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Autotoxicity of alfalfa flower extract and its allelopathy to *Echinochloa crus-galli*

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알팔파 꽃 추출물의 Autotoxicity와 돌피에 대한 Allelopathy

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ABSTRACT : The aim of this study was to separate or purify some bioactive compounds from flowers of alfalfa(*Medicago sativa* L.) and to test of the isolated compounds on alfalfa for their autotoxicity and on *Echinochloa crus-galli* for their allelopathy for seed germination and seedling weight. Using thin layer chromatography(TLC) of $CHCl_3$ extracts, the most inhibitory band to alfalfa seed germination was determined. Germination inhibition of this extract suggested a complex chemical interaction. Separation and purification of compounds with $CHCl_3$ extract of fresh alfalfa flowers were conducted by a silica gel TLC, and microcrystalline cellulose TLC (MCTLC), followed by droplet countercurrent chromatography(DCCC) bioassay. Preliminary identification was done by high perfomance liquid chromatography(HPLC) on the most inhibitory fractions in DCCC. Ferulic acid, caffeic acid, vanillic acid, rutin, narringin were identified in fraction 5 and ferulic acid, caffeic acid, vanillic acid, rutin, coumarin in fraction 6. The phytotoxicity of their individual compound was tested on alfalfa and *Echinochloa crus-galli* seed germination and seedling weight. Coumarin and ferulic acid showed the most inhibitory effect on alfalfa seed germination and *Echinochloa crus-galli* seedling fresh and dry weight. These compounds may be, at least in part, involved in autotoxicity and allelopathy.

Key words : Allelopathy, autotoxicity, flower extracts, alfalfa, *Echinochloa crus-galli*, DCCC, HPLC.

INTRODUCTION

Alfalfa(*Medicago sativa* L.) as a perennial legume forage crop has been reported to exhibit both autotoxicity[4,5,9,11,13] and allelopathy[6,8,12,15,16].

An understanding of the exact chemical nature of the compounds causing low germination and poor establishment is necessary for resolution of the complicated dynamics of alfalfa allelopathy and autotoxicity. A knowledge of the chemical nature and quantity of bioactive compounds that are related to germination and seedling growth may allow

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utilization of the compounds alfalfa extracts or alfalfa residues for weed control.

Miller[15,16] reported that extracts of alfalfa contained water soluble substances which were found in the seed coat, fresh alfalfa leaves, stems and crowns, plus in dry hay, old roots and soil residues. Chung and Miller[5] also reported that based on radicle length growth, the degree of toxicity of different alfalfa plant parts can be classified in order of decreasing inhibition as follows: leaf, seed, complete plant mixture, root, soil, flower and stem.

Identification of water-soluble allelopathic and autotoxic compounds in alfalfa has been attempted. However, few investigations have identified specific compounds responsible for autotoxic effects. Saponins, although implicated in toxicity, have been dismissed as autotoxicity agents in alfalfa[14]. Caffeic, chlorogenic, isochlorogenic, *p*-coumaric, *p*-hydroxybenzoic and ferulic acids were reported as being released from alfalfa root exudates and residues[1]. Hall and Henderlong[9] reported that alfalfa contained autotoxic compounds that were characterized as phenolic compounds. Waller et al.,[19] showed that growth and development of barnyardgrass (*Echinochloa crus-galli* L.), cheatgrass (*Bromus secalinus* L.), pigweed(*Amaranthus* spp.), dandelion(*Taraxacum officinale* L.) and coffeeweed(*Sesbania exaltata* L.) were inhibited by alfalfa root saponin. Also, Dornbos et al., identified medicarpin, sativan, 4-methoxymedicarpin and 5-methoxysativan in alfalfa leaves[8]. Among these compounds, medicarpin was found to be at least partially responsible for alfalfa autotoxicity and allelopathy by reducing their germination by 59% after 6h when exogenously used in a filter paper bioassay[17]. More recently, Chung[3] and Chung and Kim[4] detected chlorogenic acid, salicylic acid, scopoletin, rutin and quercetin from alfalfa leaves extracts.

No information is available on what kinds of autotoxic or allelopathic substances exist in alfalfa flower extracts. The objective of this study was to separate or purify compounds that might be responsible for alfalfa allelopathy and autotoxicity from fresh alfalfa flower extracts and determine their phytotoxicity on alfalfa and *Echinochloa crus-galli* seed germination and seedling weight.

MATERIALS AND METHODS

1. Extraction Procedure

The procedure previously described by Chung and Kim[4] was applied with some modifications. Fresh alfalfa(WL-320) flowers (200g) were extracted with aqueous 80% MeOH in a Waring blender for 10 min. The extracts were filtered through four layers of cheesecloth and then through filter paper (Whatman No. 1). The extracts were then concentrated under vacuum at 30℃ until most of the MeOH had been removed. The resultant aqueous solution was washed with $CHCl_3$ to extract nonpolar compounds by shaking in a separatory funnel. This procedure was repeated three times and the extracts were combined. The $CHCl_3$ extracts were then concentrated by evaporation under vacuum on a rotary evaporator and freeze dried. In result, solid dried materials(15g) were obtained from fresh alfalfa flowers.

2. General Bioassay Test Procedures

Bioassays using alfalfa seeds on the substances separation procedures were applied

by the Chung and Kim[4] method. Biological activity test on the identified compounds was evaluated with alfalfa and *Echinochloa crus-galli* seed. Pioneer-5472(5g) and *Echinochloa crus-galli* seed(3g) were surface sterilized with a solution of water and Clorox(90:10) for 25min, and rinsed several times with distilled water. One hundred alfalfa and *Echinochloa crus-galli* seeds were evenly placed on filter paper(Whatman No. 1) in sterilized 9cm petri dishes, and 10ml of the test solution was added to each petri dish which was placed in a 24℃ illuminated room. Seed germination by silica gel TLC plate and microcrystalline cellulose TLC plate, Droplet countercurrent chromatography(DCCC) fractions and identified compounds was counted as radicle protrusion through the seed coat at 96h at 24h intervals. Seedling weight on germinated seedling was recorded by drying at 60℃ for 8h at the 5th day after seeding.

Each treatment was replicated 4 times with completely randomized design and bioassays were repeated twice. Analysis of variance for the data was carried out by general linear model procedure of the SAS program[18]. Means were separated on the basis of least significant difference(LSD) at 0.05 probability level.

3. Separation and Purification Procedure

1) TLC(thin layer chromatography) Bioassay

Four silica gel TLC plates(Whatman Silica Gel 60A, 20 x 20cm) with 0.25mm thin silica gel were used for preliminary separation of the substances that might be germination inhibitions found in the $CHCl_3$ extract. One-hundred milliliter solvent(50 : 50, MeOH : double distilled H_2O) was prepared and poured into the two TLC tank for chamber saturation. TLC plates were equilibrated at 110℃ for 1h immediately before the sample was run. Based on an initial run and visualization, the samples(1.5g) which were dissolved in distilled water(10ml) were spotted several times on preparative silica gel TLC plates and the plate was developed in the chamber under a hood. When the solvent-front moved to the top of the plate, it was removed from the chamber and allowed to airdry in the hood for 25min. After visualizing under long wave UV light(366nm), each chromatographic plate was divided into 2cm bands, the bands were scraped off the plate from bottom to top, and desorbed with 50% MeOH. The resulting mixtures were filtered through quantitative filter paper(Whatman No. 42), and evaporated under vacuum. The material from each band was resuspended with acetone and methanol. The solvents were evaporated under nitrogen and the residual material dried on the freeze drier to remove traces of water. The remaining solids from each band were weighed and then dissolved in 50% methanol(10ml). The recovered sample weight in the bands 3 and 4 from the spotted original sample was 0.47g and 0.33g, respectively. The solution from each band was divided into two parts(5ml each) for the next procedure. Each band was used for bioassay to determine the presence of bioactive compounds, whereas the remaining sample was refrozen and stored in a freezer(−15℃).

2) Microcrystalline Cellulose Thin Layer Chromatography(MCTLC) Bioassay on TLC Bands

Two dimensional four MCTLC plates(0.1mm) were applied for more pure separation of compounds from the $CHCl_3$ extracts of alfalfa flowers. Half sample(0.4g) of combined

bands which were scratched from major active bands 3 and 4 exhibited high inhibition of seed germination and seedling dry weight from silica gel TLC were spotted on MCTLC after the plates were in aceton to remove contaminants. The plates were developed with the solvent *t*-butanol : acetic acid : water(TBA, 3 : 1 : 1) in the first dimension and acetic acid(15%) in the second dimension in the TLC tank. After development, the lanes on the chromatogram were divided into zones, usually corresponding to the spots seen under long wave UV light(366nm) and each lane which was scraped from the plate into a Pyrex centrifuge tube. The tubes were vortexed vigorously to suspend in the 50% MeOH, and were allowed to stand overnight in a refrigerator(5℃). The next following procedure was the same as the above silica gel thick layer chromatography(TLC) bioassay.

3) Droplet Countercurrent Chromatography (DCCC) Bioassay on MCTLC Bands

More purification was conducted by MCTLC plates. Purification of major active bands 8 and 9 obtained from MCTLC was done by DCCC carried out on an Eyela D. C. C. −300(Tokyo Rikakikai Co., Ltd., Tokyo, Japan) with 300 glass columns(2mm i.d.) with some modifications(Chung and Kim, 1994). Bands 8(86mg) and 9(47mg) were combined and applied to a DCCC after being dissolved in methanol and acetone(1 : 1). The solvent used was chloroform : methanol : water(5 : 6 : 4). The lower phase was used as the stationary phase and the system was run in ascending mode. The sample (usually 5ml) was evaporated to dryness, dissolved in about 5ml of the mobile phase, and loaded into the 20ml sample loop. Separation was carried out at room temperature with a rate of approximately 7ml/hr. Fractions of the eluant(400 drops, or 10ml, per fraction) were collected by a Gilson FC-100 fraction collector. After 20 fractions had been collected, the system was changed to descending mode. Methanol was pumped through the columns to displace the stationary phase and 20 fractions were collected(400 drops per fraction). Each fraction was concentrated under vacuum, and redissolved in methanol and water(1 : 1). The eluants with water and methanol were evaporated under nitrogen and further dried on a freeze drier. The solid materials obtained were redissolved in distilled water(10ml). All fractions were divided into parts of 5ml. One fraction was used for bioassay to determine allelopathic activity, whereas the remaining sample was refrozen and stored in a freezer at −15℃ for the next procedure.

4) High Performance Liquid Chromatography (HPLC) Analysis on DCCC Fractions

HPLC analysis to detect the presence of bioactive compounds in alfalfa flower extracts was conducted on the ascending mode 5 and 6 fractions which were collected from DCCC. Analyses of extracts and standard compounds were based on the method of Chung and Kim[4] and Banwart et al[2]. The HPLC system was employed a Beckman system with two 110A solvent metering pumps, a Beckman 25cm C_{18} Ultrasphere ODS column, and a Micromeritics 725 autoinjector with a 20㎕ sample loop. The mobile phase (solvents A and B) consisted of 98% water and 2% glacial acetic acid in 0.018M ammonium acetate, and 68% water, 25% methanol, 5% butanol and 2% glacial acetic acid in 0.018M ammonium acetate, respectively.

Both extracts and standard compounds were analyzed by the following gradient system : (a) 0.0-1.0min isocratic at 10% b ; (b) 1.0-21.0min linear gradient from 10 to 25% b ; (c) 21.0-36.0min linear gradient from 25 to 45% b ; (d) 36.0-56.0min linear gradient from 45 to 100% b ; (e) 50.0-50.15min flow increased to 1.20ml/min : (f) 82.0-82.15min linear gradient from 100 to 10% b ; (g) 92.0-92.15min flow drcreased to 1.00ml/min ; (h) at 99.0 min sample loop rinsed and gradient repeated. The UV detector was set to 254nm. Standard compounds were chromatographed alone and as mixtures. Retention times for the standard compounds and the major peaks in the extract were recorded. All chemicals used for HPLC analysis were purchased as high purity standards. These compounds were dissolved in dimethylsulfoside(DMSO) and stored in a freezer at −15℃. Solvents were HPLC spectral grade, and distilled water was deionized before use. All solvent ratios were based on a volume basis.

4. Biological Activity Test of Identified Compounds

The biological activity of suspected bioactive materials was evaluated with alfalfa and *Echinochloa crus-galli* seed. Analytical grade caffeic acid, ferulic acid, vanillic acid, rutin, narringin and coumarin were examined since these compounds had a similar retention time to one of the peaks of the purified extract. The solutions were prepared by dissolving a weighed sample of standard chemicals in distilled water and diluting the solution with distilled water to a final concentration of 10^{-3}M. The solutions were stored at 5℃.

RESULTS

1. Bioassy on Separation and Purification Procedures

As compared to the control(96%) and other bands, 3 and 4 bands of silica gel TLC

Table 1. The effect of various bands scratched from silica gel TLC plates on alfalfa germination.

Band number	Range(cm)	RSW[1]	Germination percentage(%)					TDW(mg)[4]
			24	48	72	96/h[2]	TG[3]	
Band 1(Top)	18.5~16.5	0.10	63.0	24.5	3.5	1.5	92.5	1.32
Band 2(2nd from top)	16.5~14.5	0.14	37.5	39.4	4.5	4.1	85.5	1.34
Band 3(3th from top)	14.5~12.5	0.47	5.1	18.2	29.0	3.2	55.4	0.93
Band 4(4th from top)	2.5~10.5	0.33	14.5	43.0	7.2	3.0	68.2	0.97
Band 5(5th from top)	10.5~ 8.5	0.12	24.0	46.5	21.0	2.5	93.5	1.47
Band 6(6th from top)	8.5~ 6.5	0.08	21.4	52.0	9.5	2.5	85.4	1.34
Band 7(7th from top)	6.5~ 4.5	0.01	19.5	59.0	7.0	3.5	89.5	1.40
Band 8(8th from top)	4.5~ 2.5	0.01	52.5	30.3	2.5	2.6	88.4	1.34
Band 9(9th from top)	2.5~ 0.5	0.01	48.0	30.5	3.0	1.5	83.0	1.37
Band 10(Original spot)	0.5~ 0.0	0.01	36.0	49.1	5.4	2.6	93.1	1.48
Control			87.7	2.5	2.5	4.5	97.2	1.69
CV(%)			3.1	3.3	10.1	21.4	4.2	2.4
LSD(0.05)			2.5	2.6	1.9	1.3	7.9	0.07

1, RSW: recovered sample weight (g), 2, h: hour, 3, TG: total germination percentage, 4, TDW: total dry weight per seedling.

Table 2. The effect on alfalfa germination of compounds desorbed from various bands scratched from microcrystalline cellulose TLC plates.

Band number	Range(cm)	RSW[1]	Germination percentage(%)					TDW(mg)[4]
			24	48	72	96/h[2]	TG[3]	
Band 1(Top)	18.5-16.5	0.024	82.0	12.0	2.5	2.2	98.7	1.60
Band 2(2nd from top)	16.5-14.5	0.022	69.5	22.6	1.5	3.5	97.1	1.56
Band 3(3th from top)	14.5-12.5	0.023	72.5	17.0	3.5	2.4	95.4	1.65
Band 4(4th from top)	12.5-10.5	0.019	68.5	16.3	2.5	2.0	89.3	1.60
Band 5(5th from top)	10.5- 8.5	0.020	63.0	22.5	4.5	2.5	92.5	1.56
Band 6(6th from top)	8.5- 6.5	0.021	45.5	35.5	5.5	3.0	89.5	1.56
Band 7(7th from top)	6.5- 4.5	0.020	48.0	30.5	8.5	2.5	89.5	1.58
Band 8(8th from top)	4.5- 2.5	0.086	0.5	7.8	8.5	2.5	19.3	0.99
Band 9(9th from top)	2.5- 0.5	0.047	0.5	13.0	22.4	3.5	39.4	1.05
Band 10(Original spot)	0.5- 0.0	0.021	63.5	22.5	8.0	2.5	96.5	1.65
Control			87.5	3.0	2.5	4.5	97.5	1.69
CV(%)			2.1	5.5	13.9	29.8	4.0	0.7
LSD(0.05)			2.5	2.2	1.9	1.8	7.2	0.02

1, RSW: recovered sample weight(g), 2, h: hour, 3, TG: total germination percentage, 4, TDW: total dry weight per seedling.

Table 3. Bioassay on DCCC ascending mode 5ml aliquots of $CHCl_3$ extracts.

Fraction numbers	Germination percentage(%)				
	24	48	72	96	TG*
Ascending mode					
1	86.0	7.0	0.5	1.5	95.0
2	66.5	16.5	2.5	2.0	89.5
3	46.5	37.5	3.5	2.0	89.5
4	62.0	20.5	3.0	1.5	87.0
5	7.5	14.5	17.5	14.0	53.5
6	2.5	5.5	4.5	6.5	19.0
7	57.0	14.5	3.5	1.0	76.0
8	51.5	26.5	3.5	1.5	87.0
9	72.0	19.5	3.5	2.0	97.0
10	64.5	26.5	3.5	1.5	96.0
11	54.0	29.5	3.5	0.5	87.5
12	79.0	14.0	1.5	0.5	95.0
13	73.5	17.5	3.0	0.5	94.5
14	75.5	16.5	3.5	0.5	96.0
15	75.0	16.0	2.5	0.5	95.0
16	68.5	22.0	2.0	1.5	94.0
17	74.0	18.0	3.0	0.5	95.5
18	74.0	14.5	1.5	0.5	90.5
19	75.0	16.5	1.5	1.5	94.5
20	70.5	19.5	1.5	1.5	93.0
Control	87.0	3.0	2.0	4.0	96.0
CV(%)	5.7	9.7	22.4	42.1	3.8
LSD(0.05)	7.4	3.6	1.6	1.9	6.7

*TG: total germination percentage

Table 4. Bioassay on DCCC descending mode 5ml aliquots of $CHCl_3$ extracts.

Fraction numbers	Germination percentage(%)				
	24	48	72	96	TG*
Descending mode					
1	73.0	21.5	1.5	1.0	97.0
2	64.0	21.0	1.5	0.5	87.0
3	51.5	34.5	3.5	1.5	91.0
4	84.5	3.5	0.5	0.5	89.0
5	71.5	16.5	3.5	0.5	91.5
6	81.5	11.0	2.5	1.5	96.5
7	75.0	15.5	2.5	1.5	94.5
8	76.0	14.5	4.0	2.5	97.0
9	51.0	35.0	2.5	1.5	90.0
10	57.5	33.5	2.5	0.5	94.0
11	58.0	33.5	1.5	1.0	94.0
12	67.0	24.5	1.5	0.5	93.5
13	78.0	12.5	2.5	1.0	94.0
14	70.5	23.5	1.5	1.5	97.0
15	69.0	21.5	1.5	1.0	93.0
16	67.0	25.5	2.5	1.5	96.5
17	61.5	31.5	1.5	1.5	96.0
18	69.5	21.5	2.5	1.5	95.5
19	60.5	24.0	1.5	1.5	87.5
20	79.5	14.0	2.5	1.0	95.0
Control	87.0	3.0	2.0	4.0	96.0
CV(%)	5.4	6.2	34.2	90.5	2.1
LSD(0.05)	7.8	2.7	1.5	2.5	4.0

*TG: total germination percentage

showed 55%, 68% germination of alfalfa, respectitively(Table 1). Also, 3 band 4 exhibited 0.93 and 0.97mg lowest seedling dry weight. In two dimensional MCTLC, bands 8 and 9 exhibited most inhibitory to alfalfa seed germination percentage(19% and 39%) and seedling dry weight(0.99 and 1.05mg) (Table 2). The results of bioassays on DCCC are presented in Tables 3 and 4.Fractions 5 and 6 from ascending mode were most inhibitory to alfalfa germination(Table 3). Total germination percentage on 5 and 6 band was 54% and 19%, respectitively. All fractions in descending mode showed no inhibitory effect to alfalfa germination(Table 4).

2. HPLC Analysis

The chromatogram of compounds from bands 5 and 6 are presented in Figures 1, 2 and Table 5. HPLC analysis showed the presence a large number of unknown peaks and much overlapping(Figures 1 and 2). The presence of chemicals with similar retention time which was very close such as caffeic acid, ferulic acid, vanillic acid, rutin was detected in both fractions(Table 5). However, coumarin and narringin were detected in 5 and 6 fractions, respectitively. Comparing the retention time of the sample peaks to those of the standards, it was determined that caffeic acid, ferulic acid, vanillic acid,

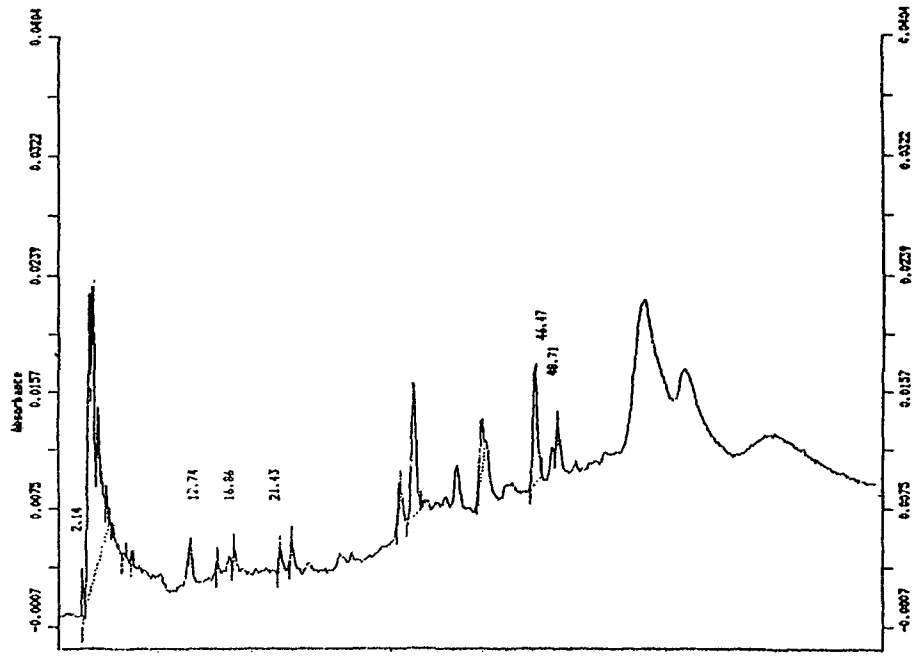

Fig. 1. Chromatogram of fraction 5 from droplet countercurrent chromatography by high performance liquid chromatography.

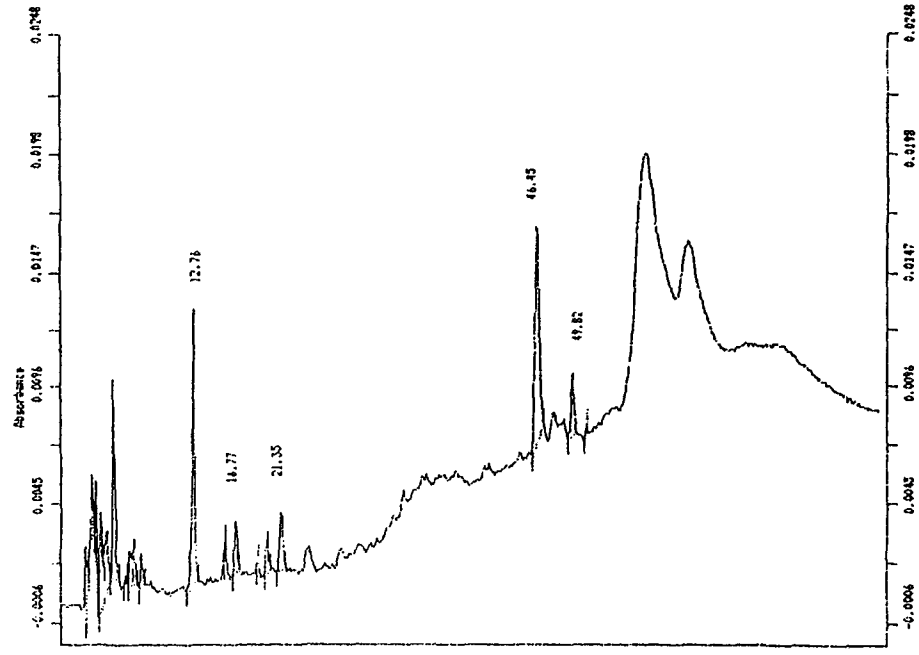

Fig. 2. Chromatogram of fraction 6 from droplet countercurrent chromatography by high performance liquid chromatography.

Table 5. Retention times for selected standards and peaks area by HPLC analysis of 5 and 6 fractions from DCC chromatography.

Chemicals	Fraction 5		Fraction 6	
	Retention time(min)	Peak area(%)	Retention time(min)	Peak area(%)
Ferulic acid	12.74	1.11	12.76	14.70
Caffeic acid	16.86	0.26	16.77	3.64
Vanillic acid	21.43	0.21	21.35	4.85
Rutin	46.47	12.08	46.45	23.05
Narringin	48.71	0.30	X	X
Coumarin	X	X	49.82	4.72
		13.88		50.96

Table 6. Biological activity test on alfalfa seed using identified compounds at 10^{-3}M concentration.

Chemicals(10^{-3}M)	Germination percentage	Fresh weight	Dry weight
	(%)	(mg)	(mg)
Ferulic acid	67.3	20.0	1.38
Caffeic Acid	73.3	22.0	1.56
Vanillic Acid	88.0	29.0	1.77
Rutin	92.0	32.3	1.88
Narringin	86.0	24.3	1.47
Coumarin	59.3	15.0	1.23
Control	96.0	36.3	1.93
CV(%)	7.9	8.1	2.90
LSD(0.05)	11.1	3.3	0.15

Table 7. Biological activity test on alfalfa seed using identified compounds at 10^{-3}M concentration.

Chemicals(10^{-3}M)	Germination percentage	Fresh weight	Dry weight
	(%)	(mg)	(mg)
Ferulic acid	54.7	12.9	0.16
Caffeic acid	58.7	16.1	0.18
Vanillic acid	69.3	16.5	0.19
Rutin	72.0	17.1	0.19
Narringin	64.7	15.4	0.18
Coumarin	45.3	9.0	0.15
Control	74.0	18.6	0.19
CV(%)	7.9	4.8	4.9
LSD(0.05)	8.7	1.3	0.02

rutin, narringin and coumarin standards closely matched peaks in fractions.

Biological Activity Test of Identified Compounds

Alfalfa and *Echinochloa crus-galli* seed germination percentage, seedling weight as inhibited by identified compound are presented in Tables 6 and 7. The most significant inhibition of seed germination, seedling fresh and

dry weight on alfalfa and *Echinochloa crus-galli* was caused by coumarin and ferulic acid treatments.

DISCUSSION

This study was attempted to separate and purify bioactive compounds from fresh alfalfa flowers that are responsible for allelopathic or autotoxic effect of alfalfa plant.

Allelopathic and autotoxic activity was identified by seed germination, seedling growth and weight. Seed germination and seedling weight were used as the key parameter to separate and purify bioactive compounds in this study. Seedling growth has been reported to be more sensitive to toxic substances than seed germination[4].

After preliminary separation using data from silica gel TLC, MCTLC, DCCC, and HPLC analysis of the two extracts separated by DCCC indicated that they had different chemical composition(Tables 1, 2, 3, 4, 5 and figures 1, 2). Although HPLC chromatogram on 5 and 6 fractions showed differently, six peaks in two fractions had similar HPLC retention times to standard caffeic acid, ferulic acid, vanillic acid, rutin, narringin and coumarin. These results which were showing different chemical composition on fraction 5 and 6 chromatogram were supported by different DCCC bioassay result.

Flavonoids are reportedly involved in alfalfa autotoxicity and allelopathy[1,3,4,8,17]. However, peaks corresponding to caffeic acid, ferulic acid, vanillic acid, rutin, narringin and coumarin on HPLC analysis, remained after fractionation. This suggests that the compounds in these extracts are fairly polar. Therefore, coumarin may, at least partially, contribute to alfalfa autotoxicity and allelopathy. Ferulic acid and coumarin were shown by bioassay to be phytotoxic to alfalfa and *Echinochloa crus-galli* seed germination(Tables 6 and 7). Among detected compounds coumarin was found to be biologically the most active compound in this study. Coumarin has been reported as being strong autotoxic agents[10]. The inhibition of seed germination and seedling weight on *Echinochloa crus-galli* suggest the possibility that alfalfa may act as a strong natural resource to some monocotyledonous weed species. The results of this study support previous conclusions by researchers that alfalfa may have potential as an allelopathic weed control[1,7]. This data suggest that allelopathic or autotoxic effect of alfalfa on alfalfa and *Echinochloa crus-galli* may be due to the presence of these substances.

After collection of bands from TLC, purification of phytotoxic compounds through microcrystalline cellulose analytical TLC plate, DCCC and HPLC analysis were useful to determine which toxic compounds were present in the extracts that inhibited seed germination and seeding growth. Unfortunately, HPLC analysis showed many unknown and overlapping peaks in both fractions (Figures 1 and 2). These unknown peaks should be further studied and biological activity of the major peaks has to be tested. Confirmation and identification of individually separated compounds is needed through liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) analysis.

Studies on the potential phytotoxicity of detected compounds on weed control as a natural herbicide are also needed to evaluate against weed species in the field and identify

specific inhibitory substances. In conclusion, the allelopathic and autotoxic substances detected in alfalfa flower extracts have the potential to reduce the productivity of established fields.

적 요

본 실험은 알파파 꽃 추출물로부터 Allelopathy와 Autotoxicity에 관련되는 활성물질을 분리, 동정하기 위하여 실시하였다. 알팔파 꽃 200g을 80% MeOH로 추출하여 $CHCl_3$층을 silica gel thin layer chromatography (TLC), microcrystalline cellulose thin layer chromatography (MCTLC)와 droplet countercurrent chromatography(DCCC)의 분획을 이용하여 생물검정을 행하고 DCCC의 생물검정에서 가장 억제력을 보였던 분획 5와 6은 HPLC로 활성물질을 동정하였다. HPLC분석 결과 분획 5에서는 ferulic acid, caffeic acid, vanillic acid, rutin, narringin 이, 분획 6에서는 ferulic acid, caffeic acid, vanillic acid, rutin, coumarin이 동정되었다. 이들 동정된 물질을 이용한 알팔파와 돌피에 대한 발아실험에서도 모두 알팔파와 돌피의 발아와 생육에 억제적으로 작용하였으며, 이중 coumarin의 처리가 가장 억제적이었다. 따라서 이들 물질이 알팔파와 돌피의 autotoxicity와 allelopathy에 관련하는 것으로 생각되었다.

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