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Investigation and isolation of the active constituents of petroleum ether fraction of *Medicago sativa* L. sprouts

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ABSTRACT
The unsaponifiable fraction from petroleum ether extract of *Medicago sativa* L. sprouts was analyzed by GLC, a series of 17 hydrocarbons and 2 phytosterols; β-sitosterol and stigmasterol were identified. Analysis of the fatty acid fraction by GLC techniques revealed the presence of 6 saturated fatty acids and 8 unsaturated fatty acids including omega 6- fatty acid lionleic acid and 2 omega 3 fatty acids Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) in a relative percent of 1.0 % and 1.12%, respectively. These two types are rarely produced in plants but produced in fish, which have protective effects against heart diseases, liver functions and inflammations.

***Key Words*:** Medicago, omega- 3, stigmasterol, β-sitosterol.

INTRODUCTION

The genus *Medicago* (Family: Fabaceae) comprises over 85 species of annual or perennial herbs rarely shrubs (Hutchinson, 1961), *Medicago* species are distributed in the temperate regions; in the Mediterranean region, west and central Asia, North America, Ethiopia and southern Africa (Boulos, 1999). In Egypt, the genus *Medicago* is represented by 18 wild species, from which only *Medicago sativa* L. is cultivated and may be found wild as escape from cultivation (Boulos, 1999) *M. sativa* is a common forage plant present all the year. Nowadays *M. sativa* sprouts is added to salad menus in several European countriesas a healthy food but no data are available for the sprouts composition and fractions so, this study was designed to determine the chemical composition of the petroleum ether fraction of *M. sativa* sprouts.

MATERIALS AND METHODS

Plant material

Aerial parts and Seeds of *Medicago sativa* L. were collected late in June from fully mature plant cultivated in keram farms, ModerayatAltahrir, Behaira, Egypt. The identity of the plant was kindly confirmed by Prof. Dr. Abdel Megeed Ali Abdel Megeed, Prof. of Plant Taxonomy, Flora and Phytotaxonomy Researches Department, Horticultural Researches Institute, Agricultural Research Center, Dokki, Cairo, Egypt. The seeds were sprouted as follows: dry seeds weight of 4.5 kg were soaked in water for 18 hours then drained off soaking water, rinsed thoroughly every 12 h for 6 days till fresh sprouts lengthen 7-12 cm with green leaflets. Finally, the sprouts were shadow dried resulted in 4.3 kg dry sprouts.

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Chromatography

Silica gel 60, particle size (0.063-0.2), 70-230 mesh, (E-Merck, Darmstadt, Germany) was used for column chromatography. Pre-coated silica gel 60 on aluminum sheets, 0.2mm thickness, 20x20cm (E-Merck, Darmstadt, Germany) was used for TLC. Solvent systems included hexane: ethyl acetate in different ratios for column chromatography and hexane:ethyl acetate (80:20) for TLC. Anisaldehyde spray and UV light at a wavelength of 365 nm was used for visualization. Apparatus used: Rotatory evaporator (Eyela Co., Japan), Centrifuge; Jouan centrifuge 1,000-10,000 rpm, France, Spectrophotometer STAT-FAX 3300 (Florida, USA). For serum glucose and serum lipid measurements, Mass spectrometer; SSQ 7000 (Finnigan), NRC, Giza, Cairo was used. GLC analysis was carried out according to the following conditions mentioned by Vogel (1975) and A.O.A.C (2000).

Conditions for (unsaponified matter) USM

Thermo TR-5MS column, coated with 3% OV-17, Column dimensions 2.8m x 0.25 inch i.d, oven temperature 70°C, temperature programming 70°C for 2 min., increased to 270°C by the rate of 10°C/min., then isothermally for 25 min. carrier gas nitrogen, flow rate 30ml/min, sample size 2µl, injector temperature 230°C, detector temperature 280°C.

Conditions for (fatty acids) FA

Thermo TR-FAME column, coated with 10% polyethylene glycol adipate (PEGA), column dimensions 2.8m x 0.25 inch i.d, oven temperature 70°C for 2 min., increased to 190°C by the rate of 8°C/min., then isothermally for 25 min, carrier gas nitrogen, flow rate 30ml/min, sample size 2 µl, injector temperature 200°C, detector temperature 220°C.

Extraction and isolation

The dry sprouts were extracted with 70% methanol by successive percolation (10 x 8 L) and concentrated under vaccum using rotary evaporator at 45°C to yield 900g residue. A weight of 500 g of the dry alcoholic extract of the

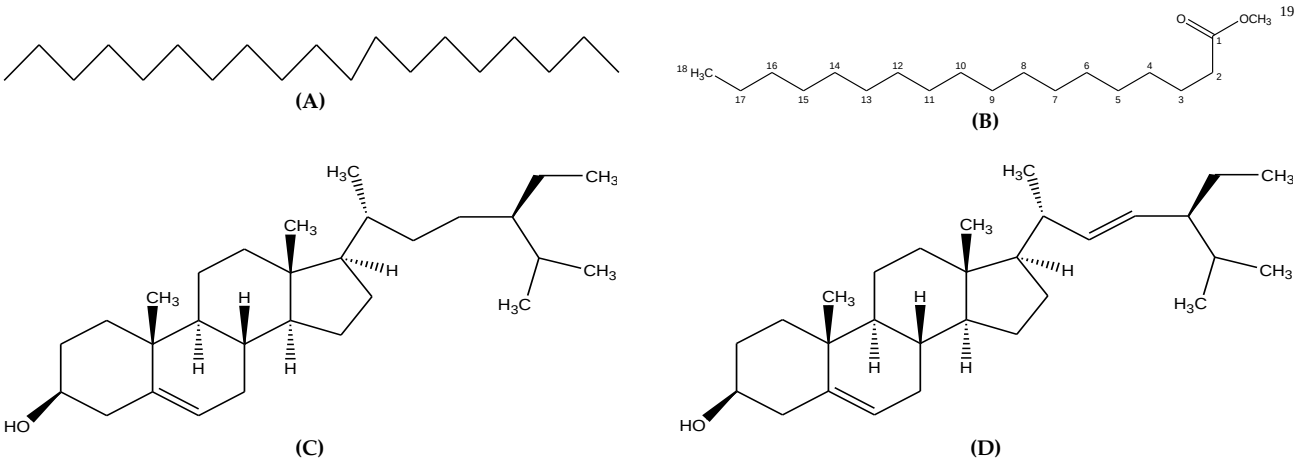

Figure 1: Isolated compounds of petroleum ether fraction of *M. sativa* sprouts: (A) n-nonadecane, (B) methyl stearate, (C) β –sitosterol, (D) stigmasterol.

sprouts of *Medicago sativa* L. was suspended in least amount of water partitioned with petroleum ether fraction yielded 32.5 g (6.5%), about 1 g of the pet. ether extract was saponified (N/2 KOH) and the unsaponified matter was separated. The liberated fatty acid mixture was extracted, methylated (methanol, 4.5% HCL). Samples of the unsaponifiable fraction and the fatty acids were subjected to GLC analysis. Petroleum ether extract (30g) was loaded on a silica gel column (8 x140cm, 800g), use mobile phase of hexane: ethyl acetate 0:100 up to 100:0 formed 88 fractions each of 250 ml which were monitored by TLC, using the solvent system hexane : ethyl acetate (80:20) the chromatograms were examined in visible and UV light at (365 and 254 nm) and sprayed with *p*-anisaldehyde reagent, similar fractions were pooled together and the solvents were separately evaporated under reduced pressure yielded 11 major fractions.

Fraction (2), eluted by 85% of hexane:ethylacetate, rechromatographed on silica gel column, use hexane:ethyl acetate (90-85%) showed compound T_1 and compound T_2.

Fraction [4], eluted by 70% of hexane:ethyl acetate, use hexane:ethyl acetate (85-70%) showed the presence of compound T_3.

Fraction [5], (420 mg): eluted by 70% of hexane:ethyl acetate showed the presence compound T_4.

These compounds were identified by spectroscopic methods (MS) and determined against reference standard compounds.

Table 1: Results of GLC analysis of the fatty acids methyl esters of saponifiable fractions of the n-hexane extracts of the sprouts of *M. sativa*.

Identified compounds	RRT*	Relative%
Decanoic (Capric) acid C10:0	0.486	1.34
Dodecanoic (Lauric) acid C12:0	0.55	1.42
Tetradecanoic (Myristic) acid C14:0	0.724	0.67
Hexadecanoic (Palmitic) acid C16:0	0.941	12.751
Hexadecanoic (Palmitoleic) acid C16:1	1	27.58
Octadecanoic (Stearic) acid C18:0	1.39	3.792
Octadecenoic (Oleic) acid C18:1	1.084	8.64
Octadecadienoic (Linoleic) acid C18:2	1.66	26.85
Octadecadienoic (Linoelaidic) acid C18:2	1.89	12.071
Octadecatrienoic (Lionleic) acid 18:3	2.31	0.53
Eicosanoic (Arachidic) acid 20:0	2.6	1.591
Eicosatetrienoic (Archidonic) acid 20:4	3.25	0.65
Eicosapentaenoic acid (EPA)	3.72	1
Docosahexaenoic acid (DHA)	4.545	1.12
Percentage of identified saturated fatty acids		21.571
Percentage of identified unsaturated fatty acids		78.429

RRT*: Relative Retention time to Palmitoleic acid with R_t=5.97mim

RESULTS AND DISCUSSION

GLC analysis of fatty acids of *M. sativa* sprouts showed the presence of 14 compounds which were identified. The identified saturated and unsaturated fatty acids were found to represent 21.1 and 76.3 relative % of FA,

Table 2: Results of GLC analysis of the unsaponifiable fractions of the n-hexane extracts of the sprouts of *M. sativa*.

Peak No.	Identified Compounds	RRT**	Relative %
1	Tetradecane	0.492	1.69
2	Pentadecane	0.506	0.726
3	Hexadecane	0.53	1.01
4	Heptadecane	0.549	0.322
5	Octadecane	0.555	1.613
6	Nonadecane	0.601	0.848
7	Eicosane	0.607	10.01
8	Heneicosane	0.637	0.906
9	Docosane	0.679	5.277
10	Tricosane	0.736	1.487
11	Tetracosane	0.753	13.254
12	Hexacosane	0.804	8.29
13	Heptacosane	0.878	1.539
14	Octacosane	0.935	3.864
15	Nonacosane	0.949	0.941
16	Stigmasterol	1	18.526
17	β-Sitosterol	1.009	9.851
18	Triacontane	1.017	5.203
19	Dotriacontane	1.023	14.54
	Percentage of total hydrocarbons		71.62
	Percentage of total sterols		28.38

RRT**: Relative retention time to stigmasterol (R_t=44.2min)

respectively. Palmitic acid (12.75%) was found to be the major saturated FA, followed by arachidic acid (1.59%), while palmitoleic acid (27.28%) was found to be the major unsaturated FA, followed by linoleic acid (26.85%) and linoelaidic acid (12.07%). The plant contains two types of Omega-3 fatty acids docosahexanoic acid (DHA) (1.12%) and Eicosapentaenoic acid (EPA) (1.0%) as illustrated in table 1. GLC analysis of USM of *M. sativa* sprouts revealed the presence of 19 compounds which were identified. The identified hydrocarbons and sterols were found to represent 71.62 and 28.38 relative % of USM, respectively. n-dotriacontane (14.54 relative %) was found to be the major hydrocarbon, followed by n-tetracosane (13.254 relative %), while stigmasterol (18.526 relative %) was found to be the major sterol in USM, followed by β-sitosterol (9.851 relative %). Stigmasterol and compactin were the most isolated sterol compounds from the plant (Shingo*et al.*, 1987) and (Huang and Grunwald, 1988) while omega 3 fatty acids were not reported before although they are detected in this research in a reasonable amounts (2.12) relative % of fatty acid methyl esters as illustrated in table 2.

Compound 1

The mass spectrum of compound T_1 showed molecular ion peak at 268, 267 m/z and other ion at m/z = 253 represented the loss of CH_2 group and other peak at m/z = 239 represented the loss of other CH_2 group. The molecular formula was estimated as $C_{19}H_{40}$. These data resembled that of a simple aliphatic hydrocarbon n-nonadecane (Guillaume *et al.*, 2003).

Compound 2

The mass spectrum of compound T_1 showed molecular ion peak at 299, 298 m/z and other ion at m/z =269 represented the loss of two methyl group and other peak at m/z = 255. The molecular formula was estimated as $C_{19}H_{38}$ O_2, The data of this compound resemble the published data of the methyl stearate (fatty acid methyl ester) (Biemann, 1962).

Compound 3

separated as yellowish white powder, Mass spectrum, showed a molecular ion peak of 414 m/z ($C_{29}H_{50}O$); 396 (M^+- H_2O), 255 (M^+- side chain) and peak at m/z 145 (18.5%) was due to fragmentation of sitosterol side chain with addition of two protons. The mass spectra of this compound were consistent with those published previously for β-sitosterol (Kamboj and Saluja, 2011).

Compound 4

Separated as white powder, mass spectrum showed a molecular ion peak of 411 m/z[M-H], [($C_{29}H_{50}O$)-H]; 383 (M- H- H_2O), 351 m/z resulted from the loss of methyl group, 255 (M^+- side chain) and peak at m/z 145 (18.5%) was due to fragmentation of sitosterol side chain with addition of two protons. The mass spectra of this compound were consistent with those published previously for stigmasterol (Kamboj and Saluja, 2011).

CONCLUSION

Petroleum ether fraction of *Medicago sativa* sprouts is a rich source of plant sterols, omega 3 fatty acids and omega 6 fatty acids like fish oils with much greater availability, so further biological studies should be performed to detect its preventive effects on atherosclerosis and heart diseases.

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