	56.7±3.34a	43.3±3.34b	43.3±3.34b	46.7±3.34a
1	46.7±6.70ab	30.0±3.34c	36.7±3.34b	43.3±3.34a
2	36.7±3.34b	5.7±3.34d	16.7±3.34c	26.7±6.7b
4	33.3±3.34b	0.0±0.00d	0.0±0.00d	23.3±3.34b
6	16.7±3.34c	0.0±0.00d	0.0±0.00d	20.0±0.00b
Conc mM	Germination % ± SE			
	(*S*)-Fenchone	Geraniol	(*S*)-Limonene	(*R*)-Linalool
0	56.7±3.34a	56.7±3.34a	56.7±3.34a	56.7±3.34a
0.5	40.0±5.80b	36.7±3.34b	56.7±6.70a	53.3±6.7a
1	36.7±3.34b	30±5.80b	43.3±6.70a	46.7±3.34bc
2	23.3±3.34c	26.7±3.34b	43.3±3.34a	43.3±3.34bc
4	6.7±3.34d	20.0±5.80b	23.3±3.34b	33.3±3.34c
6	0.0±0.00d	0.0±0.00c	16.7±3.34b	0.0±0.0d

Conc mM	Germination % ± SE			
	(*1R,2S,5R*)-Menthol	Thymol	Tribenuron-methyl	
			Conc (mM)	Germination % ± SE
0	56.7±3.34a	56.7±3.34a	0	53.3±3.34a
0.5	30.0±5.80b	30.0±5.80b	0.001	43.3±3.34ab
1	30.0±5.80b	23.3±3.34b	0.01	36.7±3.34bc
2	23.3±3.34b	3.3±3.34c	0.1	36.7±3.34bc
4	0.0±0.00c	0.0±0.00c	0.5	33.3±3.34bc
6	0.0±0.00c	0.0±0.00c	1	26.7±3.34c

[a] Data are expressed as means ± SE from experiments with three replicates of 10 seeds each.
[b] Means within a column sharing the same letter are not significantly different at the 0.05 probability level.

Table 2: Effect of essential oils on *Silybummarianum*root growth 9 d after sowing[a].

Conc mM	Camphene		(*R*)-Camphor		(*R*)-Carvone		1,8-Cineole	
	Root length (cm)	I (%)[b]	Root length (cm)	I (%)	Root length (cm)	I (%)	Root length (cm)	I (%)
0	13.1±0.56a	0.0	13.1±0.56a	0.0	13.1±0.56[a]	0.0	13.1±0.56a	0.0
0.5	6.5±0.58b	50.4	6.2±0.17b	52.9	10.2±0.41b	22.1	9.2±0.36b	29.8
1	4.2±0.33c	67.9	3.3±0.18c	74.8	8.9±0.59c	32.1	7.5±0.36c	42.8
2	3.2±0.20cd	75.6	0.7±0.33d	94.7	1.3±0.08d	89.3	6.7±0.50c	48.9
4	3.1±0.31cd	76.3	0.0±0.00d	100	0.0±0.00d	100	3.7±0.39d	71.8
6	1.6±0.52d	87.8	0.0±0.00d	100	0.0±0.00d	100	2.2±0.21e	83.2
EC_{50}	0.40		0.50		1.01		1.45	

Conc mM	(*S*)-Fenchone		Geraniol		(*S*)-Limonene		(*R*)-Linalool	
	Root length (cm)	I (%)	Root length (cm)	I (%)	Root length (cm)	I (%)	Root length (cm)	I (%)
0	13.1±0.56a	0.0	13.1±0.56a	0.0	13.1±0.56a	0.0	13.1±0.56[a]	0.0
0.5	7.2±0.23b	45.0	10.9±0.30b	16.8	7.7±0.57b	41.5	12.9±0.56a	1.5
1	6.6±0.60b	49.6	10.5±0.15b	19.8	5.6±0.07c	57.3	10.8±0.89b	17.6
2	4.5±0.22c	65.5	8.8±0.39c	32.8	4.6±0.29cd	64.9	9.3±0.30bc	29.0
4	0.8±0.42d	93.9	7.6±0.50d	42.0	3.8±0.33d	71	8.1±0.21c	38.2
6	0.0±0.00d	100.0	0.0±0.00e	100.0	0.9±0.07e	93	0.0±0.00d	100
EC_{50}	0.81		2.53		0.80		2.93	

Conc mM	(*1R,2S,5R*)-Menthol		Thymol		Tribenuron-methyl		
	Root length (cm)	I (%)	Root length (cm)	I (%)	Conc (mM)	Root length (cm)	I (%)
0	13.1±0.56[a]	0.0	13.1±0.56[a]	0.0	0	8.5±0.30a	0.0
0.5	7.7±0.33b	41.2	5.0±0.27b	61.8	0.001	5.8±0.83b	52.9
1	6.7±0.03c	48.9	1.5±0.03c	88.6	0.01	1.6±0.03c	81.2
2	2.3±0.29d	82.4	0.3±0.27d	97.7	0.1	1.2±0.03c	85.9
4	0.0±0.00e	100.0	0.0±0.00d	100.0	0.5	1±0. 30c	88.2
6	0.0±0.00e	100.0	0.0±0.00d	100.0	1	0.8±0.10c	90.6
EC_{50}	0.77		0.40		0.002		

[a] Data are expressed as means ±SE from experiments with three replicates of 10 seeds each.
[b] $I = I_{50}$ (concentration of essential oils required to cause a 50% inhibition of root length).
[c] Means within a column sharing the same letter are not significantly different at the 0.05 probability level.

Table 3: Effect of essential oils on *Silybummarianum*shoot growth 9 d after sowing[a].

Conc mM	Camphene		(*R*)-Camphor		(*R*)-Carvone		1,8-Cineole	
	Shoot length (cm)	I (%)[b]	Shoot length (cm)	I (%)	Shoot length (cm)	I (%)	Shoot length (cm)	I (%)
0	3.8±0.15a	0.0	3.8±0.15a	0.0	3.8±0.15a	0.0	3.8±0.15a	0.0
0.5	2.3±0.19b	40.4	1.8±0.17b	51.8	2.3±0.23b	40.4	1.9±0.07b	49.1
1	1.6±0.12c	58.0	1.1±0.30c	76.1	1.2±0.03c	71.1	1.5±0.10bc	60.5
2	1.4±0.09cd	63.2	0.4±0.20d	89.5	0.5±0.06d	86.8	1.4±0.06bc	63.2
4	1.4±0.12d	63.2	0±0.00d	100.0	0±0.00d	100.0	1±0.12cd	73.7
6	1±0.10d	73.9	0±0.00d	100.0	0±0.00d	100.0	0.8±0.12d	79.0
EC_{50}	0.80		0.50		0.63		0.52	
Conc mM	(*S*)-Fenchone		Geraniol		(*S*)-Limonene		(*R*)-Linalool	
	Shoot length (cm)	I (%)	Shoot length (cm)	I (%)	Shoot length (cm)	I (%)	Shoot length (cm)	I (%)
0	3.8±0.15a	0.0	3.8±0.15a	0.0	3.8±0.15a	0.0	3.8±0.15a	0.0
0.5	2.1±0.27b	44.7	2.7±0.15b	28.9	2.2±0.09b	42.1	2.7±0.09b	28.9
1	1.4±0.09c	63.2	2.2±0.06c	42.1	2.1±0.09b	44.7	2.3±0.20bc	39.5
2	1.2±0.23c	68.4	1.8±0.09d	52.6	1.6±0.1c	57.9	2.1±0.15c	44.7
4	0.5±0.25d	86.8	1.6±0.06d	57.9	1.4±0.12c	63.2	1.1±0.18d	71.1
6	0±0.00d	100.0	0.0±0.00e	100	0.8±0.06d	79.0	0±0.00e	100.0
EC_{50}	0.68		1.42		1.11		1.42	
Conc mM	(*1R,2S,5R*)-Menthol		Thymol		Tribenuron-methyl			
	Shoot length (cm)	I (%)	Shoot length (cm)	I (%)	Conc (mM)	Shoot length (cm)		I (%)
0	3.8±0.15a	0.0	3.8±0.15a	0.0	0	3.1±0.09a		0.0
0.5	1.4±0.10b	63.2	1.4±0.10b	63.2	0.001	1.8±0.03b		41.9
1	1.2±0.03c	68.4	0.9±0.03c	76.3	0.01	1.6±0.06c		48.4
2	0.8±0.06d	78.9	0.2±0.23d	94.7	0.1	1.4±0.07d		54.8
4	0.0±0.00e	100.0	0.0±0.00d	100.0	0.5	1.2±0.03e		61.3
6	0.0±0.00e	100.0	0.0±0.00d	100.0	1	1.0±0.03f		67.7
EC_{50}	0.43		0.40			0.13		

[a] Data are expressed as means ±SE from experiments with three replicates of 10 seeds each.
[b] $I = I_{50}$ (concentration of essential oils required to cause a 50% inhibition of shoot length).
[c] Means within a column sharing the same letter are not significantly different at the 0.05 probability level.

Table 4: Inhibitory effects of monoterpenes on carbonic anhydrase activity.

Conc. (mM)	Camphene		(*R*)-Camphor		(*R*)-Carvone	
	Specific activity	Inhibition (%)	Specific activity	Inhibition (%)	Specific activity	Inhibition (%)
0.0	9.0	0.0	9.0	0.0	9.0	0.0
0.3	3.0	66.7	2.13	76.3	4.0	55.6
0.6	1.78	80.2	1.09	87.8	3.0	66.7
0.8	0.67	92.6	0.82	90.9	2.34	74.0
1	0.32	96.4	0.45	95.0	1.86	79.3
I_{50}(mM)[a]	0.21		0.11		0.24	
Conc. (mM)	1,8-Cineole		(*S*)-Fenchone		Thymol	
	Specific activity	Inhibition (%)	Specific activity	Inhibition (%)	Specific activity	Inhibition (%)
0.0	9.0	0.0	9.0	0.0	9.0	0.0
0.3	2.34	74.0	1.57	82.3	4.56	49.3
0.6	1.09	87.8	0.70	92.2	4.0	55.6
0.8	0.73	91.9	0.48	94.7	2.13	76.3
1	0.62	93.1	0.34	96.2	1.63	81.9
I_{50} (mM)	0.12		0.08		0.35	

[a]The concentration causing 50% enzyme inhibition.

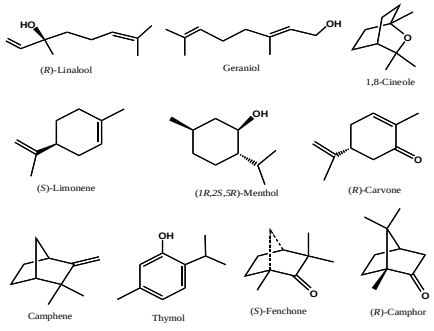

Fig. 1: The chemical structures of the tested monoterpenes.

***Effect of monoterpenes on root growth ofSilybummarianum*:**

The inhibitory effects of the tested monoterpenes on the root growth of *S. marianum* are presented in Table 2. The tested compounds showed pronounced root growth inhibition activity with varying degrees.Among the tested compounds,thymol (EC_{50} = 0.40 mM) was the most potent inhibitor of root growth at the tested concentrations. Similarly, camphene revealed a strong root growth inhibition as the EC_{50} value was 0.40 mM.In addition, (*R*)-camphor and (*1R,2S,5R*)-menthol displayed remarkable root growth inhibition with EC_{50} values of 0.50 and 0.77 mM, respectively. In contrary, (*R*)-linalool, geraniol and 1,8-cineole showed the weakest root growth inhibition activity with EC_{50} values of 2.93, 2.53 and 1.40 mM, respectively. The tested monoterpenes were less active than the herbicide tribenuron-methyl.Based on the EC_{50} values, the tested compounds were active at concentrations ranged from 0.4 to 3 mM. These data are consistent with those reported on the root growth inhibition of some tested and other monoterpenes, in which compounds such as 1,8-cineole,limonene, thymol, geraniol, linalool, α-pinene and others,have been described as potent root growth inhibitors (Reynolds 1987; Vokou*et al.* 2003; Zunino and Zygadlo 2004; Nishida *et al.* 2005; Singh*et al.* 2006).

***Effect of monoterpenes on shoot growth ofSilybummarianum*:**

The results of the inhibitory effects of the monoterpenes on the shoot growth of *S. marianum* are given in Table 3. In general, all of the tested monoterpenes inhibited the shoot growth and the degree of shoot inhibition increased as the compound concentration progressively increased from 0.5 to 6 mM.Thymol showed the highest shoot growth inhibition, followed by (*1R,2S,5R*)-menthol, (*R*)-camphor and 1,8-cineole where the EC_{50} values were 0.40, 0.43, 0.50 and 0.52 mM, respectively. (*R*)-Carvone, (*S*)-fenchone and camphene showed relative strong shoot growth inhibition, while (*R*)-linalool, geraniol and (*S*)-limonene were the less effective compounds.

It is noteworthy camphene and (*S*)-limonene showed higher root growth inhibition than shoot growth inhibition. This finding is supported by earlier studies of inhibitory effects of monoterpenes on seedling growth (Singh *et al.* 2006; Zhao *et al.* 2011; Chowhan*et al.* 2011). In contrary, (*R*)-carvone, 1,8-cineole, fenchone, geraniol, (*R*)-linalool and (*1R,2S,5R*)-menthol were more effective on shoot growth inhibition than on root growth inhibition. Similarly, Scrivanti (2010) and Acerco*et al.* (2014) found that some monoterpenes and plant extracts possessed higher growth inhibition against shoot than root.Nevertheless, thymol and (*R*)-camphor revealed similar root and shoot growth inhibition.

Inhibition of carbonic anhydrase:

The inhibitory effect of six monoterpenes, camphene, (*R*)-camphor, (*R*)-carvone, 1,8-cineole, (*S*)-fenchone and thymol, oncarbonic anhydrase activity was evaluated. Table 4 shows specific activities, inhibition percentages and I_{50} values of carbonic anhydrase isolated from the leaves of *S. marianum* after incubation with a series ofconcentrations ofmonoterpenes. The tested monoterpenes showed an inhibition of carbonic anhydrase in a concentration-dependent manner.(*S*)-Fenchone caused the highest inhibition, followed by(*R*)-camphor and 1,8-cineole, whilethymol revealed the weakest inhibition. Values of I_{50}were 0.08, 0.11, 0.12, 0.21, 0.24 and 0.35mM for (*S*)-fenchone, (*R*)-camphor, 1,8-cineole, camphene, (*R*)-carvone andthymol,respectively.

Although the mechanisms of monoterpenes action on plant growth are unknown, there are several studies describing the possible modes of action of these compounds. Monoterpenes may cause their phytotoxic effects through inhibiting DNA synthesis (Nishida *et al.* 2005), disrupting mitotic activity in the growing cells (Dayan *et al.* 2000), inhibiting cellular and mitochondrial respiration (Kohli*et al.* 1998; Macias *et al.* 2007), inhibiting of electron transfer and mitochondrial ATP production (Abrahim*et al.* 2003), causing oxidative damage through enhanced generation of ROS (Singh *et al.* 2006), inhibiting of cell proliferation and disrupting of the activity of metabolic enzymes involved in glycolysis (Kaur *et al.* 2010; Amri*et al.* 2012).The present study revealed that the monoterpenes caused a strong carbonic anhydraseinhibition for the first time. This finding indicates that, besides the other previously reported modes of action, carbonic anhydrasemay be a target enzyme for monoterpenes.This approachcanbe supported bysomeprevious studiesshowedthat themonoterpenes cause their phytotoxic effect through the sameprocesses thatoccur whencarbonic anhydrase inhibited, such as inhibition of respiration and photosynthesis, and generation of ROS.

The results showed that thymol, an alcohol, was the most potent inhibitor for seed germination and seedling growth. Moreover, (*1R,2S,5R*)-menthol, an alcohol, was among the most potent compounds. Two ketones ((*R*)-camphor and (*R*)-carvone) revealed strong phytotoxic activity. The monoterpene hydrocarbons, limonene and camphene, showed the weakest seed germination inhibition among the tested compounds. In general, the oxygenated monoterpenes have more potent phytotoxic effects on seed germination and seedling growth than monoterpene hydrocarbons. Among the oxygenated monoterpenes, alcohols and ketones displayed the highest inhibitory activities on seed germination and seedling growth. These findings are in agreement with those previously reported on the phytotoxic activities of monoterpenes (Vokou*et al.* 2003; Martino *et al.* 2010).

Thus, based on the present study it can be concluded that the monoterpenes,thymol, (*1R,2S,5R*)-menthol, (*R*)-camphor, camphene and (*R*)-carvoneexhibit a strongphytotoxicityagainst *S. marianum*. The strong inhibitory effect of monoterpenes oncarbonic anhydrase indicated that this enzyme is a target for monoterpenes.Therefore,

these monoterpenes can be utilized for weed management. Further, they can also provide lead structures for chemical synthesis of new bioherbicides.

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