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SEPARATION, IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM ALFALFA PLANT

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알팔파의 생리활성물질 분리 및 동정

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Abstract : To isolate, purity and identify of bioactive compounds involved in alfalfa allelopathy and/or autotoxicity, experiment was conducted. Isolation and separation procedures used from an 80% methanol extract of fresh alfalfa leaves(1kg), silica gel thin layer chromatography(TLC), followed by Droplet Counter Current Chromatography (DCCC). Preliminary identification was examined by high preformance lipid chromatography(HPLC). Four phenolic compounds, salicylic acid, scopoletin, rutin, and quercetin, were identified and identified all compounds were phytotoxic to alfalfa seed germination and seedling growth. Among these compounds, quercetin treatment(10^{-3}M) was most inhibitory to alfalfa seed germination and seedling growth. These compounds may be, at least in part, involved autotoxicity and allelopathy.

요약 : 본 실험은 알팔파의 Allelopathy와 Autotoxicity에 관련되는 생리활성 물질을 분리, 정제, 동정하기 위하여 실시되었다. 알팔파 생잎 1kg을 80% MeOH로 추출하여 silica gel TLC와 DCCC의 분리와 정제과정을 거쳐 HPLC로 동정하였다. HLPC 분석결과 4개의 phenolic 물질(salicylic acid, rutin, quercetin, scopoletin) 등이 동정되었다. 이들 물질을 이용한 발아실험에서도 모두 알팔파의 발아와 생육에 억제적으로 작용하였으며, 이 중 quercetin의 처리가 가장 억제적이었다. 따라서 최소한 이들 물질이 알팔파의 Allelopathy와 Autotoxicity에 관련하는 것으로 생각되었다.

Key words : Bioactive compounds, Autotoxicity, Allelopathy, Droplet counter current chromatography(DCCC)

INTRODUCTION

Alfalfa has been reported to exhibit both autotoxicity[1~4] and allelopathy[5~9]. Alfalfa aqueous extract inhibited alfalfa seed germination, seedling growth and some weeds like broad leaf species germination in a concentration-dependent manner. These autotoxic or allelopathic principles utilization may serve as natural herbicides by selectively inhibiting germination and seedling growth of cer-

tain weed species, but 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 nature and quantity of bioactive compounds that are related to germination and seedling growth may allow utilization of alfalfa extracts for weed control.

Secondary plant metabolites and their degradation products are important in all agroecosystems. The following groups of compounds, among others, can be classified as resulting in toxic effects : terpenoids and steroids, phenolics and their derivatives, coumarins, flavonoids, tannins, alkaloids, and cyanogenic glycosides[10]. Phenolic compounds are most studied with regard to their phytotoxicity[11].

Chemical analysis of the leaf extract of *Prosopis juliflora* Swartz and *P. cineraria* (L) Druce indicated that the allelopathic compounds in both the tree species are phenolic in nature[12]. Also 13 phenolic compounds were identified in the leaf extract of *Lantana camara* L. and most of these compounds are reported to be phytotoxic to ryegrass(*Lolium multiflorum* L.) seedling[13]. Lactic, succinic, malic, citric, shikimic, glyceric, fumaric, and quinic acids were identified as possible allelopathic compounds in tall fescue (*Festuca arundinacea Schreb.*)[14].

In alfalfa case, identification of water-soluble allelopathic and autotoxic compounds 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[15]. Caffeic, chlorogenic, *iso*-chlorogenic, *p*-coumaric, *p*-hydroxybenzoic and ferulic acids were reported as being released from alfalfa root exudates and plant residues[16]. Hall and Henderlong[4] reported that alfalfa contains autotoxic compounds that were characterized as phenolic compounds. Waller et al.,[17] showed that growth and development of barnyardgrass, cheatgrass pigweed, dandelion and coffeeweed were inhibited by alfalfa root saponin. Recently, Dornbos et al.,[9] identified medicarpin to be at least partially responsible for alfalfa autotoxicity and allelopathy.

The objective of this study was to characterize compounds that might be responsible for alfalfa allelopathy and autotoxicity from fresh alfalfa foliage and determine their phytotoxicity on alfalfa germination and seedlings.

MATERIALS AND METHODS

Extraction Procedure

The extraction procedure described[13] was applied with some modification(*Figure* 1). Fresh alfalfa leaves(200g) were extracted with 80% MeOH in a Waring blender for 10 min. The MeOH extract (fraction 1) obtained was first extracted 3 times with $CHCL_3$ to remove non-polar substances like lipids and chlorophyll(fraction 2). The resulting

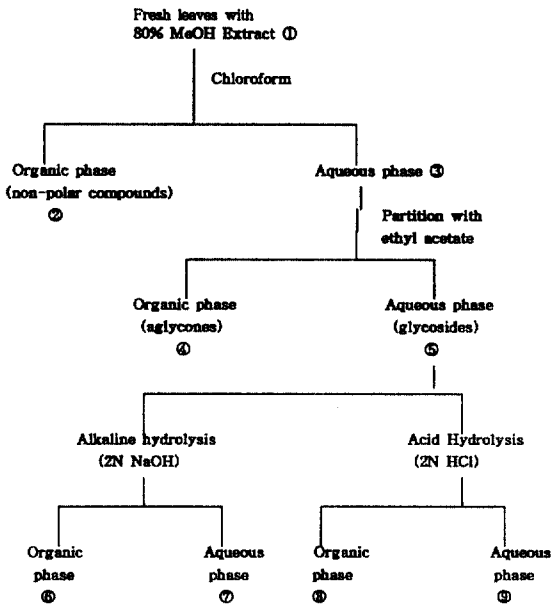

Fig. 1. Flow diagram of the procedure used for extraction and partitioning of plant materials.

aqueous solution(fraction 3) was extracted with ethyl-acetate(fraction 4). The remaining aqueous fraction(fraction 5) was subjected separately to acid and alkaline hydrolysis. Acid and alkaline hydrolysis were conducted with a 2 N HCl for 2 h at 40℃ water bath and 2 N NaOH for 2 h under nitrogen at room temperature in the dark, respectitively. The hydrolysate was extracted with diethyl ether(fraction 8) and ethyl ether(fraction 6), respectitively. Each of these fractions, including the remaining aqueous fractions from alkaline(fraction 7) and acid(fraction 9) hydrolyses, was used for bioassay at 5 and 10% and the results were presented in *Table* 1.

Table 1. Effect of various fractions obtained from aqueous extracts of fresh leaves on alfalfa.

Fraction Number	Concentration (%)	Germination Percentage(%)	TL* (cm)	TW* (mg)
Control		90.0	8.6	2.04
	5	92.5	8.4	2.00
1	10	68.0	5.7	1.62
	5	85.0	6.0	1.79
2	10	65.3	5.1	1.54
	5	64.5	4.9	1.29
3	10	50.4	3.5	1.04
	5	90.1	8.3	1.96
4	10	60.6	4.1	1.33
	5	79.5	6.0	1.58
5	10	76.0	5.6	1.50
	5	79.5	5.3	1.75
6	10	53.6	3.6	1.16
	5	65.0	4.9	1.46
7	10	54.5	3.7	1.25
	5	92.5	8.5	2.04
8	10	65.7	4.8	1.50
	5	68.5	5.5	1.54
9	10	53.5	4.7	1.33
LSD(0.05)		4.98	0.3	0.18
CV(%)		3.34	2.7	5.37

*.TL = Total Length. **.TW = Total Weight.

Purification and Identification Procedure

Since fraction 3 extract among 9 fractions was most statistically inhibitory to alfalfa germination and seedling growth in preminary phytotoxicity test, purification and identification process was conducted as following(*Talbe* 1). The fresh leaves (1kg) were extracted with 80% MeOH in Waring blender for 10 min. The extract was filtered through four layers of cheese cloth, and then through filter paper(Whatman No. 1). The extracts were then concentrated under vaccum at 30℃ to about 1/3 the original volume or until most of the methanol had been removed. The resultant aqueous solution was washed with chloroform to remove nonpolar compounds such as lipids, glycerides, terpenes, and chlorophylls by shaking in a separatory funnel. This procedure was repeated three times and the extracts combined. These chloroform washes are designated as aqueous extracts in the following discussion. The chloroform-extracted aqueous layer was then concentrated by evaporation under vaccum on a rotary evaporator and freeze dried. Finally, solid drid materials (54g) were obtained from fresh leaves (1kg).

Thin Layer Chromatography(TLC)

Solid dried material extract(3g) was dissolved in distilled(10m*l*). Dissolved samples were spotted five times on preparative silica gel TLC plates, 20cm×20cm with 250μ(Whatman Silica Gel 60A). Plates were developed methanol : water (50 : 50) and air-dried under hood. Each chromatographic plate was divided into 2cm bands after visualizing

under 366nm uv light, the bands scraped from the plate, and desorbed with 50% methanol. The resulting mixtures were filtered through quantitative filter paper (Whatman No. 42), and evaporated under vaccum. 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 (10m*l*). The solution from each band was divided into two parts(5m*l* each). One sample was used for bioassay to determine the presence of bioactive compounds, whereas the remaining sample was refrozen and stored in a freezer(−15 ℃). The bioassay test results of silica gel thin layer chromatography are presented in *Table* 2. Also, bioassay test is exhibited using two dimentional TLC(*Fig.* 2). The solvents were used as follow : first direction was TBA (butanol : reagent grade

Table 2. The effect on alfalfa germination of compounds desorbed from various bands isolated from silica gel TLC plates.

Band Number	Range (cm)	Germination (%)
Band 1(Top)	16.5~14.5	68.7
Band 2(Second from top)	14.5~12.5	75.6
Band 3(Third from top)	12.5~10.5	0
Band 4(Fourth from top)	10.5~8.5	0
Band 5(Fifth from top)	8.5~6.5	0
Band 6(Sixth from top)	6.5~4.5	87.3
Band 7(Seventh from top)	4.5~2.5	92.4
Band 8(Eighth from top)	2.5~0.5	86.7
Band 9(Original sopt)	0.5~0.0	85.4

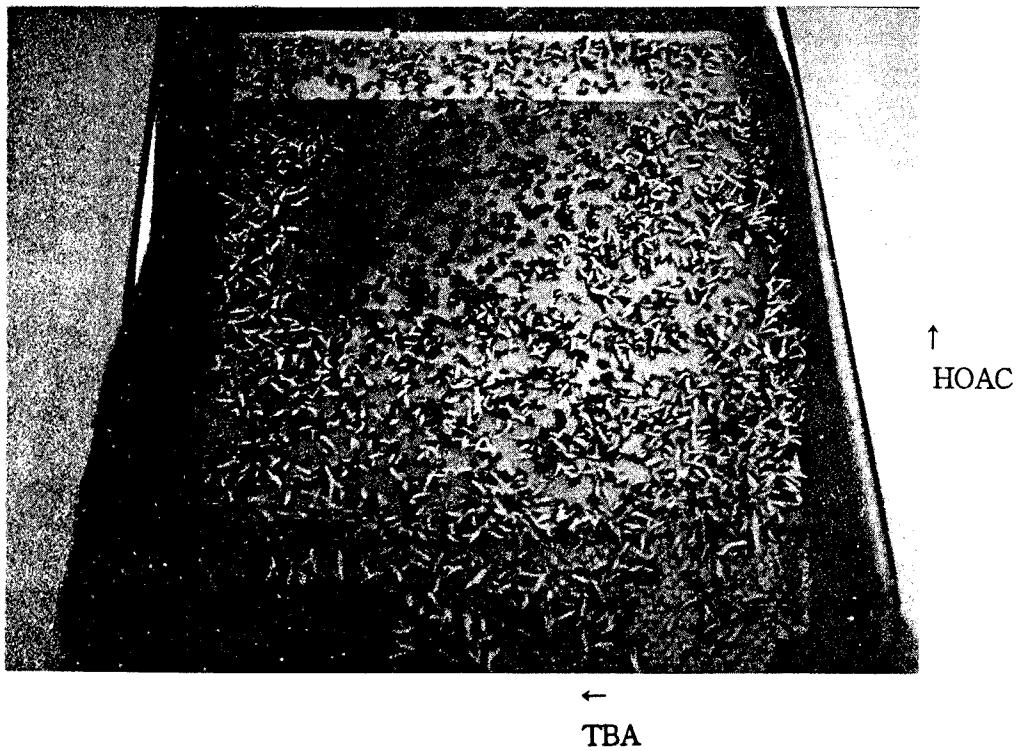

Fig. 2. The alfalfa bioassay test on a TBA/HOAC two dimentional TLC.

glacial acetic acid : water, 3 : 1 : 1) and second direction was HOAC(15m*l* reagent grade glacial acetic acid mixed with 85m*l* of H_5O).

DCCC fractionation

Purification by droplet countercurrent chromatography(DCCC) was carried out on an Eyela D.C.C. -300(Tokyo Rikakikai Co., Ltd., Tokyo, Japan) with 300 glass columns (2 mm i. d.). Since TLC plates band 3, 4, and 5 activity in the bioassay and same blue color under 366nm wavelength, the remaining sample(5m m*l*) of TLC bands were then combined and applied to a DCCC after being dissolved in methanol and aceton (1 : 1). As TLC dryness process the sample (usually 5m*l*) was evapoated to dryness, dissolved in about 5m*l* of the mobile phase, and loaded into the 20m*l* sample loop. The solvent used was chloroform : methanol : water(5 : 6 : 4). Separation was carried out at room temperature with a rate of approximately 7m*l* /hr. The lower phase was used as the stationary phase and the system was run in ascending mode. Fractions of the eluate (200 drops, or approximately 6.5m*l*, per fraction) were collected by a Gilson FC-100 fraction collector. After about 20 fractions had been collected, the system was changed to descending mode and methanol pumped through the columns to displace the stationary phase and clean the system. 6 Fractions of the stationary phase also were collected (300 drops per fraction). Each fraction was concentrated under vaccum, and redissolved in methanol and water(1 : 1). The eluants with water and methanol then were evaporated under nitrigen and further dried on a freeze drier. The solid materials obtained were redissolved in distilled water(10m*l*). Each fraction (10m*l*) was divided into two parts. One fraction was used for bioassay to determine allelopathic activity. The remaining fraction was stored in a freezer(−13℃). The results of bioassays on each fraction from DCC chromatography visualized with UV light are presented in *Table* 3. These fractions were combined for next process since fraction 10, 11, and 12 showed low seed germination and same color under visible and UV light in the ascending mode.

High Performance Liquid Chromatography (HPLC)

To augment the results of TLC and DCC chromatography and to detect the presence of unknown bioactive compounds from alfalfa fresh leaves, HPLC was employed as based on DCCC bioassays results. All chemicals like salicylic acid, rutin, scopoletin, quercetin, medicarpin and other pterocarpans used for HPLC analysis were purchased or obtained as high purity standards. Solvents were HPLC spectral grade, and distilled water was deionized before use. All solvent rations were based on a volume basis. The extracts used in HPLC analysis were collected from DCC chromatography. The HPLC system employed was a Beckman system with two 110A solvent metering pumps, a Beckman 25cm C_{18} Ultrasphere ODS column, and a Micromeritics 725 Autoinjector with a 20μl sample loop. The mobile phase (solvents A and B) consisted of 98% water and 2% glacial acetic acid in 0.018 M ammonium acetate, and 68% water, 25% methanol, 5% butanol and 2% glacial acetic acid in 0.018 M ammonium acetate, respectively. Both extracts and standard compounds were analyzed by the following gradient system : (a) 0.0~1.0 min isocratic at 10% b ; (b) 1.0~21.0 min linear gradient from 10 to 25% b ; (c) 21.0~36.0 min linear gradient from 25 to 45% b ; (d) 36.0~56.0 min linear gradient from 45 to 100% b ; (e) 50.0~50.15 min flow increased to 1.20m*l*/min ; (f) 82.0~82.15 min linear gradient from 100 to 10% b ; (g) 92.0~92.15 min flow decreased to 1.00m*l* /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.

Table 3. Bioassay and colors of DCCC fractions from 5m*l* aliquots of MeOH extracts.

Fraction Numbers	Germination percentage(%)	Visible Color	Color in UV light*
Ascending Mode			
1	87.2	–	yellow
2	92.7	–	pink
3	87.8	–	dark purple
4	90.0	orange	blue
5	84.1	–	orange
6	94.8	–	yellow
7	80.6	–	blue
8	91.7	–	white
9	96.9	–	blue
10	57.2	yellow	blue
11	55.0	yellow	blue
12	48.5	yellow	blue
13	76.2	–	yellow
14	87.0	orange	dark purple
15	97.1	–	white
16	72.2	yellow	pink
17	68.6	yellow	blue
18	97.5		green
19	91.0	–	green
20	88.2	–	blue
Descending Mode			
1	86.7	–	yellow
2	78.6	yellow	pink
3	90.2	yellow	blue
4	84.4	–	yellow
5	92.3	–	blue
6	90.5	–	blue

*366 nm wavelength

Table 4. Retention time and biological activity test of HPLC identified compounds on alfalfa germination and seedling growth.

Phenolic Compound	HPLC Retention	Germination	Total Seedling	
Common Name	Time(Min)	Percentage(%)	Lenght(cm)	Weight(mg)
Salicylic acid	14.68	74.0	5.4	1.60
Scopoletin	31.25	69.5	5.6	1.63
Rutin	46.95	82.3	5.9	1.69
Quercetin	61.84	57.3	4.9	1.42
Control		91.3	8.4	1.98
LSD(0.05)		6.8	0.2	0.2
CV(%)		5.9	2.2	7.2

*Medicarpin, Sativan, 4-Methoxymedicarpin were not detected.

Analyses of extracts and standard compounds were based on the method of Banwart et al.,[18].

Biological Activity Test of Identified Compound

The biological activity of identified compound was evaluated with alfalfa seeds (*Table* 4). Analytical grade of salicylic acid, scopoletin, rutin, quercetin was examined since these compounds had a similar retention time to one of the peaks of the purified extract. These solutions were prepared by dissolving a weighed sample of each chemicals in distilled water(5m*l*) and diluting the solution with distilled water to a final concentration of 10^{-3}M. The solutions were stored at 5℃ until used. Alfalfa seeds, Pioneer-5472(5g), were surface sterilized with a solution of water : Clorox (90 : 10) for 5 min, and rinsed with distilled water. One hundred alfalfa seeds were placed evenly in the sterilized petri dishes, and the test solution(10m*l*) added to each petri dish, and the petri dishes placed in a 24℃ lighted room. Percentage germination was defined as radicle protrusion through the seed coat and seedling growth was recorded on the 4th and 5th day after seedling, respectively. After measuring 30 seedling growth, these seedlings were dried at 85℃ for 6 h to determine the dry weight. Each treatment was replicated 5 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. Means were separated on the basis of least significant difference (LSD) at the 0.05 probability level.

RESULTS

HPLC Analysis

Fractions (10, 11 and 12) combined from DCC chromatography through silica gel TLC were analyzed by HPLC (*Talbe* 4). HPLC analysis showed the presence a large number of unknown peaks and much overlapping suggesting that further purification and fraction of the extract was needed (not shown here). However, salicyclic acid scopoletin, rutin, and quercetin were detected with retention time very close with standard chemicals. Comparing the retention time of the sample peaks to those of the standards, it was determined that salicylic acid, scopoletin, rutin, and quercetin standards closely matched peaks in combined fractions. Although DCC Chromatogrphy yielded various fractions with different inhibition effect, the combination of inhibitory fraction on HPLC was a limited success. However medicarpin, sativan, 4-methoxymedicarpin were not detected in this analysis (*Table* 4).

Bioassay Test

To verify the phytotoxic effect of the salicylic acid, rutin, scopoletin, quercetin compounds, 10^{-3}M solutions of individual authenic compounds were tested on alfalfa seed germination and seedling growth (*Table* 4). The percentage of alfalfa germination and seedling length and weight as inhibited by identified compound are presented in *Table* 4. Alfalfa seed germination and seedling length and weight were inhibited significantly by the salicyclic acid, scopoletin, quercetin and rutin chemical treatment (10^{-3}M) as compared to control. Most inhibition of seed germination and seedling length and weight of alfalfa was caused by quercetin treatment (*Table* 4). Root growth was more affected than that of shoot in total seedling length(data not shown here). The severity of germination and seedling growth inhibition depends upon compounds. These compounds detected in fresh alfalfa leaves may be involved in alfafa autotoxicity and allelopathy.

DISCUSSION

This study attempted to isolate and identify bioactive compounds from fresh alfafa leaves that are responsible for allelopathic or autotoxic activity of alfalfa. Although some compounds like

medicarpin and othe relate pterocarpans from fractions were not found in the extract in detectable amount, four peaks in combined fractions had similar HPLC retention times to standard chemicals salicyclic acid, scopoletin, rutin, and quercetin(*Table* 4). The resolution of fraction on HPLC was a limited success. The results in *Table* 4 suggest that quercetin is biologically most active : this compound inhibits alfalfa seed germination and seedling growth and increase in weight. HPLC analysis through TLC and DCC chromatography process was useful for study of extracted compounds in fractions that inhibited seed germination and seedling growth. After collection of bands from silica gel TLC plate and DCC chromatography, HPLC analysis showed some unknown and overlapping peaks. This indicates that the extract contains a complicated mixture of secondary plant compounds.

Although isoflavonoids like medicarpin are reportedly involved in alfalfa autotoxicity and allelopathy[9, 18]. Medicarpin and other related pterocarpans were not detected in detectable amounts in this research. This suggests that either these compounds could not be extractede in MeOH 80%, which was the initial solvent or even if extracted, they might have been lost in purification subsquent extraction. Preliminary characterization using data from silica gel TLC, DCC chromatography, and HPLC analysis, suggests that salicyclic acid, scopoletin, quercetin and rutin may, at least partily, contribute to alfalfa autotoxicity and allelopathy. Quercetin shown by bioassay was to be most phytotoxic to alfalfa seed germination and seedling growth(*Table* 4).

Purification of phytotoxic compounds by silica gel TLC plates and DCC chromatography may be useful for isolation of some compounds that inhibit seed germination and seedling growth, although it was not possible to determine specifically toxic compounds present in the extracts. Some fractions of DCC chromatography inhibited alfalfa seed germination in bioassays and could be used for further purification by mass spectrometry and proton nuclear magnetic resonance (^{1}H-NMR) for identification of the bioactive compounds. Thus, a method for purification and isolation of bioactive compounds from fresh alfalfa aqueous extracts could be applied further in alalyzing the extract and for identification of some compounds related to alfalfa allelopathy and autotoxicity.

Liquid chromatography-mass spectrometry(LC-MS) and proton nuclear magnetic resonance (^{1}H-NMR) analysis of the separated compound are needed to confirm identitification of individually separated compounds. Studies on the phytotoxicity of specific compounds on the crops and potential weed control are also needed.

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