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Extraction and Bioassay of Allelochemicals in Jerusalem Artichoke

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ABSTRACT: *Helianthus tuberosus* has been known to inhibit the growth of weeds and other plants sharing its habitat. This study was conducted to identify the allelochemicals of *Helianthus tuberosus* which were extracted with water and solvents. Aqueous extracts of leaf, stem, root, tuber and tuber peel of *Helianthus tuberosus* except tuber did not show significant differences in phytotoxicity to alfalfa seedlings. It was considered that *Helianthus tuberosus* contained fewer or less potential water-soluble substances that were toxic to alfalfa. Methanol extract of leaves of *Helianthus tuberosus* was sequentially partitioned in increasing polarity with *n*-hexane, ethylacetate and *n*-butanol. Each extract had a yield of 148, 12, 15.7 and 9.5 g, respectively. Inhibitory effects on germination of alfalfa seeds treated with four fractions were not significantly different. But the significant reductions on hypocotyl length were observed for all the solvent extracts. Among the four fractions, the ethylacetate fraction showed the most significant inhibition effect on bioassay with alfalfa. Further separation of the active ethylacetate fraction by open column chromatography led to the 25 sub-fractions. In bioassay of each sub-fraction with alfalfa seeds, sub-fraction No. 13 showed the most inhibitory effect on seedling growth. ^{1}H NMR and gas chromatography-mass spectrometry analysis revealed that sub-fraction No. 13 was the mixture of straight-chain saturated fatty acids.

Keywords **:** *Helianthus tuberosus* L., allelochemicals, fatty acids, bioassay, open column chromatography, Gas chromatography-mass spectrometry (GC-MS)

As synthetic agrochemicals play an indispensable role in crop production, they simultaneously contain both sides of benefit on increase in crop production and environmental pollution. Nowadays, although there is no doubt as to the role of the synthetic agrochemicals in effectively producing crop, a side effect due to long term use of these chemicals have caused a threat to human being's health and ecosystem. Chemical prevention brings about a harmful effect such as toxicity to man and livestock, residual problem in soil and food, the advent of herbicide resistance plants and the outbreak of disease tolerant insect pest. For example, a chlorinate organic compound like Benzene hexachloride (BHC) is stable one. Generally, a compound which own carbon and chlorine bond has a difficulty in decomposition by a microorganism, and remains as so-called residual pesticide. As BHC decomposes 1% per year, it thus has 99% of residue in ecosystem. Therefore, a large quantity of scattering BHC causes residual accumulation in ecosystem and will give rise to destruction of ecosystem as well as environmental problem like disaster.

Human beings must produce food on a limited land area and still has to protect crops from weeds and insects. Accordingly, there is a need to make a new strategy and trial for insect and pest control, developing agrochemical whose structure has been derived from naturally occuring substances is necessary. Endeavor for solving problems related to prominent effects in low concentration and selective function, low toxicity and resistance to livestock and environment, etc. from natural products is in progress.

Natural products are as well biodegradable in environment as stable feature to humans and livestock. In a developed country, more concerns are focused on using plants which have allelopathy for weed control, among these plant compounds some have a tendency to accumulate very specific natural products, being different from those produced by other genus and species. Pyrethrin, natural herbicide isolated from *Chrysanthemum cinetariaefolium* Visiani, is a representative instance. Alfalfa also has shown a possibility for natural herbicide potential (Chung & Miller, 1995). Besides, the bioactive compound was isolated from the leave and root of sunflower, the same genus (*Helianthus*) as Jeusalem artichoke (Macías, 1996; Park, 1991). On a aspect of utilizing inhibitory or stimulatory biochemical ineteractions among plants in agriculture, this study was conducted to investigate allelochemicals in Jeusalem artichoke.

Helianthus tuberosus has been considered to inhibit the growth of weeds and other plants sharing its habitat. On the basis of this phenomenon, this study was conducted to identify the allelochemicals of *Helianthus tuberosus* which were extracted with water and solvents. Bioassay was performed for extracts of water and solvents. Methanol extract of leaves of *Helianthus tuberosus* was sequentially partitioned

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in increasing polarity. Among the fractions, the ethylacetate fraction showed the significant inhibition effect in bioassay. Further separation of an active ethylacetate fraction by open column chromatography led to allelochemicals, which were used in bioassay for bioactive compounds.

MATERIALS AND METHODS

Plant materials

Samples were collected at the College of Agriculture & Life Sciences, Seoul National University, Suwon, Korea. Leaves, stems, tuber, tuber peel and roots were seperated. The tuber and roots were gently scrubbed with brush to remove soil. All plant components were dried in the shade and coarsely chopped.

Water extraction

Dried plant components (leave, stem, root, tuber, tuber peel) were soaked in H_2O (5% w/v) for 24 hrs with shaking at 25°C in the dark. Extracts were passed through filter paper (Whatman No. 2) under vacuum and centrifuged at 11,000 × g for 20 min at 8°C. Supernatant were filter-sterilized (0.2 μm membrane) using a aspirator (EYELA A-3S Tokyo Rikakikai Co., LTD.) to prevent any microorganism contamination. These aqueous extract were used in bioassay.

The fractionation of solvent extract and isolation of allelochemicals

Two hundred grams of leaves was extracted with methanol by boiling three times for 3 hrs, respectively. The combined filtrate was concentrated by using vacuum rotary evaporator, with the methanol removed. This extract was partitioned in increasing polarity with *n*-hexane, ethylacetate and *n*-butanol to give fractions by using a separatory funnel (Fig. 1). Allelopathic activity of the fractions were determined on the basis of bioassay.

Ethylacetate fraction which showed the most inhibitory effect in bioassay was chromatographed over a column of silica gel (Kieselgel 60, Merck 7734). The column was eluted with increasing polarity *n*-hexane, ethylacetate and methanol. The progress of elution was monitored by performing thin layer chromatography (Kieselgel 60 F_{254}, Merck 5715) of sub-fractions. These sub-fractions were collected on the basis of spot patterns on TLC plate. They were detected by exposing the developed plates to short-wave (254 nm) and long-wave (366 nm) UV light and then treated with 20% (w/w) sulfuric acid and the solvent removed.

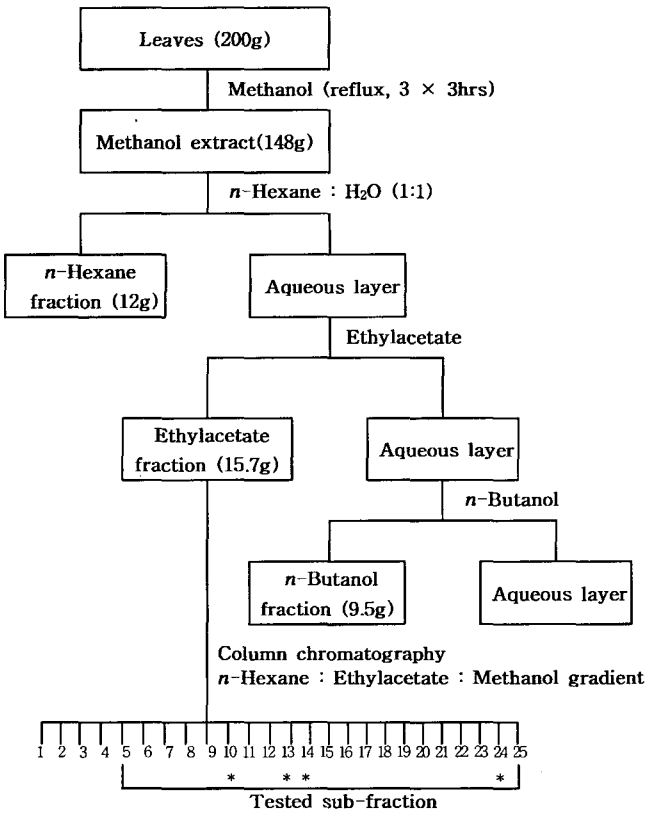


Fig. 1. Fractionation of leaves of *Helianthus tuberosus* L.

Bioassay

Allelopathic activity was determined on the basis of bioassay, which tested alfalfa seed germination and hypocotyl length. The bioassay of aqueous and solvent extract consisted of 30 seeds of alfalfa for 72 hrs at 25°C in a diameter of 5 cm petri dishes containing Whatman No. 1 filter paper and 2 ml of a test and control solution. Seed germination and hypocotyl length were measured. Four concentrations of aqueous and solvent extracts were used: 5000, 2500, 1000, 500 ppm and including blank. There were 3 replications for 30 seeds of each treatment and blank. The germination rate and hypocotyl length values were statistically tested by least significant difference (LSD) test at $P = 0.05$.

Each sub-fraction was prepared by dissolving the appropriate amount initially in small quantity of dimethyl sulfoxide (DMSO) which had no effect on the germination of alfalfa and by diluting with distilled water to a final concentration of 2500 ppm. Bioassay consisted of 10 of alfalfa seeds for 72 hrs at 25°C in well containing Whatman No. 2 filter paper and 0.35 ml of an isolated sub-fraction and control solution. Treated concentrations were 2500 ppm and control. Seed germination and hypocotyl length were mea-

sured. There were three replicates for 10 seeds of each treatment and control.

Identification of allelochemical

^{1}H NMR (300MHz) and gas chromatography-mass chromatography (GC-MS) were used to identify the sub-fraction No. 13. It was methylated by addition of 5 ml of diazomethane (CH_2N_2). The solution was kept at room temperature for 24 hrs for GC-MS analysis. The GC-MS analysis was carried out on a Hewlett-Packard 5989B mass spectrometer. Gas chromatography (GC) was performed in a HP 5890 model. The column used was a Hewlett Packard HP U2 cross-linked methyl-phenyl silicone, 25 m × 0.33 μm I.D. Helium (He) was used as carrier gas at 0.5 ml/min. A flame ionization detector which operated at 320°C was used for detection of sub-fraction No. 13. The injector operated at 230°C. The sample volume was 2 μl. The temperature for the oven was as follows: start at 170°C with an increase of 5°C/min up to 310°C, then hold for 1 min.

RESULTS AND DISCUSSION

The bioassay of water extract

Bioassay was done by treating water extract of Jerusalem artichoke according to plant organs and concentrations (Table 1). Water extract of leaf did not show any inhibitory effect against seedling growth. Water extract of stem showed a tendency to inhibit germination rate and hypocotyl length of alfalfa in accordance with increased conentration. But there was no significant differences in treated concentrations. Water extract of root had a tendency to stimulate the germination of alfalfa seeds according to the concentrations, but there was no significant difference in statistical analysis. So it is necessary to examine this result in detail. Also, water extract of root did not show significant difference in inhibitory effect on hypocotyl length. Very thin roots were used in this experiment for water extract and they contained few allelochemicals. Hypocotyl length of alfalfa was slightly reduced in aqueous extract of tuber at 5000 ppm. It can be estimated that allelochemical contained in water extract of tuber is not major compound, for this effect occurred at 5000 ppm, or relatively high concentration. It might be due to osmotic potential rather than allelopathic compound.

No significant difference was observed for the inhibition effect on alfalfa seedling growth between treated concentrations of water extract of tuber peel. Tuber peel used for this experiment was a brown, it is essential to study further whether a purple-colored have allelochemicals.

Table 1. Germination rate and hypocotyl length of alfalfa treated with aqueous extract of *Helianthus tuberosus* L.

Extraction part	Concentration (ppm)	Seed germination (%)	Hypocotyl length (cm)
Leaf	0	90.0	2.9
	500	92.2	2.7
	1000	85.5	2.6
	2500	94.4	3.1
	5000	93.3	3.2
	F value	1.2^{ns}	2.3^{ns}
Stem	0	95.5	3.0
	500	93.3	3.1
	1000	91.1	3.1
	2500	91.0	2.9
	5000	90.0	2.8
	F value	0.5^{ns}	1.8^{ns}
Root	0	86.6b	2.9
	500	94.4a	2.9
	1000	87.7b	3.0
	2500	92.2ab	2.9
	5000	95.5a	2.9
	F value	4.0*	0.2^{ns}
	LSD	6.3	
Tuber	0	91.0	3.3a
	500	87.7	3.2a
	1000	91.0	3.0a
	2500	92.2	3.3a
	5000	92.2	2.7b
	F value	0.5^{ns}	4.9*
	LSD		0.4
Tuber peel	0	91.1	3.5
	500	83.3	3.6
	1000	91.1	3.5
	2500	83.3	3.1
	5000	90.0	3.0
	F value	1.2^{ns}	1.9^{ns}

ns : not significant at P=0.05; *significant at the 0.05 probability level
The same letters are not significantly different at the 5% level of probability.

Bioassay of water extract from *Helianthus tuberosus* except root and tuber showed that inhibitory or stimulatory effect on germination rate and hypocotyl length of alfalfa was not significant (Table 1). Therefore, it was concluded that *Helianthus tuberosus* contained few or less potential water-soluble substances that were toxic to alfalfa.

The bioassay of solvent extract

It was reported that leaves of sunflower, a plant belonging to the same genus (*Helianthus*) as Jerusalem artichoke, had allelochemicals such as sesquiterpene lactone, flavonoid, etc. (Macías *et al.*, 1996; Macías *et al.*, 1997). Heliangine, a

sesquiterpenic lactone isolated from the leaves of *Helianthus tuberosus,* inhibits the elongation of *Avena* coleoptile sections (Shibaoka, 1961; Morimoto *et al.,* 1966). Based on these results, the extraction part was focused on the leaves.

Of the methanol extract of *Helianthus tuberosus* with the yield of 158 g, ten grams was prepared for bioassay. Methanol extract of 148 g was then sequentially partitioned in increasing polarity with *n*-hexane, ethylacetate and *n*-butanol. Each extract has a yield of 12, 15.7 and 9.5 g, respectively (Fig. 1).

Among the four fractions, seed germination seemed to be inhibited at 2500 ppm of the ethylacetate fraction, however all four fractions did not show inhibitory effect on seed germination of alfalfa (Table 2). Compared to the results of inhibitory effect on seed germination, the significant reduction in hypocotyl length of alfalfa was observed in all the solvent extracts. Among the four fractions, the ethylacetate fraction showed the greatest inhibitory effect on alfalfa (Table 2). When the concentration of ethylacetate fraction increased to 2500 ppm, hypocotyl length was significantly reduced. In treating ethylacetate fraction, there might be a possibility that it contained a very small amount of ethylacetate which was used for extraction. The inhibitory effect of ethylacetate, or organic solvent itself, was not significant through the experiment. In methanol fraction, the concentration of 2500 ppm from which inhibition of hypocotyl length of alfalfa emerged and increased with increasing concentration. In *n*-hexane fraction, inhibitory effect on hypocotyl length of alfalfa came out at the concentration of 2500 ppm, yet this effect remained steady with increasing concentration. In *n*-butanol fraction, the inhibitory effect was shown at 500 ppm, which was lower concentration than the other fractions and continued to increase as the concentration increased (Table 2). *n*-Butanol fraction was prepared by using vacuum rotary evaporator. *n*-Butanol has the feature of rather high viscosity, so the complete removal from fraction seems to be difficult. Therefore, it is essential to evaluate the net effect of organic solvent.

Table 2. Germination rate and hypocotyl length of alfalfa treated with solvent fraction of *Helianthus tuberosus* L.

Extraction Solvent	Concentration (ppm)	Seed germination (%)	Hypocotyl length (cm)
Methanol	0	88.8	3.1ab
	500	80.0	3.0a
	1000	85.6	3.0ab
	2500	90.0	2.3b
	5000	86.7	1.0c
	F value	1.2^{ns}	18.0**
	LSD		0.6
n-Hexane	0	88.9	2.8a
	500	86.7	2.7a
	1000	88.9	2.3a
	2500	84.4	1.7b
	5000	83.3	1.5b
	F value	0.6^{ns}	14.8**
	LSD		0.5
Ethylacetate	0	92.2	3.0a
	500	90.0	2.7a
	1000	88.9	2.9a
	2500	77.8	1.6b
	5000	83.3	0.6c
	F value	2.0^{ns}	58.7**
	LSD		0.4
n-Butanol	0	84.5	3.3a
	500	86.6	3.1b
	1000	82.2	2.7b
	2500	92.2	2.0c
	5000	81.1	1.1d
	F value	2.5^{ns}	50.0**
	LSD		0.4

ns : not significant at P=0.05; **significant at 0.01 probability level
The same letters are not significantly different at the 5% level of probability.

Bioassay of solvent extracts showed that all extracts contain solvent-soluble substances that might have the ability to inhibit the hypocotyl length of alfalfa.

The bioassay of ethylacetate sub-fraction

Further separation of the active ethylacetate fraction by open column chromatography led to the 25 sub-fractions, which were used for bioassay. Test solutions were prepared using DMSO (1% w/v) as initial solubilizing agent and diluting the previous solution. Bioassay was not affected by the concentration of DMSO (1% w/v). But, Some of sub-fractions (sub-fraction No.1~4) could not be solubilized. The possible reason was that they might be changed to insoluble compound through oxidation and other factors. Therefore, additional progress is finding proper solvent. In bioassay of 21 sub-fractions, there was no stimulary effect on the seed germination and hypocotyl length of alfalfa (Table 3). Sub-fraction No. 10, 13, 14, 24 reduced the seedling growth of alfalfa, and sub-fraction No. 13 showed the most inhibitory effect (Table 3). Sub-fraction No. 10 inhibit the hypocotyl length but did not reduce seed germination. The inhibitory effect of sub-fraction No. 14 was graded as medium on seed germination among four sub-fractions and hypocotyl length was greatly reduced. Sub-fraction No. 24 showed inhibitory effect both on seed germination and hypocotyl length of alfalfa (Table 3). Thin layer chromatography of sub-fraction No. 24 showed several spots on TLC plate, suggesting that synergistic inhibition, combined action of mixture of allelochemicals.

Table 3. Germination rate and hypocotyl length of alfalfa treated with sub-fraction of ethylacetate of *Helianthus tuberosus* L.

Concentration (2500 ppm)	Seed germination (%)	Hypocotyl length (cm)
control	93.3a	2.5ab
fr. No. 5	70.0b	2.1abcde
fr. No.6	80.0ab	1.3ef
fr. No.7	80.0ab	2.0bcde
fr. No.8	80.0ab	1.8bcde
fr. No.9	80.0ab	1.5def
fr. No.10	93.3a	0.2gh
fr. No.11	86.7ab	2.2abcde
fr. No.12	96.6a	1.4ef
fr. No.13	0.0d	0.0h
fr. No.14	43.3c	0.4gh
fr. No.15	90.0ab	1.8bcde
fr. No.16	76.7ab	2.5abc
fr. No.17	70.0b	1.4ef
fr. No.18	76.7ab	2.3abcd
fr. No.19	83.3ab	2.0bcde
fr. No.20	93.3a	2.9a
fr. No.21	90.0ab	2.3abcd
fr. No.22	76.7ab	1.7cdef
fr. No.23	96.7a	2.3abcd
fr. No.24	3.3d	0.01gh
fr. No.25	76.7ab	0.9fg
F value	11.3**	7.8**
LSD(.05)	2.2	0.8

†fr.- fraction
ns : not significant at P=0.05; **significant at the 0.01 probability level
The same letters are not significantly different at the 5% level of probability

The identification of allelochemicals

The solid obtained from the sub-fraction No. 13 which showed the most inhibitory effect was repeatedly recrystallized from chloroform and ethylacetate to yield white amorphous crystalline substance. Identification of this substance was carried on by ^{1}H NMR and gas chromatography-mass spectrometry (GC-MS).

The ^{1}H NMR spectrum was characteristic of straight-chain saturated fatty acid. The signal at δ 0.94 was assigned to a primary methyl. Two signals at δ 1.6 and 2.4 were assigned to methylene group attachted to β and α position of carbonyl group, respectively. The most intense signal at delta δ 1.3 was assigned to methylene aliphatic protons. The proposed structure was further substantiated by a comparison of the Aldrich library of ^{13}C and ^{1}H FT-NMR Spectra data with those for fatty acid (Fig. 2).

Confirmed from ^{1}H NMR, MS also showed several features of straight-chain saturated fatty acid ester (Fig. 3). Losing alkoxy group from an ester, the acylium ion ($R\text{-}C\equiv \overset{+}{O} \leftrightarrow R\text{-}\overset{+}{C} = O$) gives an easily recognizable peak for esters. In fatty acid methyl ester it occurs at M-31. Cleavage at each C-C bond gives an alkyl ion (*m/z* 29, 43, 57,...), thus there are hydrocarbon clusters at intervals of 14 amu. Carbomethoxy ions $CH_3OCO(CH_2)_n^+$ are observed at *m/z* 87, 101, 115, 129, 143, 157, 171, 185, 199, etc. The most characteristic peak at *m/z* 74 results from the McLafferty rearrangement (Fig. 3).

In order to elucidate the structures of fatty acid, IR, NMR and MS can be possible but fatty acids typically exist as complex mixtures in nature. Therefore, GC-MS is required

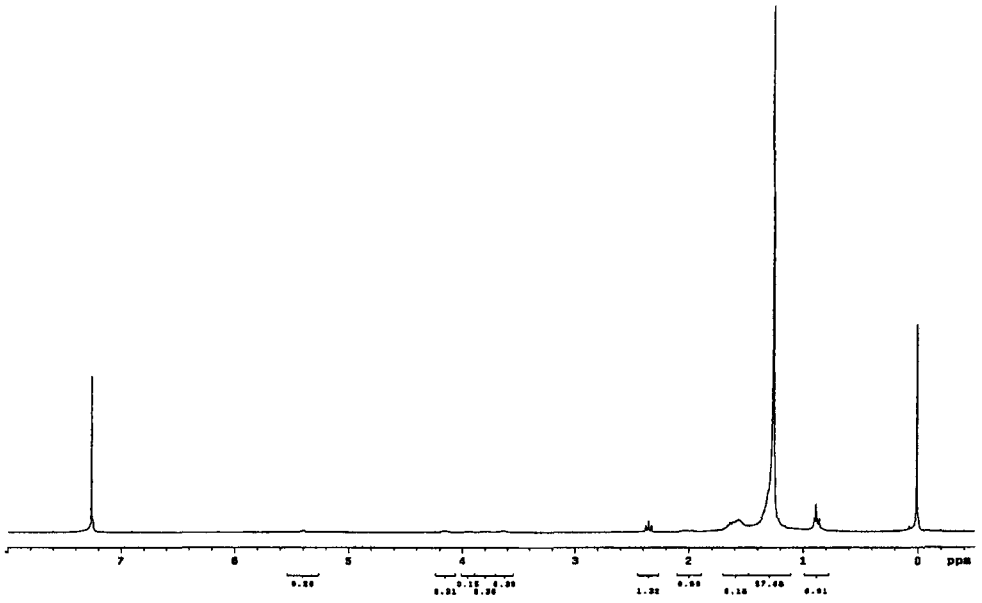


Fig. 2. ^{1}H NMR of sub-fraction No. 13(300MHz, $CDCl_3$).

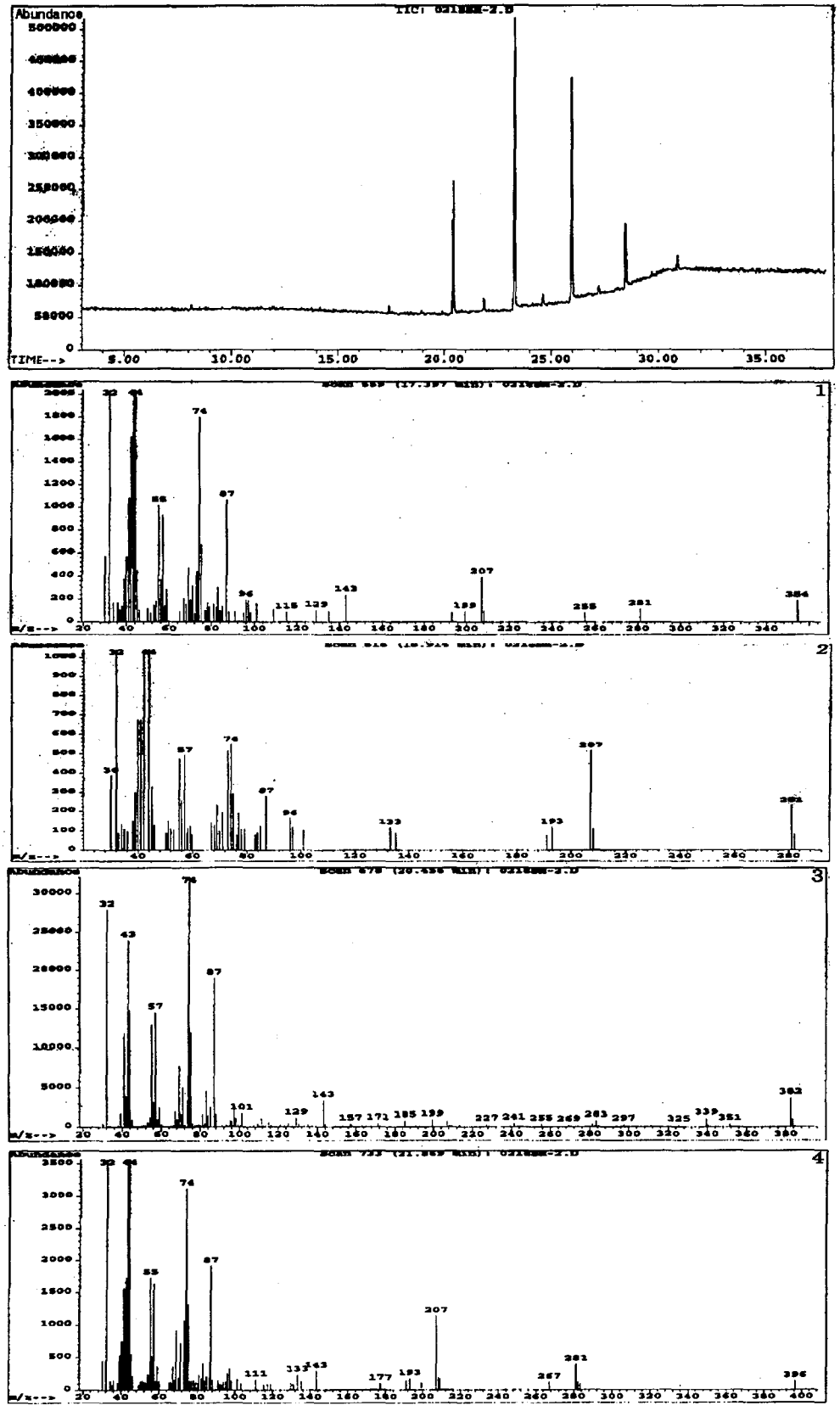
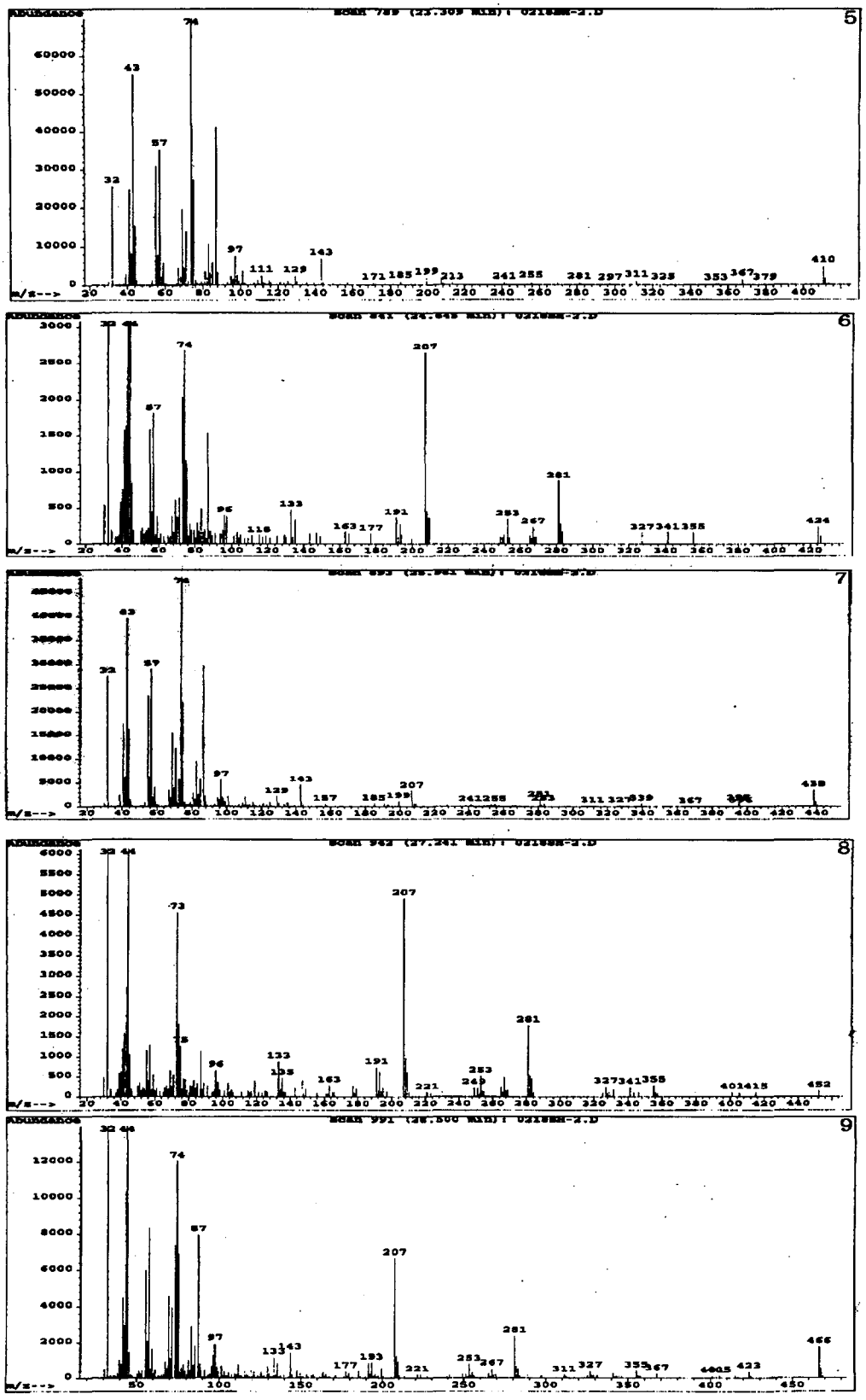


Fig. 3. Gas chromatography-mass spectrometry of sub-fraction No. 13



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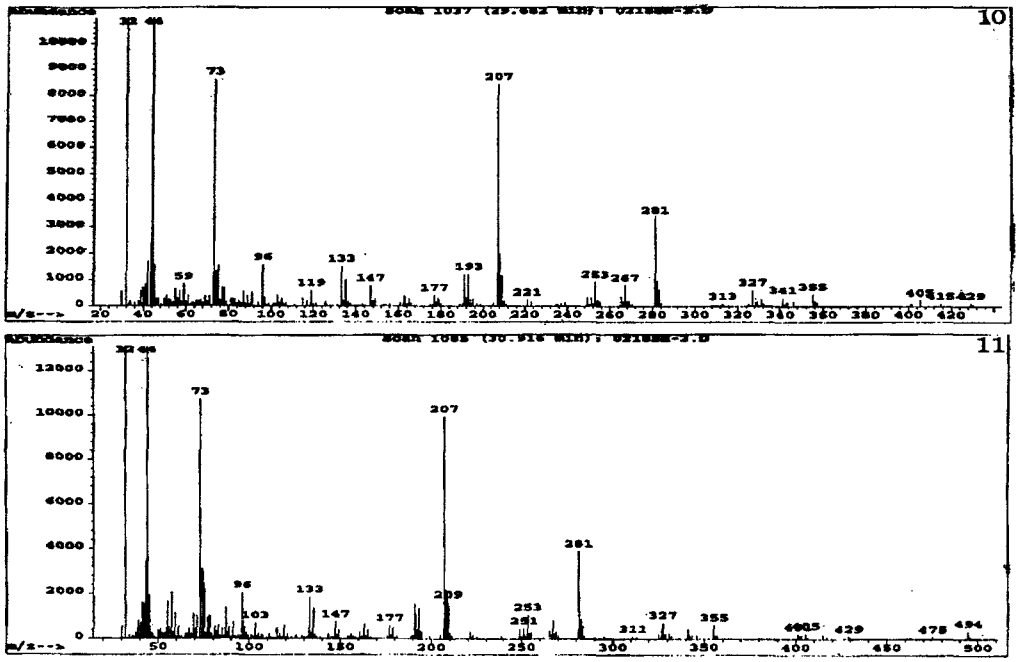

Fig. 3. Continued.

to separate many of the components (Murphy, 1993).

GC-MS analysis revealed that these allelochemicals are straight-chain saturated fatty acids. Analysis of the peaks by MS revealed that the eleven compounds with 22-32 carbons. The peaks were identified as follows with the molecular weight in parentheses: docosanoic (340), tricosanoic (354), tetracosanoic (368), pentacosanoic (382), hexacosanoic (396), heptacosanoic (410), octacosanoic (424), nonacosanoic (438), triacontanoic (452), hentriacontanoic (466) and dotriacontanoic acid (480).

All spectra showed molecular ions except tricosanoic and hentriacontanoic acid. That might be related to minute amounts. Their molecular weight thus were inferred by an interpretation of gas chromatogram showing equivalent interval corresponding same chain length of 14 amu.

It has been reported that fatty acids isolated from *Polygonum aviculare* residues and soil under *Polygonum* stands were myristic, palmitic, linolelaidic, oleic, stearic, arachidic, 11,14-eicosadienoic, heneicosanic and behenic acid. And all nine isolated fatty acids significantly inhibited growth of bermudagrass seedlings even in the low concentration of 5 ppm (Alsaadawi *et al*, 1983). The investigation above and literature from other studies make it clear that sub-fraction No. 13 has the potential for allelochemical. Potential also goes with sub-fraction No. 24, because of strong inhibitory effect on the seedling growth of alfalfa. It was considered that sub-fraction No. 24 was at least three compounds mixture rather than single compound by the thin layer chromatography developed with chloroform-methanol-water (70 : 30 : 4).

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