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Isolation and purification of allelochemicals from *Cephalaria syriaca* plant

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Abstract

This study was conducted to investigate the existence of allelochemicals in *Cephalaria syriaca* endemic weed plants, the process of purification and isolation of allelochemicals started from the plants root, shoot, seeds 70% methanolic extracts then it passed series of solvent solvent extractions with indicating the inhibitory effect of each fraction. Finally the 100% active fraction that caused 100% germination inhibition were HPLC system was employed for indicating the allelochemicals in the three studies plant parts and results indicated ten phenolic compounds which were, gallic acid, P-hydroxy benzoic acid, protocatechuic acid, Vanillic acid, Syringic acid, Sinapic acid, phluroglucinol, Chlorogenic acid, Xanthotoxine, and Chlorocatechol. Eight of these compounds were identified for the first time beside 27 unknown compounds. These results indicated the importance of that plant not only as medicinal plant but also as an alternative of herbicides for controlling weed plants.

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Introduction

Cephalaria is a plant genus of about 65 species of flowering plants in the family Dipsacaceae native to western and central Asia, southern Europe, and northern and southern Africa, they are annual or perennial plants, *Cephalaria syriaca* is an endemic weed and considered as a noxious weed in Iraqi Kurdistan ((Ali and Aziz, 2002)). There is no study to indicate its sole effect on yield loss but it is the most dangerous weed plant due to its ability of giving the bread a bitter test if wheat seeds were contaminated with 2% (Ali, 2001and Gorge, 1983). *Cephalaria transsylvanica* flowers contain Transsylvanoside A and B, both compounds were new triterpene glycoside by PLC and NMR methods (Kirmizigul and Anil, 1994). Then the chemical structure of cephalaria saponin B was identified from *Cephalaria transsylvanica* (Galiskan and Anil, 1995). Other triterpenoid glycosides has been documented and isolated from same plant species named saponin G and H (Kirmizigul et al, 1995). In China, on the basis of field investigations and taxonomic researches on medicinal plants of the genus Dipsacaceae, studies indicated the discovery of eighteen species and four varieties, among which some were new medicinal plant resources (Chen and Ai, 1997).

New lignin glycoside named ambrosidine with seven known compounds (four iridoids and three hydroxycinnamic esters) were isolated from the roots of *Cephalaria ambrosioides* by the use of NMR and MS techniques, then there cytotoxic activity was evaluated against five solid human tumour cell lines (Pasi *et al.* 2002). Also three new triterpenic saponins were isolated from aerial parts *Cephalaria transsylvanica* with indicating their structures by the use of chemical spectroscopic (Kirmizigul and Anil, 2002). While, there was a new discovery of two new Flavonoids from the flowers of *Cephalaria pastricensis* which have luteolin structure then they were identified by NMR and spectroscopy, where both structures showed significant antiradical activity (Godjevac *et al.*, 2004). The same team has published another scientific paper on the isolation of triterpenoid saponins and iridoid glycosides from the aerial parts of the previous studied plant with giving a condense review of studies that deal with the genus Cephalaria (Godjevac *et al.*, 2006). Another discovery was also declared when three new saponins, along with eight known ones, were isolated from *Cephalaria gigantea* followed by the determination of their cytotoxic activity as well as their capability to act as anti-proliferative when investigated in vitro using three cancer cell lines (Tabatadze *et al.*, 2007). Write the aim of your study here!!

Materials and methods

This part of study was conducted in the laboratories of molecular bioscience and bioengineering department of the University of Hawaii at the United States of America. In these experiments, we focused on isolation and purification of allelochemicals from the 70% methanolic MeOH extracts of different plant parts of *Cephalaria syriaca,* extracts were reduced by flash evaporator to get rid off the alcoholic remains into semi-dryness where then dissolved in distilled water and broad to 100ml. these crude extractions were then subjected to a liquid–liquid extraction processes for obtaining dichloromethane DCM, Ethyl-acetate EtOAc, 1-Butanol BuOH, and H_2O dissolved organic matters for shoot and root partitions, while for the seeds there was a different sequence for the organic solvents: Hexane, DCM, EtOAc, BuOHl, and H_2O. liquid partitions were dried by using rotary evaporator Buchi Rotavapor-R, Germany under reduced vacuum pressure at 40°C. After that the dried residues were weighted and redissolved in 100% MeOH to be used for bioassay technique. The bioassay started by a pouring carefully 1 ml of each partition solution that contains 8mg of the residue, to a sterilized filter paper Whatman #1 disposed in a disposable plastic petri-dish 9 cm in diameter, while control petri-dishes, contains only 1ml of pure organic solvents that was prepared in vivo. After evaporation of the organic solvents, 5 ml of double distilled water was added for each prepared petri-

dishes with 25 lettuce seeds distributed randomly to investigate partitions allelopathical effects in what is called bioassay guided investigation (Fig. 1) (Rimando *et al.*, 2001, Kpoviessi *et al.*, 2006, Zahida *et al.*, 2005).

The two partition solutions that caused 100% inhibition of lettuce seed germination were then further fractionated by utilizing vacuum liquid chromatography (VLC) (Pelletier *et al.*, 1986,Coll and Bodwen.1986) with silica gel (TLC grade) by using solvent combination for each partition alone.For shoot EtOAc partition the solvents sequence were DCM : MeOH were mixed in different percentages: 97.5:2.5, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100, while for shoot BuOH partition, the sequence of solvents were DCM : EtOAc : l MeOH were mixed in different percentages as followed: 50:50:0, 25:75:0, 0:97.5:2.5, 0:95:5, 0:90:10, 0:80:20, 0:60:40, 0:50:50, 0:40:60, 0:30:70, 0:20:80, 0:10:90, 0:0:100. Thus, 24 fractions of these two partition solutions were obtained from the above procedure where (VLC) was applied for such purpose. The sub fractions were then bioassayed to elucidate their effectiveness on lettuce seed germination. At all steps, TLC was running for all fractions by Whatman TLC flexible plates covered with 250 µm layer of silica gel 20×20 cm made in Germany. The only allelopathic active fractions (AAF) were the sub fraction #5 of EtOAc, fraction #6 and fraction #7 of BuOH fraction. It is well documented that some allelochemicals existed in plants in minute amounts, where it is difficult to be extracted and identified by classical chemical methods. Because of that, high performance liquid chromatography (HPLC) was used as an effective tool for identification of such chemicals. Thus this study was started and initiated with the preparation of the standard chemicals which were run through the HPLC to make a standard library used as a map for the identification of such chemicals (Sakakibara., *et al* 2003, Macias, 1999). The certainty of the standards results was assigned by purchasing the standard chemicals from Sigma-Aldrich company and they were as shown in table-1.

The HPLC system employed in this study was an auto sampler Agilent 1100 DAD system, Germany, made instrument, equipped with chemstation revision B.02.01 SRS 2006 and DAD for monitoring all wave length from 200 to 600 nm. The column, Phenomenex USA (C18, 250 ×4.60mm 5 µm) at 25°C was implemented to such fractionation of allelochemics. Gradient elution's were performed with solution A composed of 50mM sodium phosphate (PH3.3) and 10% methanol, while the solution B was comprising of 70% methanol and were delivered at a flow rate of 0.500mL/min as follows:-

Initially started with 100% of Solution A was used for 5 minute. Followed by 70% A and 30% of solution B for 15 minutes. Then with 65% A and 35% of solution B for the next 30 minutes. Followed by 60% A and 40% of solution B for 20 minutes. Then 50% A and 50% of solution B for next 5 minutes. Finally the column was washed 0% A and 100% of solution B for 30 minutes.

Only 10µL volume for each sample of fraction was injected into the HPLC each time. For the preparation of HPLC library every 5 mg chemical standard was dissolved in 50 ml of 100% MeOH to obtain the concentration of 100ppm. After that, the standard vials each contains 1ml of sample, were run in HPLC to provide the Retention time for each standard. Then samples of the active allelopathic fractions (AAF) were analyzed by HPLC, and according to retention time, only the standards which gave the same retention time, were reanalyzed with different concentrations of 100ppm, 80ppm, 60ppm, 40ppm, 20ppm, 10ppm, and 5ppm to provide a wide scope of information to evaluate the contained of the same chemicals present or existed in plant sample. The unknown compounds of the allelopathic active fraction (AAF) #5 were analyzed by the use of Bruker quadrupole time of flight mass spectrometry (micro TOF)

Germany made instrument to indicate the molecular weight of the unknown compounds. The other allelopathic active fractions (AAF) according to the results of (HPLC), they were not run through (micro TOF) because of their minute amounts.

Results and discussion

The first step followed the process of isolation of allelochemicals from the crude extracts of *C. syriaca* was bioassay guided investigations (Rimando *et al*., 2001, Zahida *et al*., 2005, Kpoviessi *et al*., 2006) and lettuce *Lactuca sativa* L. var anuenue was used as test plant (Dayan *et al*., 2000, Piechowski et al., 2006, Anaya, 2006). From table (2) it is obvious that the solvent extraction and partitioning for *C. syriaca* shoot, root and seeds partitions caused significant differences for all registered parameters. Germination percentages were zero at six partitions which means the diagnosis of six allelopathic active partitions capable of causing inhibition of seed germination by 100%, while for both seed and shoot extractions of *C. syriaca* allelopathic active partitions, the solvents were ethyl acetate and BuOH, whereas for root parts only, the allelopathic active partitions came from the solvent BuOH and water (Figure 2). It is worth mentioning here that non polar components could be extracted with DCM, while the relatively polar components would go to EtOAC and BuOH extraction solvents. The main purpose for using such different chemical solvents for the extraction processes was for gathering more precise information to clarify that the partitions possesses more inhibitory effects to illustrate and reveal the way to conduct further purification steps on the active partitions (Cia, 1997 , Wickenden, 2001). Thus, the two most active partitions of shoot parts, EtOAc and BuOH partitions were further fractionated by the vacuum liquid chromatography (VLC) (Pelletier *et al*., 1986, Coll and Bodwen, 1986) technique, using different solvent percentages as demonstrated previously. Subsequently, there were twelve fractions for each of the EtOAc and BuOH partitions. All fractions were dried and redissolved in MeOH 100% and prepared for another step of bioassay in order to indicate the most allelopathic affective fraction (AAF). The data obtained from the bioassays of all the twelve fractions for each partition showed significant differences between all studied parameters as shown in (table 3 and 4). The most activate part in the EtOAc fractions was only fraction 5, which was dissolved in the mixture of (methanol 30:70 dichloromethane), while for BuOH partitions the most allelopathic active fractions were fraction 6 and 7, whereas dissolved in the mixture of (MeOH and EtOAc) with the rate of 20:80 and 40:60 respectively. Thus, from all twenty four fractions of both EtOAc and BuOH that have been bioassayed with lettuce seeds as an indicator, only three fractions caused 100% inhibition of the tested plants. The three fractions mentioned previously might be the main source of the allelopathic potent of *C. syriaca* shoot extracts. As proceeding the purifications, the bioassay response may decrease the inhibitory effects due to the separation of multiple compounds which might act at the same metabolic pool or at different biochemical and physiological sites. During any study the possible additive, synergistic and antagonistic effects of multiple allelochemicals need to be considered, especially in crude extracts.(Inderjit,1996, Macias *et al*., 2000, Hoagland and Williams, 2004). However the results obtained from the isolation and analysis of the shoot, root, and seeds of *C. syriaca* through various successive partitioning and purifications, and through the testing of the biochemics dissolved in each partition, have indicated that the shoot parts of *C. syriaca* have preserved and contain more allelochemicals which have been synthesized by the plant during periods of growth and development compared to what have been found in the root parts. These variations in the existence of such biochemics might be due to two factors 1) the location of the root part under the soil and the position of the shoot parts in the free environment where it exposed to the process of photosynthesis and accumulation of chemicals. Secondly 2) it might be also attributed to the functions and specializations of cells in both shoot

and root with regarding to the type of metabolites, taking in mind that development of fruits comes from the translocation of biochemics and food energy of the shoot parts (Lambers *et al*., 2008). Almost every plant secondary metabolites class has been implicated in allelopathy under normal conditions 20% of fixed carbon flows through the shikimic pathway, while the weight percentage of plant secondary metabolites depends on the plant species and tissue type such as fruit, seeds, stem, bark, wood, flowers, and leaves but it is normally less than 10% (Rice, 1984, Wickenden, 2001, Macias *et al*., 2007). However, and depending on physiological functions of plant parts, still leaves are regarded as the main site for production of these regulatory compounds including the secondary metabolites as well as the inhibitory and promotive biochemics (Hess, 1975, Taiz and Zeiger, 2006, Lambers *et al*., 2008).

This analytical study has being initiated with the analysis of the twenty one previously mentioned chemical standards through the HPLC to make a standard library that would be used as a map for identification of *C. syriaca* allelochemicals. An auto sampler Agilent 1100 DAD HPLC system, Germany made instrument, was employed in this study, and equipped with chemstation revision B.02.01 SRS 2006 and DAD for monitoring all wave length from 200 to 600 nm. The column, Phenomenex USA (C18, 250 ×4.60mm 5 µm) at 25°C was implemented to such fractionation of allelochemics. Gradient elution's were preformed with solution A composed of 50mM sodium phosphate (PH3.3) and 10% methanol, while the solution B was comprising of 70% methanol and delivered at a flow rate of 0.500mL/min as demonstrated in the methodology chapter. The standard vials, each contained 1ml of sample, were run in HPLC to provide the retention time for each standard. Then the three *C. syriaca* shoot, root, and seed part samples were run through the HPLC, whereas in shoot parts there were more compounds than the other two plant parts (table 4). Shoot parts of *C. syriaca* plants showed peaks for three known compounds vanillic acid, sinapic acid, and chlorogenic acid with six peaks for unknown compounds. Such results mean that the shoot parts showed nine peaks for different compounds compared to seed plant parts which revealed five peaks and root parts showed only one peak. High pressure liquid chromatography using C18 columns, results in polar compounds which is eluted first, followed by non polar compounds which is due to the fact that C18 is known as a reversed-phase chromatography whereas the solvents used in reversed-phase chromatography were the (reverse) polarity to the solvents used to elute compounds from the normal phase chromatography, whereas the stationary phase was the silica derivatized with non polar octadecyl 18 chains, while the mobile phase were the polar solvents which included two solutions of methanol 10% (A) and methanol 70% (B) where scheduled to be mixed under computerized program (Fifield and Kealey, 2000, Cooke and Poole, 2000). The partitions of the shoot, seed, and roots of *C. syriaca* run through the HPLC, pursuing the same procedure, showed more total or aggregate compounds in root partitions compared to seed and shoot partitions (table 5), whereas root partitions showed total of 42 peaks compared to shoot partitions 38 peaks and seed partitions 40 peaks (table 5). Further fractionation for *C. syriaca* shoots, two allelopathic active partitions resulted in the twenty four fractions that have been run by the HPLC and pursuing the same procedure. Table (6) shows the result of the HPLC analysis illustrating that the active fraction ethyl acetate partitions (F5) shows only three peaks for unknown compounds, while the allelopathic active fractions of number (6) and (7) for BuOH partition did not show any peaks (table 3). These results about the BuOH fractions might be attributed to the minute amount of allelochemicals in each fraction or to the concentrations of the extracts which were insufficient to make any response. Therefore, the original and reference spectra for all the three peaks of the allelopathic active fraction were recorded, after that the fraction which gives the positive results was subjected to the Bruker quadrupole time of flight mass spectrometry (micro TOF) in order to

indicate the molecular weight of the active allelopathic compounds. The results showed ambiguous response of the sample which might be due to the special characteristic of such allelochemical compounds for the three unknown peaks or to the minute amounts of the extracted allelochemicals. Thus, it is desirable to minimize the overlapping peaks with further fractionation techniques for further studies (Chernushevich *et al*., 2001, Griffiths *et al*., 2001).

Table 1. The Pure standard compounds used in the study.

1.	***P*-hydroxy benzoic acid**	**2.**	**Chlorogenic acid (1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid)**
3.	Vanillic acid (4-hydroxy-3-methoxybenzoic acid)	4.	Phloroglucinol (1,3,5-trihydroxybenzene)
5.	Ferulic acid (4-hydroxy-3-methoxycinnamic acid)	6.	Digitoxin (3β,5β)-3-[(*O*-2,6-dideoxy-β-D-ribo-hexapyranosyl-(1->4)-2,6-dideoxy-β-D-ribo-hexopyranosyl)oxy]-14-hydroxycard-20(22)-enolide)
7.	Umbelliferone (7-hydroxy cumarin)	8.	Gallic acid (3,4,5-hydroxy benzoic acid)
9.	Caffeic acid (3,4-Dihydroxy-cinnamic acid)	10.	3,4- dihydroxy benzoic acid **(**protocatechuic acid**)**
11.	Sinapic acid (4-Hydroxy-3,5-dimethoxycinnamic acid)	12.	Xanthotoxin (8-methoxy psoralen)
13.	Coumarin	14.	Xanthene (9H-Xanthene)
15.	2-Hydroxycinnamic acid	16.	Catechol (2-hydroxyphenol)
17.	Syringic acid (4-hydroxy-3,5-Dimethoxybenzoic acid*)*	18.	Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1- benzopyran-4-one)
19.	Scopoletin (7-Hydroxy-6-methoxycoumarin)	20.	Salicylic acid (2-Hydroxybenzoic acid)
21.	Chlorocatechol		

Allelochemicals that found in *C. syriaca* plant parts in this study are mentioned for the first time in a precise compact, the only study on the plants chemical analysis utilizing HPLC was done by Gafoor (2002), with crude extracts only, therefore the results were not covered all aspects and nature of these biochemics. In fact this is the first study about utilizing allelopathical potential of a weed plant, especially *C. syriaca*, in which extracts were sprayed on tested plants. Conversely, the weed plant *C. syriaca* contains allelochemics which showed peaks when treated with different solvent.

The compounds which were found in the different *C. syriaca* parts were:

1) Gallic acid (3,4,5-trihydroxy benzoic acid): found in seeds crude extracts and its partitions, except in dichloromethane partition, as well as with shoot DCM partition. It is found in sumac, tea leaves, oak bark, eucalyptus trees, and act as an antioxidant which seems to have an anti-fungal and anti-viral properties. Gallic acid is causing the reduction of growth on bahiagrass plants when used in high concentrations while it has stimulatory effects in low doses. On the other hand it is known as an antioxidant and pharmaceutical commodity (Moreiras-sanchez *et al*., 2004, Narwall, 2006, Weidenhamer, 2006).

Table 2: The effect of solvent extractions on some registered parameters

Treatment	germination %	inhibition %	shoot length (cm)	root length (cm)	total seedling length (cm)	shoot dry weight (mg)	root dry weight (mg)	total dry weight (mg)
Hexane Seed	61.67	38.33	1.45	1.33	2.77	0.60	0.05	0.65
DCM Seed	43.33	56.67	1.43	1.33	2.76	0.53	0.07	0.60
DCM shoot	30.00	70.00	1.38	0.53	1.91	0.75	0.07	0.81
DCM Root	21.67	78.33	1.32	0.41	1.73	0.71	0.08	0.79
EtOAc seed	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
EtOAc Shoot	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
EtOAc Root	33.33	66.67	0.36	0.29	0.65	0.31	0.02	0.34
BuOH Seed	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
BuOH Shoot	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
BuOH Root	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
H_2O Seed	31.67	68.33	2.31	1.87	4.18	0.71	0.05	0.76
H_2O Shoot	13.33	86.67	0.46	0.24	0.69	0.68	0.05	0.73
H_2O Root	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
Control	100.00	0.00	2.76	3.06	5.82	0.82	0.12	0.94
Tukey's HSD	8.49	8.49	0.16	0.13	0.17	0.05	0.01	0.05

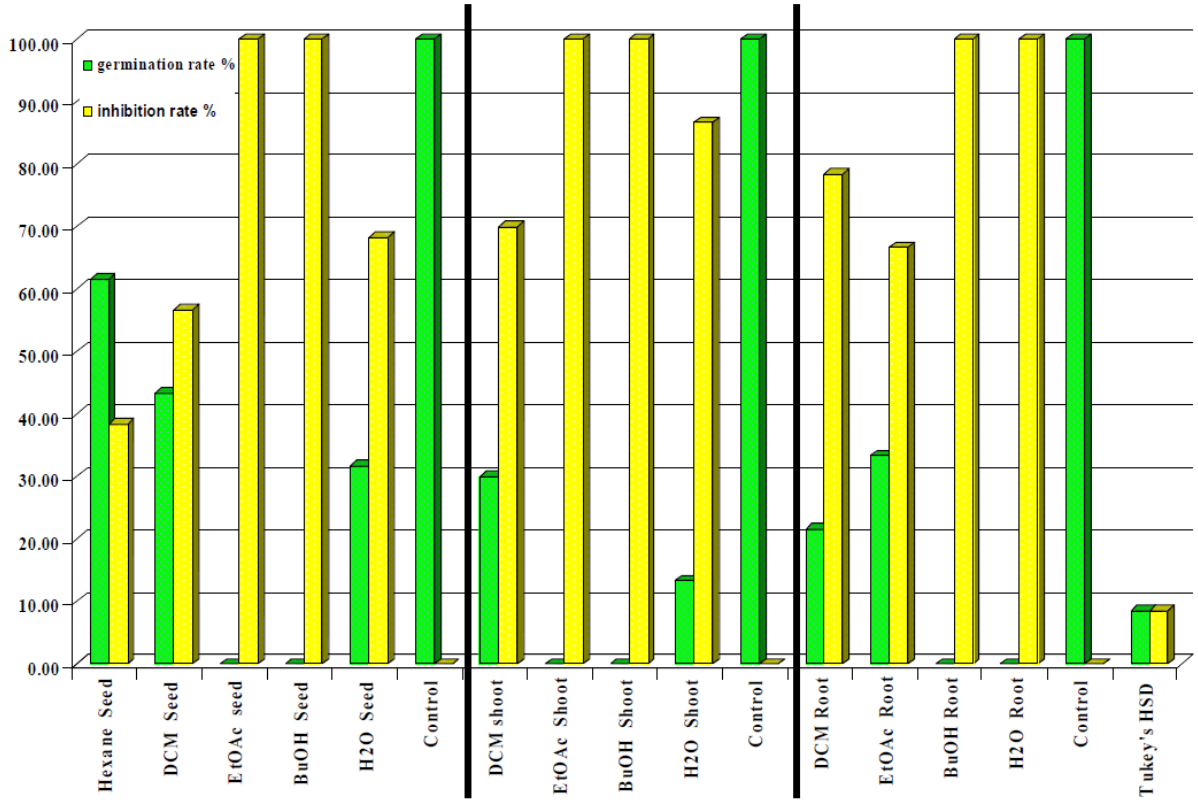

Figure2: The *C. syriaca* plant parts solvent extraction and partitioning effect on germination and inhibition rate of Lettuce *Lactuca sativa*.

Table 3: The effect of ethyl acetate VLC fractionation by the solvents (DCM and MeOH) on some registered parameters

Shoot EtOAc Fractions	germination %	inhibition %	shoot length (cm)	root length (cm)	total seedling length (cm)	shoot dry weight (mg)	root dry weight (mg)	total dry weight (mg)
Fraction 1	66.67	33.33	2.77	2.41	5.18	0.40	0.05	0.45
Fraction 2	21.33	78.67	1.95	1.73	3.69	0.58	0.06	0.64
Fraction 3	33.33	66.67	2.41	1.49	3.90	0.65	0.06	0.71
Fraction 4	21.33	78.67	1.80	1.38	3.17	0.68	0.07	0.75
Fraction 5	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
Fraction 6	49.33	50.67	3.97	3.02	6.99	0.61	0.08	0.69
Fraction 7	36.00	64.00	3.33	2.74	6.07	0.65	0.07	0.72
Fraction 8	40.00	60.00	3.11	2.21	5.32	0.57	0.07	0.64
Fraction9	46.67	53.33	2.65	1.88	4.54	0.53	0.07	0.60
Fraction10	34.67	65.33	3.48	2.22	5.70	0.78	0.06	0.84
Fraction 11	44.00	56.00	3.24	1.73	4.97	0.69	0.07	0.77
Fraction 12	46.67	53.33	3.67	1.57	5.24	0.55	0.07	0.63
Control	100.00	0.00	2.95	3.13	6.08	0.81	0.11	0.92
Tukey's HSD	13.58	13.58	0.34	0.38	0.51	0.07	0.02	0.07

Table 4: The effect of butanol VLC fractionation by the solvents (DCM, EtOAC, and MeOH) on some registered parameters

Shoot BuoH Fractions	Germination %	inhibition %	shoot length (cm)	root length (cm)	total seedling length (cm)	shoot dry weight (mg)	root dry weight (mg)	total dry weight (mg)
Fraction 1	78.67	21.33	1.83	1.44	3.27	0.65	0.04	0.69
Fraction 2	85.33	14.67	2.14	1.86	4.00	0.65	0.05	0.70
Fraction 3	93.33	6.67	1.73	0.92	2.66	0.61	0.04	0.65
Fraction 4	92.00	8.00	1.63	0.97	2.60	0.57	0.04	0.61
Fraction 5	76.00	24.00	1.96	1.22	3.18	0.55	0.05	0.60
Fraction 6	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
Fraction 7	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
Fraction 8	33.33	66.67	1.25	0.83	2.08	0.55	0.03	0.58
Fraction9	76.00	24.00	2.14	1.56	3.69	0.60	0.04	0.64
Fraction10	42.67	57.33	1.89	1.75	3.64	0.53	0.06	0.59
Fraction 11	61.33	38.67	1.66	1.44	3.10	0.48	0.04	0.52
Fraction 12	64.00	36.00	1.84	1.53	3.37	0.50	0.05	0.55
Control	100.00	0.00	2.95	3.13	6.08	0.81	0.11	0.92
Tukey's HSD	12.68	12.68	0.26	0.25	0.29	0.06	0.01	0.02

Table :5 The compounds and their retention time that recorded by HPLC for *Cephalaria syriaca* different plant parts

#	Standar	Retention Time (minute)	Seed					Shoot				Root				Seed	Shoot	Root
			H	DCM	EtOAc	BuOH	H_2O	DCM	EtOAc	BuOH	H_2O	DCM	EtOAc	BuOH	H_2O			
1	Gallic acid	17.38	+		+	+	+								+	+		
2	3,4-dihydroxy benzoic acid	19.342			+	+		+	+				+					
3	p-hydroxy benzoic acid	24.825				+							+	+				
4	Phloroglucinol	28.216						+				+	+					
5	Vanillic acid	29.378		+	+			+	+				+				+	
6	Sinapic acid	29.663		+	+													
7	Syringic acid	30.146	+					+	+			+	+			+	+	
8	Chlorogenic acid	40.745															+	
9	Xanthotoxine	87.997		+	+	+		+		+		+						
10	Chlorocatechol	93.437				+		+				+	+		+	+		
11	Unknown	109.191										+						
12	Unknown	108.901						+										
13	Unknown	107.515										+						
14	Unknown	106.802						+		+								
15	Unknown	105.893						+				+						
16	Unknown	104.501	+					+				+						
17	Unknown	103.536		+	+	+		+		+		+						
18	Unknown	101.613		+	+	+		+	+	+			+	+				
19	Unknown	98.745										+	+				+	
20	Unknown	97.955	+	+	+			+	+			+	+		+		+	
21	Unknown	95.453		+	+	+		+				+	+			+		
22	Unknown	92.378										+						
23	Unknown	91.906		+	+	+		+		+		+	+		+	+	+	
24	Unknown	89.150		+		+		+		+		+					+	
25	Unknown	85.408						+	+			+			+			
26	Unknown	83.577		+					+									
27	Unknown	82.325		+				+				+			+			
28	Unknown	79.224						+										
29	Unknown	73.92										+						
30	Unknown	46.072			+			+										
31	Unknown	44.977		+								+	+					+
32	Unknown	42.326											+					
33	Unknown	36.461							+									
34	Unknown	34.543	+	+					+								+	
35	Unknown	26.842											+					
36	Unknown	22.644						+	+				+					
37	Unknown	13.433								+							+	
	Total		5	13	11	10	1	21	10	7	0	19	15	2	6	5	9	1

Table :6 The compounds and their retention time that recorded by HPLC for *Cephalaria syriaca* shoot allelopathic active partitions

#	Standar compound	Retention Time (minute)	Shoot Ethyl acetate Fractions												Shoot Butanol Fractions											
			F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
1	Gallic acid	17.38																					+	+		
2	3,4-dihydroxy benzoic acid	19.342									+	+	+										+			
3	p-hydroxy benzoic acid	24.825			+																					+
4	Xanthotoxine	87.997									+	+	+													+
5	Chlorocatechol	93.437																								
6	Unknown	101.613				+	+	+							+											
7	Unknown	103.536													+											
8	Unknown	105.893						+																		
9	Unknown	108.901							+																	
10	Unknown	98.914					+																			
11	Unknown	94.698					+	+	+	+																
12	Unknown	91.906																					+	+		
13	Unknown	22.644																						+	+	

2) P-hydroxy benzoic acid: found in *C. syriaca* seed EtOAc and BuOH partitions, and root BuOH partitions. it is one of the phenolic derivatives of benzoic acids and has been classified as the most common phenolic acid that possesses an important allelopathic agent, causing reduction of growth in *Lactuca sativa*, *Amaranthus retrofelxus*, *S*olanum nigrum, *Cirsium* sp, and *Rumex crispus*. Also it causes inhibition of radical growth of wheat, establishing the autotoxicity in annual bluegrass and buffalo grass due to it is prevailing in such weeds and crop plants. (Moreiras-sanchez *et al*., 2004, Macias *et al*., 2007, Harborne, 1980).

3) 3,4-dihydroxy benzoic acid (protocatechuic acid): it is one of the best known allelopathic agents, where it causes inhibition of photosynthesis process with the major effect on chlorophyll a as being illustrated in rice plants (Einhellig, 2004, Vokou *et al*., 2006, Skulman *et al*., 2004).

4) Vanillic acid (4-hydroxy-3-methoxybenzoic acid): found in the *C. syriaca* shoot extracts within its DCM and EtOAc partitions. Also it exists in roots EtOAc partition. It is a benzoic acid derivatives used as a flavoring agent. Also it was reported that it causes the inhibition of shoot and root growth in wheat, inhibition of root growth, autotoxicity in annual bluegrass and rice, as well as inhibition of seed germination and the reduction of aerial and root parts in lettuce plants. (Einhelling, 2004, Ramanathan *et al*., 2006, Zhou and Yu, 2006).

5) Syringic acid (4-hydroxy-3,5-Dimethoxybenzoic acid): found in seed and shoot extracts, seeds hexane and DCM partitions, shoots and roots DCM and EtOAc partitions, Syringic acid is correlated with high antioxidant activity. It was reported that it causes autotoxicity of rice plants with the reduction of root and shoot growth (Al saadawi *et al*., 1998, Al-Mezori *et al*., 1999, Zeng, 2008, Einhelling, 2004).

6) Sinapic acid (4-Hydroxy-3,5-dimethoxycinnamic acid): found in seeds DCM and EtOAc partitions. It is identified as an allelopathic potential agent. This compound rarely occurs free but rather occurs as glycosides or as asters.

7) Phluroglucinol (1,3,5-trihydroxybenzene): found in the shoots DCM partition and in roots DCM and EtOAc partitions. It is an organic compound used in the synthesis of pharmaceuticals and explosives (Rizvi and Rizvi, 1992). Besides it causes the reduction of shoot and root growth of radish, mustard, wheat, and pea plants when used as test plants (Bohm, 1998).

8) Chlorogenic acid (1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid): existed only in shoot part extracts. It was reported that leachates from *pluchea lanceolata* contains chlorogenic acid, affects the seedling growth of *Brassica juncea* (Inderjit and Dakshini, 1996), also it shows high phytotoxic effect on the aquatic weed plants when used at concentrations of 50ppm (Ramanathan *et al*., 2006, Chou, 1998).

9) Xanthotoxine (8-methoxy psoralen): found in shoot and seed partitions of DCM, EtOAc, and BuOH. While for root parts it was found in DCM partition only. It is reported that the celery plant, infected with the fungi *Sclerotinia sclerotiorum*, stimulated the production of xanthotoxins by the fungi (Wu *et al*., 1972, Sajjadi and Noroozi, 2007). It is being documented that it causes the lowering of oxygen uptake by plant roots (Kupidlowska, 1994).

10) Chlorocatechol: it was found in the seed extracts, seed butanol partition, shoots, roots DCM and EtOAc partitions. Unfortunately no sufficient information is available on such biochemicals. It is one of the plants phenolic compounds which might derive from catechol through annexation of one of the ionic chlore.

Conclusion

the phytotoxicity of *Cephalaria syriaca* plant parts was associated with the allelochemicals existed and caused germination inhibition which was due to the biological active allelopathic compounds released from *C. syriaca* plant parts during the study, could be utilized in controlling weeds in plant nurseries or within orchard lines.

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