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Phytotoxic activity of *Capparis spinosa* L. and its discovered active compounds

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ABSTRACT

We evaluated the phytotoxic activity of extracts from *Capparis spinosa* organs (leaves, stems and roots) on germination and seedlings growth of *Lactuca sativa*, *Raphanus sativus*, *Silybum marianum* and *Peganum harmala.* We also isolated and identified the bioactive compounds in donor plant. Leaf extracts were most phytotoxic to lettuce germination and growth, complete inhibition (100%) occurred with aqueous extract at 20 g L^{-1} and 65.9% inhibition with methanolic extract at 6 g L^{-1}. The methanolic residue was sequentially extracted with petroleum ether, ethyl acetate and methanol-water. The ethyl acetate extract caused 75.5% inhibition in lettuce germination and growth at 6 g L^{-1}, while other two extracts caused inhibition of 60.1%. Twelve subfractions were obtained from the ethyl acetate extract, among which two (A_9 and A_{10}) were most toxic (31.7 and 64.4% inhibition). The bioactive ethyl acetate subfractions were chromatographed and subjected to NMR techniques. Based on bio-guided chromatographic fractionation, three bioactive allelochemicals were identified as: quercetin-3-O-β -D-glucopyranoside, which was the most toxic followed by quercetin and kaempferol 3-O-β -D-glucopyranoside. Understanding the action of single allelochemicals or their mixtures on physiological processes is critical for fully explaining the phytotoxic effects.

Key words: Allelopathic activity, bioassay-guided isolation, *Capparis spinosa, Lactuca sativa*, *Peganum harmala*, phytotoxic compounds, *Raphanus sativus*, *Silybum marianum*.

INTRODUCTION

The intensive use of synthetic herbicides in agriculture is of increasing concern due to contamination of environment and has induced the resistance in weeds to herbicides. Hence, scientists have focussed on searching for plant compounds to develop bio-herbicides as alternative strategy for weed control (6,14). Allelopathy refers to the chemical inhibition of one plant by another and various methods have been suggested to evaluate the allelopathic potential of plants (27,41). The allelochemicals are present in all plant parts (61) and they are released in the soil through volatilization, root exudation, leaching and decomposition of plant residues (32,62). Besides, the environmental conditions and genetic characteristics enhances the synthesis and exudation of

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allelochemicals (13,46). Active substances of medicinal plants have strong allelopathic properties (21,33) that could be used safely in agroecosystems. Indeed, medicinal plants are inhibitory to selected weeds (30,34) and some of their herbicidal allelochemicals have been identified (27,52). It is easier to screen the allelopathic plants from medicinal plants because they are rich in metabolic compounds (21). Contemporary allelopathy research focusses on isolation, identification and quantification of specific active allelochemicals. It is estimated that there are about 400,000 secondary metabolites, of which, till 1988 (19) only 3% have been identified. Once these substances are identified and characterized they could be used as natural herbicides or to develop new ecofreiendly herbicides (6,14,15).
Capparidaceae family has 40-45 genera (700-900 spp.) having wide diversity in habitat, fruit and floral features (39). Among these species, *Capparis spinosa* L. (caper) is common aromatic and medicinal plant growing in wild and dry regions in Asia and the Mediterranean basin (55). *C. spinosa* is a perennial winter deciduous specie, has round fleshy leaves and white to pinkish-white flowers (49). It is best known for the edible buds and fruits (caper berry), consumed as pickles (44), hence has great economic importance in Mediterranean area. In Tunisia, this plant grows in wild and is also widely cultivated in central and southern coastal areas. Its plant contains the phytosterols, tocopherols, carotenoids, flavonoids and glucosinolates in different parts (24,58). But, flavonoids [kaempferal, quercetin, isorhamnetin and their O-methyl derivative, thomnocitirin, rhamnetin and rhamnozin (31)] are major constituents in leaves. Its roots also contains flavonoids and glycosides [kaempferal-7-rhamnoside, kaempferol-3-rutinoside, kaempferol-3-glucoside-7-rhamnoside, quercetin- 3-rutinoside and isorhamnetin-3-7-dirhamnoside (20)]. Some of these isolated compounds have medicinal uses [anti-oxidative (24,52), antifungal (38), antihepatotoxic (22), anti-inflammatory (2), anti-diabetic (17), anti-hypertensive (22), anti-hyperlipidemic (17), antibacterial, antiparasital (60), immunostimulant and antitumor activities (3)]. Despite its popularity as an aromatic and medicinal plant, there are no studies on allelopathic effects of *C. spinosa* on other plants.

This study aimed to investigate (i). the phytotoxic effects of different *C. spinosa* organs (roots, stems and leaves) on 4-dicotyledonous species: 2-most sensitive plants (*Lactuca sativa* L., *Raphanus sativus*) and two common weeds (*Silybum marianum* L. and *Peganum harmala* L.) under laboratory conditions and (ii). the bioactivity of allelochemicals through bioassay-guided phytotoxic tests. Activity of organic fractions was tested only on *Lactuca sativa*, a most sensitive plant to herbicidal molecules (21).

MATERIAL AND METHODS

Plant material

Plants of wild caper (*C. spinosa*) were collected from 5-years old plants in May 2010 in Northern Tunisia. These plants were partitioned into roots, stems and leaves. They were washed several times with tap water and dried in hot-air oven at 60°C for 72 h. Then, they were cut into 1 cm pieces, powdered in blender and sieved through 40 mesh (420 µm) sieve.

EXTRACTION

Aqueous extracts

The powdered roots, stems and leaves were soaked each at 50 g in 1 L distilled water at room temperature for 24 h, followed by filtration through 3-layers of cheesecloth to remove any debris. The supernatant was then filtered through Whatman No. 1 filter paper several times and kept at 4°C in the dark until use.

Organic extracts

Sequential extraction was done with hexane, chloroform ($CHCl_3$) and methanol (MeOH). One hundred g dried powder of roots, stems and leaves was extracted, successively in organic solvents for 24 h at room temperature. The organic extracts were evaporated to dryness under reduced pressure at 45-50°C, using Rotavapor R-114 (Buchi, France). The residue was weighed and the yields were expressed in percentage of residue on dry weight basis. Residues were stored at 4°C until use.

LABORATORY BIOASSAYS

Aqueous extracts

Aqueous extracts (root, shoot and leaves, at 10, 20, 30, 40 and 50 g L^{-1}) were tested on *Raphanus sativus* L., *Lactuca sativa* L. and on two weeds (*Silybum marianum L.*, *Peganum harmala* L.). Seeds were surface sterilized with 0.525 g L^{-1} sodium hypochlorite for 15 min, then rinsed four times with deionized water, imbibed at 22°C for 2 h and carefully blotted using a folded paper towel. Twenty imbibed seeds of target species were separately placed on the filter paper in 9 cm dia plastic Petri dishes, 5 mL of respective extracts was applied per petridish as per treatments. The seeds were irrigated with distilled water in control. The Petri plates were then placed in a growth chamber [400 µmol photons.m^{-2} s^{-1} photosynthetically active radiation (PAR) at 24/22°C for 14/10 h light and dark periods]. Treatments were arranged in a completely randomized design with three replications. Cumulative germination was determined by counting the number of germinated seeds at 24 h intervals during 6 d. Shoot and root length of receiver species were measured 7 d after sowing. Data were transformed to percent of control for analysis.

The index of germination GI was determined as under (8):

$$GI = (N1) * 1 + (N2-N1) * 1/2 + (N3-N2) * 1/3 + ... + (Nn-Nn-1) * 1/n$$

Where, N1, N2, N3, …., Nn: the number of germinated seeds observed after 1, 2, 3,…., n-1, n days. This index measures the delay in germination induced by the extract (16).

The Inhibition/Stimulation (%) was calculated as under (11):

$$\text{Inhibition (-)/Stimulation (+) \%} = [(\text{Extract} - \text{Control})/\text{Control}] \times 100$$

Where, Extract: Growth parameter measured in presence of *C. spinosa* extract and Control: Growth parameter measured in presence of distilled water.

All growth assays were conducted on pre-germinated seeds.

Organic extracts

The dry residues of hexane, $CHCl_3$ and MeOH extracts from roots, stems and leaves were dissolved in MeOH at 6 g L^{-1}. There were two controls : distilled water and MeOH, to eliminate the effects of organic solvents. Filter papers placed in Petri dish, were soaked with distilled water, MeOH or various organic extracts. Solvents were evaporated for 24 h at 24°C, then 5 mL distilled water was added and 20 soaked seeds were used for germination for 7 d. Germination, shoot and root length of target species were estimated as before. Treatments were arranged in a completely randomized design with three replications and data were transformed to percent of control for analysis.

Isolation and identification of bioactive compounds

Dried powder of *C. spinosa* leaves was extracted twice at room temperature with MeOH for 48 h. After filtering, extracts were combined and dried at reduced pressure. The obtained residues were re-dissolved in MeOH-water (1:1) and partitioned in a separator funnel with petroleum ether and ethyl acetate (EtOAc). The most active EtOAc extract (5g), was separated by Sephadex LH-20 (Pharmacia) and was eluted with MeOH:CH_2Cl_2 (5:1). According to TLC (Merck Kieselgel 60 F254 plates with 0.2 mm film thickness) analysis, chromatograms were viewed under UV light at 254 nm and sprayed with (EtOH:H_2SO_4 (93:7). Collected fractions were combined in 12 homogenous fractions (A1-A12) which were tested for their allelopathic activity. Bioactive fractions (A9: 40 mg and A10: 37 mg) were next chromatographed on semi-preparative TLC eluted with MeOH:CH_2Cl_2 (2:8). The structures of allelochemicals were elucidated by NMR and three pure compounds were identified. The phytotoxic activity of these fractions was determined using a lettuce bioassay as described above, at 6, 0.6 and 0.06 g L^{-1} concentrations for screening extracts, and at 0.6 g L^{-1} for fractions and 0.06 g L^{-1} for pure compounds. The bioassay of lettuce germination and seedling growth was arranged in a completely randomized design with four replications.

Statistical analysis

The laboratory bioassays was done in complete randomized design with three or four replications. Data were subjected to ANOVA and a post hoc LSD tests (p=0.05) with PASW Statistics 18, for Windows, to analyze differences among means.

RESULTS AND DISCUSSION

Solutions of PEG Polyethylene glycol having the same pH and osmotic potential of most concentrated extracts were prepared and tested on lettuce and peganum. PEG is widely used in lab bioassays to regulate water potential (54). Under the same conditions, experiments with extracts of roots, stems and leaves of *C. spinosa* and PEG experiment were concurrently done to distinguish between the inhibitory effects of substances and osmotic potential of most concentrated extract. Indeed, all results were similar or better than control. PEG solutions had no effects on germination index or growth (Table 1).

Table 1. Effects of 50 g L^{-1} aqueous extracts of *C. spinosa* roots, stems and leave and polyethylene glycol 4000 (PEG) solutions at same pH and same osmotic potential (Ψ_Π) (bar) on germination index and root/shoot length (% of control) of lettuce and peganum

Parameter	Root extract		Shoot extract		Leaf extract	
	Extract	PEG solution	Extract	PEG solution	Extract	PEG solution
pH	6.05	6.05	5.68	5.68	6.23	6.23
Ψ_Π	0.00058	0.00051	0.00068	0.00073	0.00252	0.00247
Germination Index						
Lettuce	0±0.0c	106.1±4.7a	12.4± 8.5b	110.9±9.3a	0±0.0c	110.1±4.4a
Peganum	0±0.0c	108.8±6.8a	27.4± 7.2b	113.9±7.9a	0±0.0c	102.6±2.8a
Root length						
Lettuce	0±0.0c	78.4± 4.8a	3.8±1.0c	87.4±4.8a	0±0.0c	85.3±4.2a
Peganum	0±0.0c	122.6±2.9a	11.59±2.0b	105.0±8.4a	0±0.0c	112.0±7.2a
Shoot length						
Lettuce	0±0.0c	96.2±2.4a	11.1±2.4b	114.3±5.3a	0±0.0c	113.6±6.5a
Peganum	0±0.0c	112.9±5.7a	28.2±3.1b	109.5±1.7a	0±0.0c	101.9±5.0a

Means with the same letter in a column are not significantly different at $P < 0.05$.Values (N=3±S.E.).

I. AQUEOUS EXTRACTS

Germination: The aqueous extracts of *C. spinosa* inhibited the germination of all test species (Table 2). Inhibition increased with extract concentrations and varied with target species and organs. Root and leaf extracts were most inhibitory to germination (4.08 and 36.3% germination in sensitive plants) and 17.1 and 38.6% in weed species. They induced total inhibition at 20 g L^{-1} for lettuce and at 40 g L^{-1} for other test species. Except thistle, which germinated up to the highest concentration (50 g L^{-1}) of root 91.4% extract (germination 11.6%). It did not germinate at 30 g L^{-1} leaf extract. The stem extract was less inhibitory to radish (91.4 % germination) and 35.3 - 47.5% germination in other spp. But at the highest concentration (50 g L^{-1}), the thistle germination was completely stopped, indicating large differences in target species behaviour (Table 2). Similar finding was reported by Sodaeizadeh and Maybodi (53), who found that *C. spinosa* aqueous extract at 100% (w/v) caused 86% inhibition in germination of wheat and alfalfa. Furthermore, aqueous extract of *C. decidua* induced total inhibition of germination in *Calligonum polygonoides* under laboratory conditions (23). Such drastic effects on germination has been earlier reported (37), the root aqueous extract of *Corrigiola telephiifolia* (aromatic and medicinal plant) inhibited the germination of *Raphanus sativus* and *Triticum aestivum*. This phytotoxic effect was due to the presence of higher concentrations of specific types of allelochemicals in aqueous extracts (9), which prevented the growth of embryo, or caused its death due to chromosomal aberrations in dividing cells (48).

Seedling Growth: The root and leaf extracts proved most toxic to seedlings growth of test crops. They induced total inhibition at 20 g L^{-1} for lettuce, at 30 g L^{-1} for thistle and at 40 g L^{-1} for radish and peganum (Fig. 1). The lowest concentration (10 g L^{-1}), inhibited the seedling growth of all target species. The stem extract at the highest concentration (50 g L^{-1}) reduced the seedling growth of lettuce and thistle by 92% and 100%. There was great variability in reduction of radish and peganum seedling growth [40% and 80% respectively

Table 2. Influence of *C. spinosa* root, stems and leaves aqueous extracts concentrations on Germination index (GI) (% of control) of test species: lettuce, radish, peganum and thistle

Aqueous extracts/ concentration (g L^{-1})		Germination Index (%)			
		Lettuce	Radish	Peganum	Thistle
Root	10	20.4±0.3b	96.2±4.4d	72.8±1.3d	53.2±2.2d
	20	0.0±0.0a	59.5±5.2c	46.8±2.9c	54.2±2.5d
	30	0.0±0.0a	25.9±2.9b	26.7±0.5b	43.8±2.7c
	40	0.0±0.0a	0.0±0.0a	0.0±0.0a	30.5±1.2b
	50	0.0±0.0a	0.0±0.0a	0.0±0.0a	11.6±1.7a
	Mean	4.08	36.3	29.2	38.6
Shoot	10	68.1±1.5c	94.7±3.3b	91.7±2.7d	70.9±5.01c
	20	39.4±2.1b	90.7±1.9ab	78.5±2.1c	55.3±2.8b
	30	39.9±1.7b	94.9±3.1b	63.2±1.3b	51.6±3.8b
	40	16.9±3.0a	89.9±2.2ab	30.6±1.9a	59.8±3.4b
	50	12.4±1.7a	88.0±2.4a	27.4±2.4a	0.0±0.0a
	Mean	35.3	91.4	58.2	47.5
Leaf	10	24.6±2.6b	90.4±0.9d	34.8±1.7c	49.1±2.4b
	20	0.0±0.0a	43.7±1.8c	41.1±3.4d	50.4±1.9b
	30	0.0±0.0a	12.7±2.1b	10.0±1.03b	0.0±0.0a
	40	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
	50	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
	Mean	4.9	29.3	17.1	19.9

Means with the same letter in a column are not significantly different at $P < 0.05$. Values (N=3±S.E.).

at 40 g L^{-1} (Fig. 1)]. This initial bioassay was necessary to evaluate the allelopathic potential of plant species (10). *C. spinosa* drastically inhibits (98%) the alfalfa and wheat growth at the highest concentration of 100% (w/v). Also, Gautam and Bishnoi (23) found similar results with *C. decidua* aqueous extracts, which showed significant allelopathic activity on *Calligonum polygonoides*. Leaf and root aqueous extracts of *Anisomeles indica* inhibits the growth *Phalaris minor* (12), while *Piper sarmentosum* induced significant inhibition on alfalfa seedling growth (47). The phytotoxic effect of *C. spinosa* aqueous extracts may be due to water soluble substances in its extract. The allelochemicals present in plants aqueous extracts, affects the various physiological processes by affecting the enzymes of phytohormone synthesis and inhibiting the nutrients and ion absorption by affecting plasma membrane permeability.

II. ORGANIC EXTRACTS

Organic extracts yields of *C. spinosa* parts

Leaves contained the highest yields (2.9%), followed by $CHCl_3$ and hexane (2.2 and 1.0%, respectively). Roots and stems $CHCl_3$ extracts (1.4 and 2.2%, respectively) gave higher yield than MeOH (1 %) which were double than hexane extracts yield (0.5% for two extracts).

Germination: Methanol, where residues were dissolved, had no effect on germination; hence effects could be attributed to allelochemicals present in organic extracts. The ortgan-

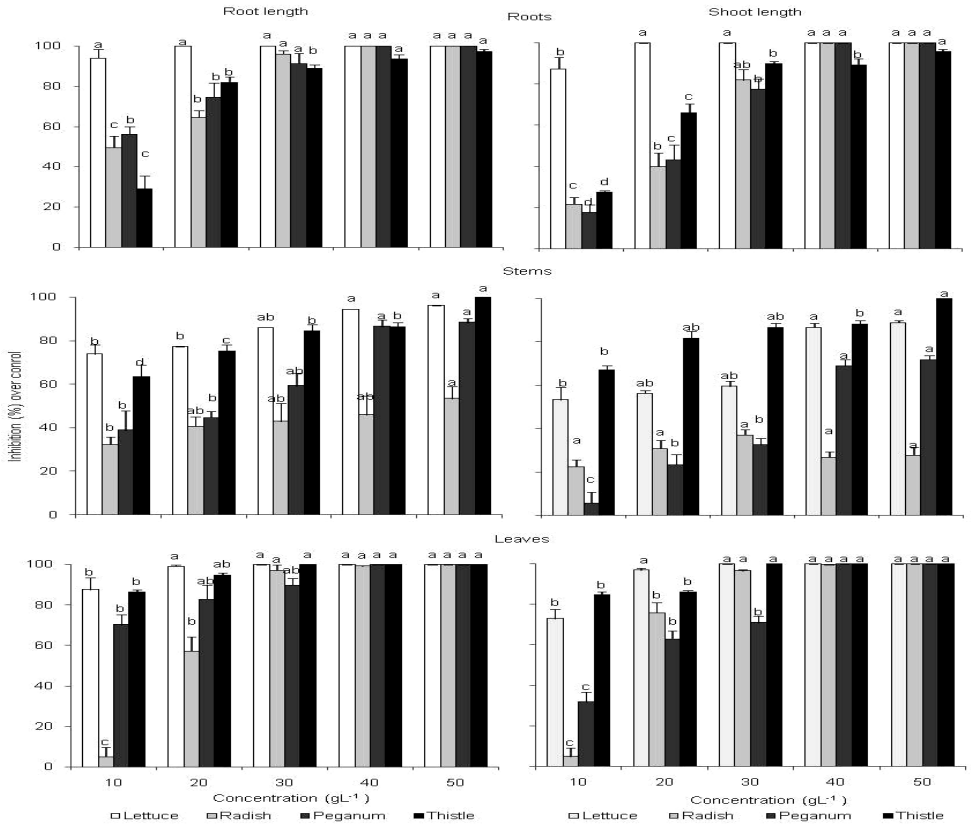


Figure 1. Inhibition (% of control) of root and shoot length of target species, 7 d after germination, in presence of different concentrations of *C. spinosa* roots, stems and leaves aqueous extracts. The bars on each column show standard error. Values (N=3±S.E.). Different letters in columns indicate significant differences among treatments at P<0.05 (LSD test).

-ic extracts were moderately toxic to germination (67.4% and 92.1%) of target species. The germination of target species with leaves organic extracts was 67.4 - 92.1%. Besides, the MeOH extract of leaves was most toxic to lettuce germination (58.9%). Root and shoot organic extracts were less inhibitory (68.7 and 90.1% germination) (Table 3). The most common explanation for this variation in response would be the allelochemicals selectivity for target species (28). Indeed, inhibition of seed germination was attributed to some allelopathic compounds that interacts with the mitochondrial membrane (1,45) and was strongly correlated with the inhibition of glycolysis enzymes activities and the oxidative pentose phosphate pathway (OPPP) (42).

Seedling Growth: The MeOH extract of leaf was most toxic to lettuce (90.8% inhibition). In peganum, the leaves hexane and $CHCl_3$ extracts caused 61.6% inhibition, while the

Table 3. Germination index GI (expressed in % of control) of test species: lettuce, radish, peganum and thistle in the presence of organic extracts (at 6 (g L^{-1}) of *C. spinosa* roots (R), stems (S) and leaves (L)

Organic extracts (6 g L^{-1})		Germination Index (%)			
		Lettuce	Radish	Peganum	Thistle
Root	Hexane	75.7±1.3a	75.5±2.7a	88.3±3.9a	63.3±6.6a
	Chloroform	86.0±0.7b	95.7±2.4b	98.1±7.8b	66.8±5.9a
	Methanol	78.9±1.9a	89.1±3.9a	83.9±4.3a	76.0±5.1a
	Mean	80.2	86.7	90.1	68.7
Shoot	Hexane	74.4±4.2a	80.8±8.6a	99.3±9.4b	88.3±7.4a
	Chloroform	76.5±1.6a	79.8±7.3a	79.1±7.3a	76.4±5.5a
	Methanol	77.4±3.2a	81.2±3.9a	88.5±6.2ab	85.2±3.2a
	Mean	76.1	80.6	88.9	83.3
Leaf	Hexane	72.3±2.2b	74.5±4.9a	86.0±7.6a	66.0±4.2a
	Chloroform	71.1±3.1b	78.2±8.2a	89.9±6.9a	76.2±4.3b
	Methanol	58.9±4.1a	72.3±9.2a	100.4±8.4b	61.7±6.6a
	Mean	67.4	75.0	92.1	67.9

Means with the same letter in a column are not significantly different at $P < 0.05$. Values (N=3±S.E.).

inhibition by methanol extract was only 46.4% (Fig. 2). With stems extracts, the most phytotoxicity was registered by hexane and methanol extracts which induced 76.7% inhibition in peganum seedling and 60.2% in thistle seedling, respectively. However for roots, $CHCl_3$ extract was most toxic to thistle, inducing inhibition of 84.8% for roots length and 69.8% for shoot length. This phytotoxicity variability could be attributed to the change in the chemical composition of different organic extracts and/or to the combined action of their chemicals, indeed several combinations of allelochemicals have either additive or synergistic effects (18). The mixture of organic extracts gave different inhibitory effects than when they are applied separately (36). Concerning the explanation of their effects, it was reported that allelochemicals reduce the auxin inducing root growth (57) affects the respiration, the oxidative phosphorylation and thereby reduces the growth (25). However, the phytotoxins that inhibits the weed species growth at certain concentrations, might cause less or no growth inhibition in another specie (59). Therefore the separation of components fractions of organic extracts and separating their allelochemicals are necessary to find the active ingredients.

Isolation and identification of leaf bioactive compounds

The testing of *C. spinosa* aqueous and organic extracts revealed that leaves extract was most active, hence, it was selected for further chemical study by testing its fractions bioactivity on lettuce (43,50).

III. PETROLEUM ETHER, EtOAc AND MEOH-WATER EXTRACTS

Germination: Methanolic extract of leaves was separated into three fractions: petroleum ether, ethyl acetate and MeOH-water extracts, their bioassays were tested at 0.06, 0.6 and 6 g L^{-1} on lettuce germination and seedling growth (Fig. 3). Results exhibited a significant

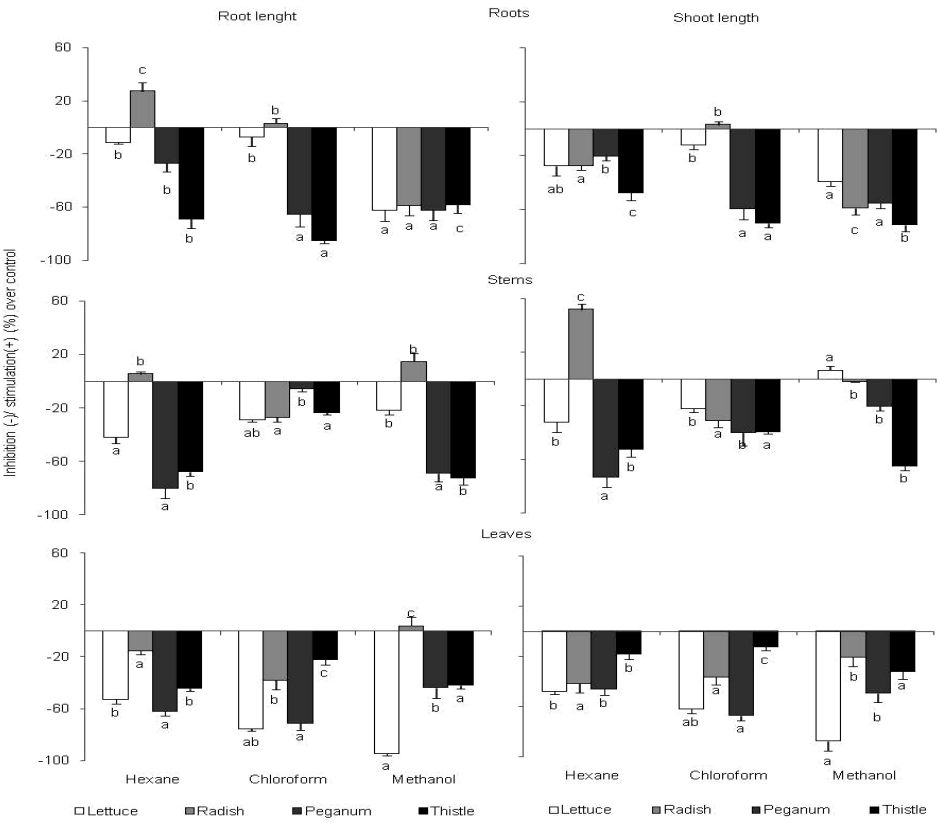


Figure 2. Inhibition (-)/stimulation (+) (% of control) of root and shoot length of target species, 7 d after germination, in presence of three organic extracts of *C. spinosa* roots, stems and leaves, at 6 g L^{-1}. The bars on each column show standard error. Values (N=3±S.E.). Different letters in columns indicate significant differences among treatments at P<0.05 (LSD test).

inhibition in which magnitude increased with the increasing concentration. The most toxic extract was EtOAc, giving 51.8% germination at the lowest concentration (0.06 g L^{-1}) and 27.8% at the highest concentration (6 g L^{-1}), while other two extracts gave 40.9% germination. MeOH-water was slightly more inhibitory than petroleum ether with 76.5 and 87.0 % germination at 0.06 g L^{-1}.

Seedling growth: All concentrations of EtOAc extract were very toxic to lettuce seedling growth (45.9% and 80.3% inhibition). The MeOH-water extract of roots caused 32.1% to 59.6% inhibition, while, shoot extracts caused 16.3% to 43.6% inhibition. However, petroleum ether extract was phytotoxic only at 6 g L^{-1}, which caused 81.4% inhibition of seedling length. Below 0.6 g L^{-1} concentration, the inhibition by root extracts was 5.8% for roots and 28.1% by shoots extracts (Fig. 4).

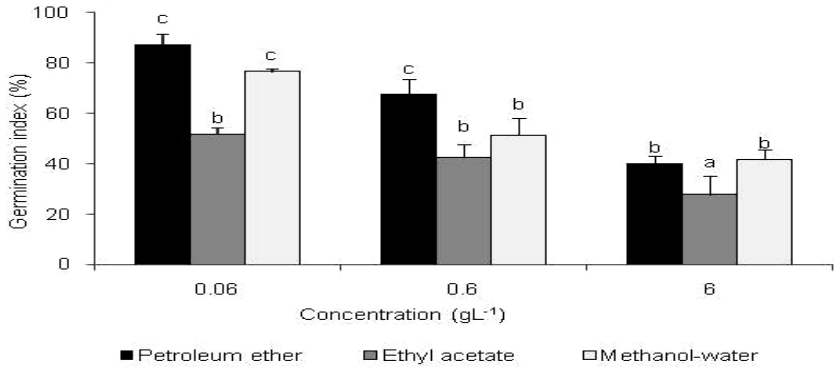


Figure 3. Variation of lettuce germination index GI, (% of control) in presence of *C. spinosa* petroleum ether, ethyl acetate and methanol-water extracts (at 0.06, 0.6 and 6 g L^{-1}). The bars on each column show standard error. Values (N=4±S.E.). Different letters in columns indicate significant differences among treatments at P<0.05 (LSD test).

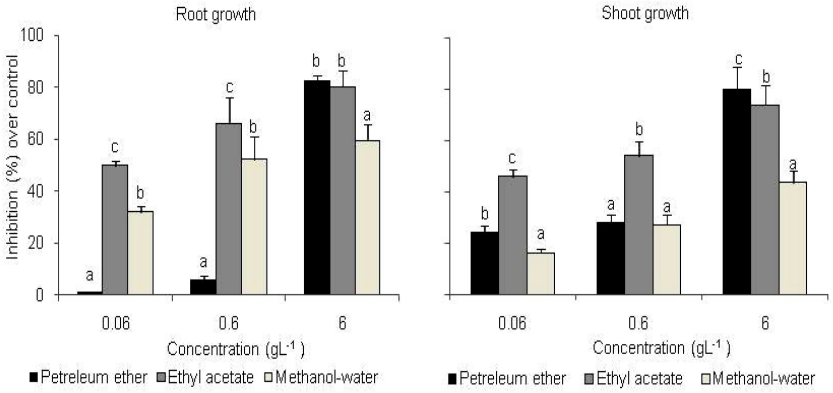


Figure 4. Inhibition (% of control) of lettuce root and shoot length, 7 d after germination, in presence of *C. spinosa* petroleum ether, ethyl acetate and methanol-Water extracts (at 0.06, 0.6 and 6 g L^{-1}). The bars on each column show standard error. Values (N=4±S.E.). Different letters in columns indicate significant differences among treatments at P<0.05 (LSD test).

III. EtOAc FRACTIONS

Germination: The EtOAc extract was chromatographed over Sephadex LH 20 eluted with MeOH:CH_2Cl_2 (5:1) and the obtained fractions were tested on lettuce at 0.6 g L^{-1}. The most toxic fraction was A10, followed by A9, with respective inhibition of 44.7 and 31.7% in seed germination. The other fractions induced slight inhibition in lettuce germination with great variability [2.1% and 26.4% (Fig. 5)].

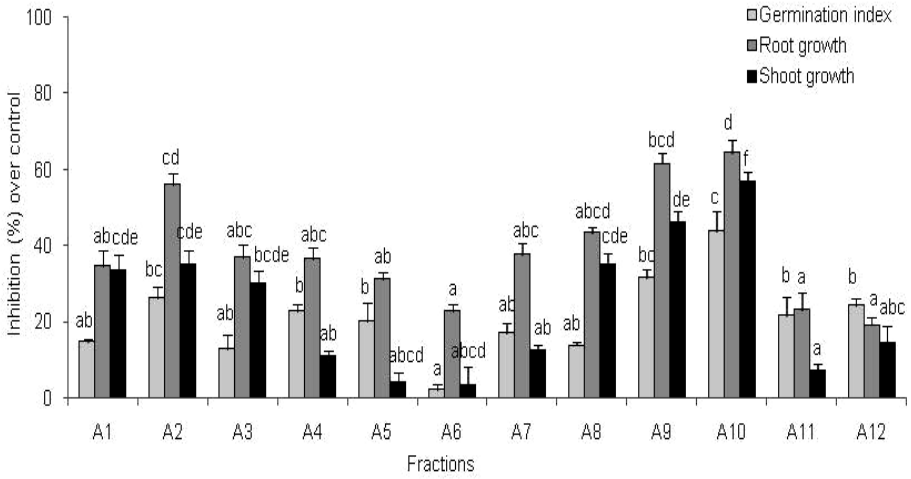


Figure 5. Inhibition (% of control) of lettuce germination index, root and shoot length, 7 d after germination, in presence of *C. spinosa* ethyl acetate fractions (A1–A12) at 0.6 g L^{-1}. Values (N=4±S.E.). Different letters in columns indicate significant differences among ethyl acetate fractions at P<0.05 (LSD test).

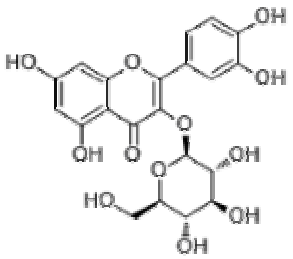
Seedling growth: The similar results to seed germination were obtained on seedling growth. The fractions A9 and A10 were most toxic and the roots were more sensitive than shoots. These fractions induced inhibition of 63.1 and 54.5% in root and shoot length, respectively. The other fractions, caused 19.2 and 43.3% inhibition in roots and 3.4 and 35.2% in shoots. The fraction A2 reduced the root length by 55.9% (Fig. 5).

Among bioassay-guided isolation, EtOAc extract was most toxic to lettuce germination and growth. The bioactivity-guided fractionation of EtOAc extract yielded twelve fractions, two of which (A9 and A10) were most active, suggesting that the active molecules should exist in these two fractions. Roots were more sensitive than shoots, and it is attributed to direct contact of roots with allelochemicals, which did not happen in shoots (4).

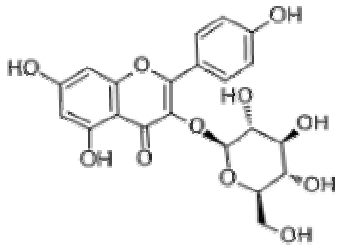
Identification of growth inhibitory allelochemicals

The fractions A9 and A10 were purified by TLC semi preparative and three allelopathic active compounds were isolated as yellow residues (Fig. 6).

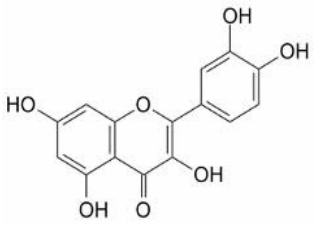
Compounds 1: The ^{1}H NMR (500 MHz, CD3OD) δ 7.71 (1H, s, H-2'), 7.59 (1H, dd, J = 8.5 and 2.0 Hz, H-6'), 6.87 (1H, d, J = 8.5 Hz, H-5'), 6.37 (1H, s, H-8), 6.19 (1H, s, H-6), 5.23 (1H, d, J = 7.6 Hz, H-1"), 3.71 (1H, dd, J = 11.8 and 2.1 Hz, H-6"a), 3.58 (1H, dd, J = 11.8 and 5.6 Hz, H-6"b), 3.49 (1H, m, H-3"), 3.43 (1H, m, H-2"), 3.35 (1H, m, H-4"), 3.22 (1H, m, H-5"). The ^{13}C NMR (125 MHz, CD3OD)□δ□79.9□ C□-4), 167.3 (C-7), 163.5 (C-5), 159.4 (C- 9), 159.0 (C-1), 150.4 (C-4'), 146.4 (C-3'), 136.1 (C-3), 123.7 (C-1'), 123.5 (C-6'), 118.0 (C- 5'), 116.5 (C-2'), 105.9 (C-10), 104.9 (H-1"), 100.7 (C-6), 95.4



Compound 1
Quercetin 3-O-β-D-glucopyranoside



Compound 2
Kaempferol 3-O-β-D- glucopyranoside



Compound 3
Quercetin

Figure 6. Chemical structure of allelochemicals from *C. Spinosa* leaves

(C-8), 78.9 (H-5"), 78.6 (H- 3"), 76.2 (H-2"), 71.7 (H-4"), 63.0 (H-6"). From comparison of these data with those reported in the literature (40), the compound was identified as quercetin-3-O-β-D-glucopyranoside (Fig. 6).

Compound 2: The ^{1}H NMR (500 MHz, CD3OD) δ 7.73 (1H, d, J = 2.0 Hz, H-2'), 7.63 (1H, dd, J = 8.4 and 2.0 Hz, H-6'), 6.88 (1H, d, J = 8.4 Hz, H-5'), 6.39 (1H, s, H-8), 6.19 (1H, s, H-6). From comparison of these data with those reported in the literature (40), the compound was identified as kaempferol-3-O-β-D-glucopyranoside (Fig. 6).

Compound 3: The ^{1}H NMR (500 MHz, CD3OD) δ 7.73 (1H, d, J = 2.2 Hz, H-2'), 7.63 (1H, dd, J = 8.5 and 2.2 Hz, H-6'), 6.88 (1H, d, J = 8.5 Hz, H-5'), 6.39 (1H, s, H-8), 6.18 (1H, s, H-6), the compound was identified as quercetin by comparison with authentic sample (Fig. 6).

Phytotoxicity of purified compounds

Isolated compounds were tested for their phytotoxicity on lettuce at 0.06 g L^{-1} (Table 4). Isolated flavonoids were more potent inhibitors of growth than germination. Quercetin-3-O-β -D-glucopyranoside and quercetin induced similar effects on lettuce germination with 23.4% inhibition, followed by kaempferol-3-O-β-D-glucopyranoside (9.1% inhibition). However, quercetin-3-O-β -D-glucopyranoside was most toxic to seedlings growth (66.1% inhibition). It was followed by Quercetin (55.6% inhibition) and kaempferol-3-O-β-D-glucopyranoside (41.4% inhibition) (Table 4). However Reigosa and

Table 4. Germination index, root and shoot growth (% of control) of *Lactuca sativa* in presence of the three identified compounds (at 0.06 g L^{-1}) from leaves of *C. spinosa.*

Compounds	Germination Index (%)	Root length (%)	Shoot length (%)
1 (Quercetin 3-O-β-D-glucopyranoside)	76.4±9.1a	30.4±0.9a	37.4±2.4a
2 (Kaempferol 3-O-β-D-glucopyranoside)	90.9±2.6b	51.4±3.7b	65.7±3.1c
3 (Quercetin)	76.9±6.5a	34.8±3.6a	54.1±3.7b

Means with the same letter in a column are not significantly different at $P < 0.05$. Values (N=4±S.E.). Different letters in lines indicate significant differences among treatments at $P<0.05$ (LSD test).

Pazos-Malvido (51) did not observe toxic effects of quercetin on *Arabidopsis* plants, several flavonoids (quercetin, isoquercitin and rutin) are phytotoxic to plants (29). The quercetin inhibits the seed germination and growth of *R. sativus* (5). The phytotoxic effects of identified flavonoids on lettuce roots and shoots growth could be due to decreased cell division, elongation and expansion rate which are growth pre requisites. Indeed, the inhibitory effects of allelochemicals on mitochondrial respiration after reduction in ATP levels, decreases the seedling growth (26) and blocks the chloroplast functions (18). Quercetin may act on electron transfer steps between quinone pool and oxygen (56). Overall, flavonoids do not effect the seed germination, but reduced the cotyledon and root size, thereby inhibiting the seedling development, as smaller cotyledons develops in to smaller and less competitive plants (7). The allelochemicals that inhibits the cell division acts in two ways: (i). affecting the synthesis or the structure of DNA-RNA and (ii). inhibiting the energy production for mitosis (35). Both these processes are important for cell division and interfere with these, inhibits the growth. Allelochemicals may produce multiple effects on the cellular processes leading to decreased plant growth.

CONCLUSIONS

C. spinosa leaves were potent phytotoxic. Further bioassay-guided fractionation of leaf extracts led to the isolation of three flavonoids; Quercetin-3-O-β-D-glucopyranoside, quercetin and kaempferol-3-O-β-D-glucopyranoside with potent phytotoxic activity. Quercetin-3-O-β-D-glucopyranoside was most active, followed by quercetin and kaempferol-3-O-β-D-glucopyranoside. These phytochemicals could play important role in the allelopathic potential of *C. spinosa* and must be considered in studies seeking actives biomolecules. More searches are in progress to evaluate (i) their interactions at different concentrations, (ii) their herbicidal activities on other targets weeds and (iii) to understand the allelopathic mechanisms at the cellular level.

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