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Identification of Biologically Active Substances from Lilac(*Syringa vulgaris* L.)

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라일락 잎에 함유된 생리활성물질의 동정

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ABSTRACT

Inhibitory substance in the water extracts from lilac(*Syringa vulgaris*) leaves was determined in terms of the allelopathic chemicals. The water extracts from *S. vulgaris* leaves inhibited the germination and root growth of *Digitaria sanguinalis* and *L. sativa*, indicating that a biological substances are presented in the lilac leaves. The phenolic acids were separated and tentatively identified from *S. vulgaris* leaves by gas chromatography and there were composed of higher contents of *p*-coumaric acid, salicylic acid, pyrogallol, and catechol. Polyphenolic compounds such as rutin (5.3%), scopoletin (3.3%), kaempferol (2.9%), and other polyphenolic compounds were detected from lilac leaves. The mixtures of 10^{-6}M of pyrogallol with all the concentrations of catechol had high inhibition of the shoot growth on *D. sanguinalis* and *E. crus-galli* regardless of the catechol concentrations.

Key words : Allelopathic substances, phenolic compounds, polyphenolic compounds, *Syringa vulgaris*

INTRODUCTION

Recently the importance of allelopathy in agricultural practices is recognized, and thus application of biological control of weeds is gradually increased[20,21,22]. Many researches have investigated the effect of the water extracts from trees on the germination and growth of plant species, but there is little information on the allelopathic effect of *Syringa vulgaris* L. (lilac). It has been known that lilac tree is not infected with any pathgen and escapes the attack of insects in natural habitat. Thus, we would be very interested in investigation if these naturally synthesized chemicals may be presented and related to the allelopathic activity[30,31,32]. One of the most potential allelochemicals is the phenolics which are wide spread in the most of plants as major classes of ecologically important secondary compounds of plants. These includes simple phenolic acids, flavonoids, polyphenols such as tannins, other classes of compounds common in plants, and many compounds of more restricted distribution.

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Mega and Lorenz[7] reported that phenolic acids can contribute objectionable flavors, especially astringency, at taste threshold levels of 40-90 ppm. High content of phenolic compounds in leaves in the Cameroon site may explain the general avoidance of leaves and great selectivity in leaves feeding shown by *Colobus satanas*. It is interesting that seeds contain consistently lower amounts of phenolic compounds than that of leaves on this site[1]. We would believe that the high content of tannins and other phenolic compounds in vegetation of the Douala-Edea site has major implications for the ecology of animals dwelling there. *Colobus guereza*, for example, feeds heavily on leaves of *Celtis durandii*, an abundant tree in which the contents of tannins and other phenolics are low[9]. Generally, allelochemicals present in all plant tissues may be released in different ways such as root exudation, leaching, decomposition, and volatilization[10] in natural habitat.

Our considerable attention has been given to the investigation of the role on allelopathic interactions between crop plants and phytotoxins produced by *S. vulgaris* leaves extracts especially in the water soluble extracts and leaching condition which is similar to the common natural state.

This experiment was conducted to determine the presence of inhibitory substances on plants in the water extracts from lilac leaves and to detect the allelopathic chemicals. In addition, we determined the effects of authentic phenolic acids and their combining effects on the growth of some weeds.

MATERIALS AND METHODS

The *Syringa vulgaris* leaves were collected on September and October of 1995 in the campus of Kyungpook National University. The leaves were air dried for several weeks under room temperature and ground using a Wiley mill to pass through 40 mesh screen. The ground samples were stored in glass bottles in refrigerator at 5℃ until experimental use. Fresh samples were immediately used immediately after collection for water extraction.

Experiment 1. Inhibitory effects of the extracts from fresh *Syringa vulgaris* leaves on the growth of plants

To investigate an allelopathic activity of the extracts from lilac leaves, soaking and leaching methods were employed. For the soaking method, the fresh lilac leaves were soaked in distilled water at final concentrations of 2, 5, 7.5, 10, and 20%(w/v) under room temperature conditions. Leaching method was designed for getting naturally water soluble extracts under artificially rainy condition. For this method, three-layer plastic pot with small holes in the bottom of the pot was prepared and distilled water was passed and repeated this procedure for the increase of extract concentration at different leaching times of 6, 12, 18, and 24 hours. The concentrations of leaching extracts were 10 and 20%(w/v).

For the bioassay, ten seeds of rice, *Echinochloa. crus-galli*, *Digitaria. sanguinalis*, *Cyperys. amuricus*, and lettuce were placed on Whatman No. 2 filter papers moistened with 10 ml of the lilac water extracts in a petridishes. Each treatment was replicated with 3 times.

In order to detect allelopathic substances and inhibitory effect of *Syringa vulgaris* leaves, germination rate, shoot length, root length, and fresh weight of plants tested were measured 9 days after treatment. Data were analyzed using Duncan's Multiple Range Test (DMRT). Seeds were considered as a germinated ones when the coleoptile was 2 mm in length.

Experiment 2. Chemical analysis of *Syringa vulgaris* leaves

General components analysis

General components were measured by common methods.

Extraction and isolation of phenolic acids

Extraction and fractionation of phenolic acids : For analysis of phenolic acids from the lilac leaves, the samples were defatted 5 times with hexane. The final procedures adopted for the isolation of phenolic acids were outlined in Fig. 1.

Ten grams of defatted flour were extracted 3 times with 100 ml distilled water at room temperature. After filterating, the combined supernatants were analyzed for free phenolic acids and soluble phenolic acid esters, and the residue was reserved for determination of insoluble-bound phenolic acids. The combined supernatants were evaporated under vacuum at 45±2℃ to a 50 ml volume. The aqueous suspension was adjusted to pH 2 and filtered to separate a cloudy precipitate. The free phenolic acids were then extracted 5 times with diethylether at a solvent to water phase

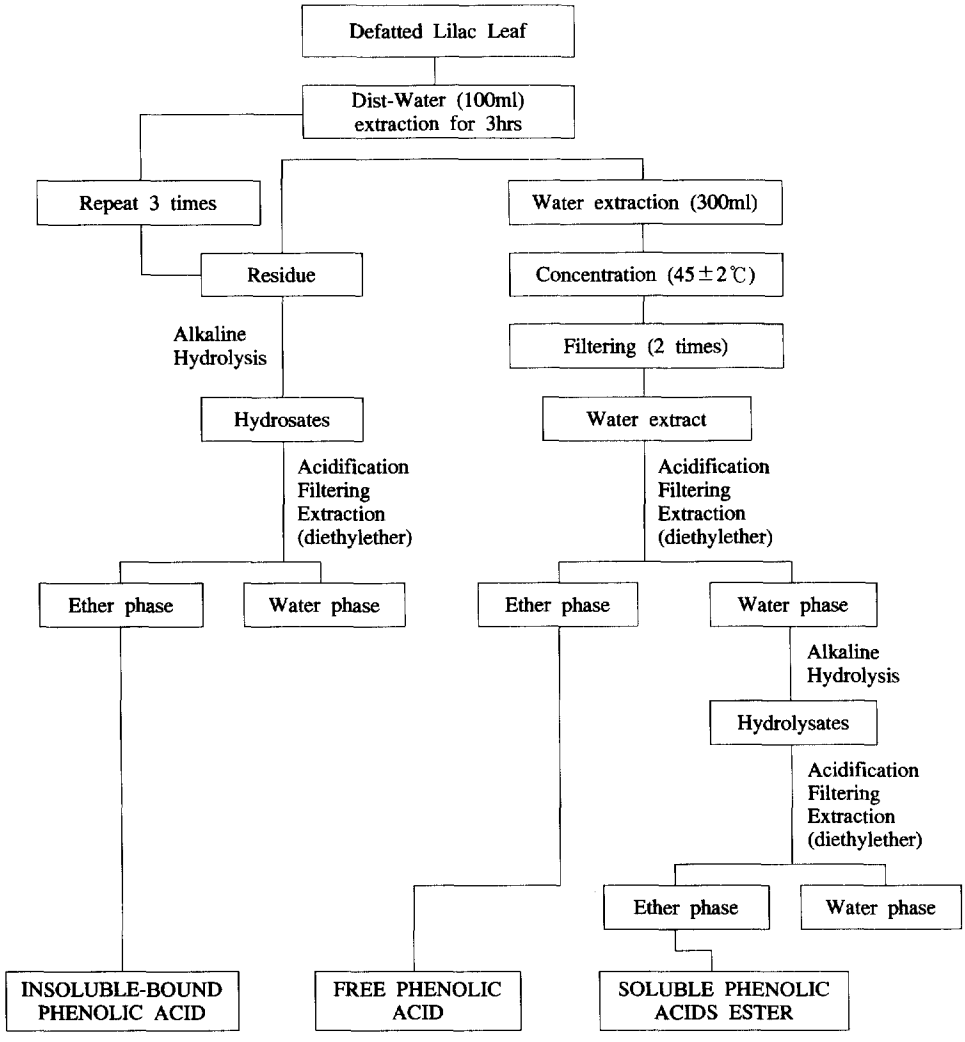

Fig. 1. Procedures for isolation of water extract and free-, ester-, and insoluble phenolic acids.

ratio of 1 : 1. The diethylether extracts were dehydrated with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum at 40 ℃. The dry residues were transferred into vials by using methanol as a solvent and dried before silylation under nitrogen at room temperature. The moist sodium sulfate was washed free of phenolic acid esters, and the washings were combined with the above water phase and the cloudy precipitate. The esters were hydrolyzed with 40 ml of 2N NaOH for 6 hours under nitrogen at room temperature. The hydrolysates were acidified to pH 2 and the liberated phenolic acids were then extracted with diethylether as described above. The residues from the distilled-water extractions were hydrolyzed directly with 40 ml of 2N NaOH under the same conditions as the esters. After acidification and filteration, the clear supernatants were extracted diethylether as described above.

Since phenolic acids are nonvolatile compounds, they must be converted to volatile derivatives before they can analyzed by GLC. The most used derivatives are trimethylsilyl ether(e.g., Kuwatsuka and Shindo, 1973 ; Hartley and Jones, 1975) and methyl ethers(Erickson *et al.*, 1973).

Gas-Liquid Chromatography : Phenolic acids were silylated by slight warming with Tri-Sil/BSA Formula D (Pierce Chemical Co.). The Me_3Si derivatives of the phenolic acids were separated on a Varian STAR 3400 CX Series gas chromatograph equipped with a flame ionization detector and peak area integrator. The 2.0×2.0 mm o.d. glass column was packed with SE 30 on 80-100 -mesh chromosorb W(HP) in Alltech. The flow rate of the carrier gas was 30 ml/min of nitrogen. The injector and detector temperatures were 270 ℃ and 280℃, respectively. The oven temperature was programmed at the start of the run from 130 to 245℃ at 3℃/min. Phenolic acids were identified by comparing the retention times of the Me_3Si derivatives with the values of retention times of the Me_3Si derivatives of standard acids.

Analysis of Polyphenol Compounds by HPLC

Polyphenol compounds which were not separated in the GLC were isolated by Snook's method. Five hundred mg of lilac flour supplemented with 0.1 mg of I.S.T.D (7-OH-coumarin) was extracted with 10 ml of MeOH and let the sample in a sonicator for 30 minutes. The extracts were filtered several times with Whatman No. 2 and 0.5 ㎛ membrane. The purified compounds were analyzed by HPLC (Waters Model 246 Liquid Chromatograph) with authentic standards. As a final step, separated solution by membrane filtering was completely filtered by using Sep-pak C_{18} Cartridge and the analysis conditions were as followed.

Items	Conditions
Column	μ Bondapak C_{18}
Detector	Model 441 absorbance (340nm)
Mobile phase A	MeOH/H_2O/Acetic acid (10 : 88 : 2, v/v/v)
Mobile phase B	MeOH/H_2O/Acetic acid (88 : 10 : 2, v/v/v)
Flow rate	0.5 ml/min
Injection volume	10 ㎕

Experiment 3. Effect of authentic standard substances on germination and growth of plant species

Effect of authentic phenolic acids

After phenolic acids identification, pyrogallol and catechol which have high proportions in the lilac leaves were selected to investigate the own effects of the authentic phenolic acids. The standard phenolic acids were prepared at different concentrations of $10^{-1} \sim 10^{-6}$M. The bioassay methods were the same as the above. For the combinig effects, two authentic phenolic compounds at different concentrations were mixed and applied on the growth of *D. sanguinalis* and *E. crus-galli*.

RESULTS AND DISCUSSION

1. Inhibitory effects of the extracts from lilac leaves on the plants

From a bioassay of *O. sativa*, *E. crus-galli*, *D. sanguinalis*, *C. amuricus* and *L. sativa*, the water extracts from *Syringa vulgaris* leaves markedly inhibited the germination of *D. sanguinalis* and *L. sativa*, indicating that a biological substances are presented in the lilac leaves. A little inhibitory effects of the water extracts on the germination of the several plants were detected in the both of extraction methods(Table 1 and Table 2). Although there were a little differences within two different extraction methods, soaking method have 20% more inhibitory effect than leaching. At the treatment of water extracts of 72 hrs soaking, the highest inhibition was observed. Generally, the germination of *D. sanguinalis* and lettuce were greatly affected by the treatment of extracts. This implied that the concentration of inhibitor in the exudates was low enabling the test species to recover with time[10].

Table 1. Effect of water extracts from *Syringa vulgaris* leaves by different extraction methods on germination of different plant species[1].

Extraction method	Duration (hours)	Plant species				
		O. sativa	*E. crus-galli*	*D. sanguinalis*	*C. amuricus*	*L. sativa*
Soaking	0	100 a	82 b	99 a	45 a	99 a
	12	99 a	86 ab	99 a	41 ab	93 a
	24	100 a	89 a	93 ab	36 b	95 a
	36	99 a	80 bc	91 b	37 b	99 a
	48	99 a	85 ab	84 c	38 ab	80 b
	72	99 a	74 c	66 d	36 b	83 b
Leaching	0	99 a	82 ab	99 a	45 a	99 a
	6	99 a	87 a	94 ab	45 a	100 a
	12	94 b	84 ab	99 a	46 a	94 b
	18	98 ab	77 b	86 b	43 a	100 a
	24	94 b	75 b	92 ab	35 a	91 b

1) % germination, determined 9 days after incubation under conditions of 26±2℃ and 2,000 lux ; In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Table 2. Effect of several concentrations of water extracts from *Syringa vulgaris* leaves on germination of different plant species[1].

Extraction method	Conc.[2] (%, w/v)	Plant species				
		O. sativa	*E. crus-galli*	*D. sanguinalis*	*C. amuricus*	*L. sativa*
Soaking	0	100 a	82 a	99 a	45 a	99 a
	2.0	100 a	85 a	97 a	38 ab	99 a
	5.0	99 a	83 a	94 ab	39 ab	95 ab
	7.5	99 a	83 a	90 bc	36 b	94 ab
	10.0	99 a	84 a	84 c	38 ab	89 b
	20.0	99 a	80 a	67 d	36 b	74 c
Leaching	0	99 a	82 a	99 a	45 a	99 a
	10.0	95 b	75 b	96 a	39 a	96 ab
	20.0	96 ab	86 a	87 b	44 a	94 b

1) % germination, determined 9 days after incubation under conditions of 26±2℃ and 2,000 lux ; In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.
2) Concentrations; based on w/v, g of fresh leaves / ml of distilled water.

Seedling growth of *E. crus-galli* and *D. sanguinalis* were inhibited by aqueous extracts of fresh *S. vulgaris* leaves. The inhibition was increased as the extraction time was prolonged from 12 hours to 72 hours in the both of extraction methods showing differential effects according to the species. A complete inhibition in germination and seedling growth of plants was caused by 70 % (w/v) methanol extracts of *Syringa vulgaris* leaves (data not shown).

There are a number of reports on the inhibitory effects of plant tissue extracts on the germination and growth of plants. Top and root aqueous extracts of lambsquarter(*Chenopodium album* L.), giant foxtail(*Setaria faberii* Herrm.), redroot pigweed(*Amaranthus retroflexus* L.), velvet leaves (*Abutilon theophrasti* Medic.), crabgrass(*Digitaria sanguinalis* L.), canada thistle(*Cirsium arvense* L.), and prostrate knotweed(*Polygonium aviculare* L.) significantly inhibited the germination and seedling growth of alfalfa. Germination, seedling length, and weight of alfalfa were more inhibited as the concentration of dried velvet leaves extracts was increased. Greater toxic effects were observed from the dried extracts compared to the fresh extracts[8].

Park *et al.*[10] also reported that the leaves exudates of sunflower had significant inhibitory effects on the lengths of the shoots and roots of all the test species, and differential effects on the plant species were observed showing that there was a greater inhibitory effect on *Digitaria sanguinalis* and radish than rice. There was about 90% inhibition on germination and seedling growth of sesame due to 2%(w/v) methanol extracts of *A. asistica.* The water extracts of leaves, stems, and roots significantly inhibited germination and seedling growth in *E. colona* and radish[20,21,22]

The inhibitory effect of 10% water extracts from lilac leaves on the shoot and root length of plants was shown in Fig. 2 and 3. There were little effects of the extracts on the shoot growth

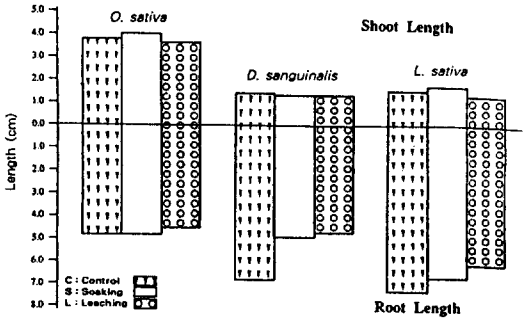

Fig. 2. Effect of the water extracts for 24 hours from *Syringa vulgaris* leaf on the growth of different plant species.

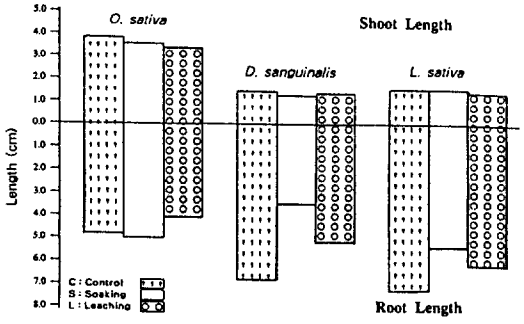

Fig. 3. Effect of 10% concentration of water extracts from Syringa vulgaris leaf on the growth of different plant species.

of plants used in the both of extraction methods, but an inhibition was observed in the root growth of *D. sanguinalis* and lettuce.

Based on the germination, shoot and root growth of plants affected by the water extracts of lilac leaves, some biologically active substances may be presented in the leaves of lilac and phytotoxic effects of the extracts varied with extraction solvents and test plants used.

2. Chemical analysis of lilac leaves

General components analysis

The general components of lilac leaves were shown in Table 3. Higher content of moisture and much lower contents of ash, fat, and protein were detected in comparision with the common medicinal plants such as *Lycium chinensis miller, Angelica acutiloba kitag, Schizandra chinensis*

bailon, and *Acanthopanax sessiliflorum seeman*[34].

Isolation and detection of phenolic acids

Water extracts, and free-, ester- and insoluble phenolic acids were extracted from lilac leaves. The biochemical substances of phenolic compounds such as ferulic acid, *p*-coumaric acid, salicylic acid, vanillic acid, *p*-hydroxybenzoic acid which may be responsible for exhibiting inhibitory effects were detected in the lilac leaves in large amounts (Table 4).

The phenolic acids separated and tentatively identified from *S. vulgaris* leaves by gas chromatography were composed of higher contents of *p*-coumaric acid, salicylic acid, pyrogallol, and catechol.

Salicylic acid(SA) is a natural inducer of disease resistance in some dicotyledonous plants. SA levels have a correlation with generalized blast resistance in 28 rice varieties, indicating that SA may play a role as a constitutive defense compound. SA in rice and tobacco is synthesized from cinnamic acid via benzoic acid. Subsequently, SA was shown to be a signal in acquired resistance to pathogens in tobacco(Malamy *et al.*, 1990; Gaffney *et al.*, 1993; Vernooij *et al.*, 1994) and cucumber(Mettraux *et al.*, 1990)[2]. While rape seed(Krygier *et al.*, 1982) and the potato were almost devoid of insoluble-bound phenolic acids, the cereal residues contained quite high levels, especially in corn. Most of the phenolic acids in the cereals were bound to insoluble residues and would have little immediate effect on the color or flavor of aqueous slurries of cereal flours[7].

Table 3. General components of *Syringa vulgaris* leaves.

Moisture	Ash	Fat	Protein	Fiber
		%		
33.67	1.96	3.16	3.26	3.14

Analysis of Polyphenolic compounds by HPLC

Polyphenolic compounds such as chlorogenic acid, kaempferol, scopoletin, and rutin which were not detected by GLC were analyzed by HPLC.

Table 4. Constitution of free, esterified and insoluble-bound phenolic compounds identified from *Syringa vulgaris* leaves[1].

Phenolic compounds	*Syringa vulgaris* leaves			
	Free fraction	Soluble fraction	Insoluble fraction	Total
p-Cresol	0.5[1]	1.9	0.1	2.5
Catechol	7.6	1.1	0.4	9.1
Hydroquinone	0.7	0.5	tr	1.2
Orchinol	3.5	1.2	0.9	5.6
Salicylic acid	9.8	2.3	0.4	12.5
trans-cinnamic acid	1.6	0.9	0.6	3.1
Pyrogallol	1.1	8.6	2.7	12.4
p-OH-benzoic acid	1.3	0.3	0.8	2.4
Phloroglucinol	2.2	0.4	0.1	2.7
Vanillic acid	1.1	1.2	0.3	2.6
Umbelliferone	3.2	0.3	0.4	3.9
Syringic acid	1.4	0.7	0.4	2.5
p-Coumaric acid	3.4	10.0	1.9	15.3
Tannic+gallic+tyrosine	1.5	1.5	0.1	3.1
Ferulic acid	0.8	2.6	tr	3.4
Caffeic acid	1.1	2.1	tr	3.2

1) Percent of total GLC analyzed phenolic acids, tr; trace.

Rutin(5.3%), scopoletin(3.3%), kaempferol(2.9%), and other polyphenolic compounds were detected from lilac leaves(Table 5).

During analyzing, there were small kinds and low amounts of polyphenolic compounds were detected in lilac leaves. So we tentatively concluded that the inhibitory effects of lilac leaves had little to do with polyphenolic compounds.

3. Effect of several authentic phenolic acids on the germination and growth of plants

Authentic phenolic acids effects

To demonstrate if inhibitory effects of lilac leaves on the plant growth were connected with high contents of phenolic acids of lilac leaves, two authentic phenolic acids were treated on the plants at different concentrations. The mixtures of 10^{-6}M of pyrogallol with all the concentrations of catechol had high inhibition of the shoot growth on *D. sanguinalis* and *E. crus-galli* regardless of the catechol concentrations used showing that there were no combining effects in the mixtures(Table 6 and 7). The retarded shoot growth of *D. sanguinalis* and *E. crus-galli* influenced by the compounds was shown.

Consequently, these phenomenons were difficult to be explained in respect to the general relations between concentrations and inhibitory effects in the mixture treatments of the all inhibitory substances. But it was obviously detected that the low level of 10^{-6} M of pyrogallol was needed for inhibitory effect on the used weed species and it was thought to be the synergistic effects between two phenolic compounds. Further studies are demanding or demanded to find out the pathway and metabolism of the phenolic compounds for the inhibitory effects on the growth of plants.

Table 5. Polyphenolic compound contents extracted from *Syringa vulgaris* leaves[1)].

Chlorogenic	Kaempferol	Scopoletin	Rutin
	%		
2.4	2.9	3.3	5.3

1) Polyphenolic compounds contents were determined by HPLC.

Table 6. Combined effect of phenolic substances on the shoot growth of *Digitaria sanguinalis*.

Phenolic acids	Conc. (M)	Pyrogallol				
		0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Catechol	0	1.5 a[1)]	0.2 c	1.2 a	0.8 b	1.2 a
	10^{-6}	1.4 b	0.4 c	1.3 b	1.6 a	1.5 ab
	10^{-5}	1.4 b	0.6 c	1.8 ab	1.6 ab	1.5 b
	10^{-4}	1.6 b	2.0 a	1.7 b	1.8 b	1.6 b
	10^{-3}	1.5 c	0.4 d	1.3 c	1.8 b	2.4 a

1) In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Table 7. Combined effect of phenolic substances on the shoot growth of *Echinochloa crus-galli*.

Phenolic acids	Conc. (M)	Pyrogallol				
		0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Catechol	0	2.8 b[1)]	0.6 d	2.5 c	2.9 ab	3.0 a
	10^{-6}	2.4 d	1.9 e	3.7 a	2.7 c	3.3 b
	10^{-5}	2.0 b	1.8 b	3.2 a	2.5 ab	3.1 a
	10^{-4}	2.4 d	0.8 e	4.0 a	3.2 b	2.8 c
	10^{-3}	2.6 a	2.0 b	2.6 a	2.5 a	1.9 b

1) In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

要　約

라일락 잎에 함유되어 있는 生理活性物質을 檢定코저 수행한 본 實驗의 結果를 要約하면 다음과 같다.

1. 침적과 Leaching법으로 抽出한 라일락 잎 수용 추출물의 식물 생육 억제효과를 검정해 본 결과, 바랭이와 상추의 발아율에 영향을 주었으며, 초장보다 뿌리에 더 큰 억제현상을 보여 1차적인 생리활성효과를 검정할 수 있었다.
2. 라일락 잎의 페놀, 그리고 폴리페놀성 성분을 분석한 결과, *p*-coumaric acid, salicylic acid, pyrogallol 및 catechol의 높은 비율이 동정되었고, 폴리페놀성 물질로는 rutin, chlorogenic acid, kaempferol 및 scopoletin 등이 각각 검정되었다.
3. 표준페놀물질인 pyrogallol과 catechol은 공시식물의 생육에 통계적으로 유의한 억제효과를 보였고, 특히 수용성인 pyrogallol 10^{-6}M과 catechol의 혼합처리에서 억제효과가 크게 나타났다.

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