Comparative Analysis of the Fatty Acid Pattern of *Silybum marianum* with *Nigella sativa* by Gas Chromatography-Mass Spectrometry

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Abstract

Many studied were conducted to evaluate the antihepatotoxic and antioxidant activities of Silybum marianum and proved these actions. The Naturally grown seed in Iraqi-Kurdistan Region also were studied for its chemical contents and biological activities. Vegetable oils occur in various plant parts mainly concentrated in the seeds.

In this study comparison was made between the fatty acid patterns of two plant seeds, *Silybum marianum* and Nigella sativa. Seed sample of *Silybum marianum* and *Nigella sativa* were exposed for extraction and isolation of the fatty acid contents using two different solvents (petroleum ether and *n*-hexane) at 60-80^{o}C using soxhlet apparatus and the oily extract were purified and analysed by GC-MS triplet system.

Result showed that extraction with petroleum ether yielded 23% and 24% of total weight of the seeds of *Silybum marianum* and *Nigella sativa* respectively and comparable results with *n*-hexane extract yielded 17% and 22% of total seed weight of *Silybum marianum* and *Nigella sativa* respectively showing significant differences in the fatty acids patterns of both plants under research.

Key words: Fatty acids, GC-MS, *Silybum marianum*, natural products, cardiovascular diseases.

دراسة تحليلية مقارنة لأنماط الأحماض الدهنية في نباتي السليمارين والحبة السوداء بواسطة تقنية الفصل بالكروماتوغرافيا الغازي ذا الطيف الكتلي

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الخلاصة

أجريت العديد من الدراسات لتقييم فاعلية السليمارين كدواء فعال لحماية الكبد و كمضاد للأكسدة, وقد تم اثبات فاعليتها في هذا الشأن. دراسات أخرى تم اجراءها على بذور السليمارين النامية طبيعيا في أقليم كردستان العراق للكشف عن مكوناتها الكيمياوية والفاعلية الحيوية لتلك المكونات. فكا هو معلوم ان الزيوت النباتية عادة ما تتواجد في أجزاء عديدة من النبات و تتركز بصورة مكثفة في البذور.

تمت في دراستنا هذه مقارنة نوعية الأحماض الدهنية لبذور النباتين وهما الكعوب و الحبة السوداء باسخدام نوعين من المذيبات (أيثرات البترول و الهكسان العادي) حيث تم استخلاص وعزل الأحماض الدهنية لنماذج من بذور النبتتين في درجة حرارة 60-80 درجة مؤية بإستخدام جهاز السوكسليت, و بعدها تمت تنقيةالمستخلصات الزيتية و تحليلها بواسطة جهاز GC-MS.

أظهرت النتائج بأن عملية الأستخلاص بإستخدام أيثرات البترول أعطت 23% و 24% من الزيوت الثابتة نسبة الى وزن نم اذج البذور لكل من للكعوب و الحبة السوداء على التوالي وأكبر من نسبها المستحصلة بواسطة مذيب الهكسان العادي واللتي بلغت 17% و 22% على التتابع وهذا ينعكس على محتوى الأحماض الدهنية في كلتا النبتتين.

الكلمات المفتاحية: الأحماض الدهنية, الأستشرابية الغازية-الطيف الكتلي الشامل, سليمارين, المنتوجات الطبيعية, الأمراض القلبية.

Introduction

Despite the advances and developments in synthetic chemistry contributing to the production of large number of drugs, natural products remain an attractive source for many new active metabolites [(1,2,3)]. These biologically active molecules have contributed in the last fifty years to pharmaceutical industries with unique drugs that exhibit potent pharmacological activities. Natural products are known as robust sources of new drugs which are involved in production of 60% of the anticancers, and 75% of the antimicrobials [(1,2)]. Prior to 2007 natural products accounted for approximately one-half of all licensed drugs in the world [(3)]. Extensive studies on plant secondary metabolites revealed their potential biosynthetic capacity for production of biologically active molecules that are extensively used for the treatment of several acute and chronic disorders [(4)].

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Research in epidemiological fields showed that free radical-induced oxidative damage of cell membranes, DNA, and proteins played essential role in aging, degenerative diseases, such as cancer, atherosclerosis, and cataracts [5]. It has been confirmed that antioxidants metabolites extracted from plants, such as α-tocopherol, L-ascorbic acid, and β-carotene, have protective effects against the free radicals [6,7,]. In addition, the secondary metabolites, flavonoids showed wide range of therapeutic efficacy that contributed in management and supportive treatment of various diseases like cancer, liver damage, bacterial infections and diabetes [5], for example; the Silymarin which is composed mainly from flavonolignan silybins, is a component of the fruit and seed of the variegated milk thistle *Silybum marianum* L. Gaertn extract.

This plant is a wild biennial herb which grows in many parts of the world including Iraqi Kurdistan-Region (IKR) as an indigenous plant [8]. The fatty acid composition of Silybum marianum seed oil was investigated by El-Mallah et al., 2003 showing oil rich in unsaturated fatty acids, including linoleic acid, oleic acid as well as saturated fatty acids including palmitic and stearic acids [33].

The seeds of *Nigella sativa* Linn. (Ranunculaceae), which is commonly known as black seed, have been widely used in herbal medicine for the treatment and prevention of various diseases including diabetes, asthma and dyslipidaemia [11]. The seed contains both fixed and essential oils. Researches show different pharmacological activities attributed to the crude extracts of the *Nigella sativa* seeds namely antiinflammatory, analgesic, antimicrobial, antihepatotoxic and antinephrotoxicity [11,12]. The fixed oil pattern of *Nigella sativa* have been analysed, eight fatty acids were identified in the extract including saturated and unsaturated fatty acids (Lauric acid, Myristic acid, Palmitic acid, Stearic acid, Oleic acid, Linoleic acid, Linolenic acid and Eicosadienoic acid) [11].

The primary functions of fixed oils in the biologic system are to serve as energy storage [14]. The fixed oils are important products used pharmaceutically, industrially, and nutritionally [2, 3]. Many drugs contain fixed oils as their principal constituents exemplified by Omega-3 and Omega-3,6,9, which are used for the treatment of variety of disorders including hypercholesterolemia [15].

Vegetable oils occur in various plant parts, but they are mainly concentrated in the seeds [16]. Dietary supplements in form of unsaturated fatty acids has shown the potential to reduce both the progression of cardiovascular diseases and related mortality including the sudden cardiac arrest, through lowering triglycerides levels in serum, acting as antithrombotic, decreasing the blood and plasma viscosity and showing improvement endothelial dysfunctions [17,18,19]. WHO data reveals that cardiovascular diseases (CVD) contribute to the highest mortality rates among population worldwide [20]. Measurements have been taken to decrease the risks of CVD through changing the life style, dietary habits and exercise [21], one of the important measurements that are employed in this manner is dietary supplements of food and medications rich in unsaturated fatty acids, these drugs are imported, putting burdens on the economy, meanwhile; they could be obtained from the available sources in the nature around us. *Silybum marianum* seeds showed significant amounts of unsaturated fatty acids (table 2) making it a good source for these elements that could be obtained from the plant.

Materials and Method

Soxhlet apparatus (Pyrex), capacity 350ml. Rotary evaporator (Heidolf). All the solvents were of analytical grade.

(GC-MS)(Agilent 6890N gas chromatograph with a 5973 electron impact mass selective detector (Agilent, Waldbronn, Germany) according to Bode and Ring [25,26]. The column temperature was kept at 130°C for 2.5 min and then increased to 240°C at 5°C/min. The mass selective detector was operated in scan mode, with a scanning mass range of *m/z* 40 to 500.

Sample preparation

The wild *Silybum marianum* plant was harvested during June 2011 from south east (Industrial area) of Al-Sulaimaniah city, 5km far from the city centre. Morphological features were used for characterization of the collected plant. Nearly 600gm of the plant seeds were collected and stored under low temperature. In addition 1 kg of *Nigella sativa* seeds were purchased (Black Seeds (Kalonji) A1-Quality, Pakistan). According to the company's literature it was originated from western Asia. Two Hundred grams of *Silybum marianum* and *Nigella sativa* seed samples were harvested dried under shade and kept frozen at 20^{o}C until use. The seeds were ground to coarse particles using

an electric mill. Then the product was extracted using petroleum ether and *n*-hexane at 60-80^{o}C for six hours in a soxhlet apparatus [22,23]. The extract was then dried over anhydrous sodium sulphate, filtered and the solvent was removed from the filtrate by rotary evaporator under reduced pressure at 45^{o}C. The oily extract was subjected to three vacuum distillations in series to remove the debris [24]. The oil sample was stored in a vacuum desiccator for seventy two hours then centrifuged for ten minutes at 5000 rpm. Finally, the samples were aliquoted into 1.0 ml portions and stored at -20°C. Later, samples were analysed by GC-MS in triplicates.

GC-MS spectrometry

To 250 µl of each sample, equal volumes of methanol/toluene/Sulfuric acid H_2SO_4 (to separate the free fatty acids from the triglycerides) (50:50:2 V/V/V) mixture was added, and incubated overnight at 55°C. After cooling down, 400 µl of 0.5M Sodium bicarbonate NH_4HCO_3 solution was added. The mixture was centrifuged at 5000 rpm for 5 minutes at room temperature. 75 µl of the upper organic phase was mixed with 25 µl of the derivatization reagent, N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, Germany) (to esterify the free fatty acids which ease the separation by GC) and incubated at 37°C for 30 minutes. 5 µl of each sample was analysed by gas chromatography coupled to mass spectrometry (GC-MS)(Agilent 6890N gas chromatograph with a 5973 electron impact mass selective detector (Agilent, Waldbronn, Germany), using a dimethyl- (5% phenyl)-polysiloxane capillary column (Agilent HP-5ms, 0.25 mm by 30 m by 0.25 µm) and helium as the carrier gas at a flow rate of 1 ml/min according to Bode *et al.,* (2006) and Ring et al., (2006). Samples were injected in split mode (split ratio, 10:1). The column temperature was kept at 130°C for 2.5 min and then increased to 240°C at 5°C/min. The mass selective detector was operated in scan mode, with a scanning mass range of *m*/*z* 40 to 500.

Fatty acids were identified using the National Institute of Standards and Technology (NIST) mass database and MassBank database and based on mass spectral and retention index libraries for metabolomics and time of flight gas chromatography/gas chromatography. As control , FAME mix reference (fatty acids essential standards) from (Sigma-Germany) was run under the same experimental condition.

Results and Discussion

In this work we have determined the fatty acid contents of wild *Silybum marianum*, and compared to that of *Nigella sativa*. Fatty acids represent a chemically inert class of organic compounds that are easy to extract from biological material. Normally, fatty acids are acids produced in cell after catabolism break down of fat. These compounds are hydrophobic and not water soluble. They are important part of a healthy diet and body requires them for organs and tissues and utilize them in many cellular activities [14]. Fatty acid compounds found usually either in saturated or unsaturated state. Saturated branched chain fatty acids are present as complex mixtures in numerous biological samples. Branched chain fatty acids are usually saturated and the branch is normally a methyl-group. The common constituents of the lipids in plants are branched fatty acids, and they are rarely found in the integral lipid. Unsaturated branched-chain fatty acids are normally found in marine animals, and branches other than methyl may be present in microbial lipids.

The high-throughput gas chromatography/mass spectrometry (GC/MS) technology enables analysing a huge number of chemicals and biological samples. One of the important analyses of GC/MS data is compound identification. We characterized the fatty acid profiles of *Silybum marianum* to show its difference from that of *Nigella sativa* seeds. Extraction solvent has great impact on fatty acid extraction yield [27]. Petroleum ether and *n*-hexane have low polarity index of 0.1, therefore in our work we aimed to use them as extracting solvents for the fatty acid content of the *Silybum marianum* and *Nigella sativa* seeds. As shown in table 1, the use of petroleum ether in extraction of 200 grams of each *Silybum marianum* and *Nigella sativa* seeds yielded 23.5% and 24% of the total weight respectively, while 17% and 22% of the seeds weight were yielded from *Silybum marianum* and *Nigella sativa* respectively by extraction using *n*-hexane (Table-1).

Table1: Oil yields obtained from extraction of *Silybum marianum* (L.) Gaertn, and *Nigella sativa* seeds using organic solvents petroleum ether and *n*-hexane

Plant seeds	Extraction yield using organic solvents	
	Petroleum Ether	*n*-hexane
***Silybum marianum* (L.) Gaertn**	23%	17%
Nigella sativa	24%	22%

The differences in lipid content between *Silybum marianum* and *Nigella sativa* by extraction with different organic solvents might resulted from genetic regulation of fatty acid biosynthesis machinery as well as differences in physiological and environmental effects. Identification of the fatty acids contents was evaluated by comparison to spectra of highest similarity to the proposed substances in the NIST Chemistry WebBook mass database library maintained by the National Institute of Standards and Technology (NIST) as a library of reference spectra and repetitive mass spectral data as query spectra [28,29,32]. The analysis of the plants seeds extract the fatty acids of variable chain length including both saturated and unsaturated structures (14:00, iso-15:0, 16:1ω7, 16:00, 18:2ω6,9, iso-17:0, 18:02, 18:01, and 18:00) were identified (Figure 1).

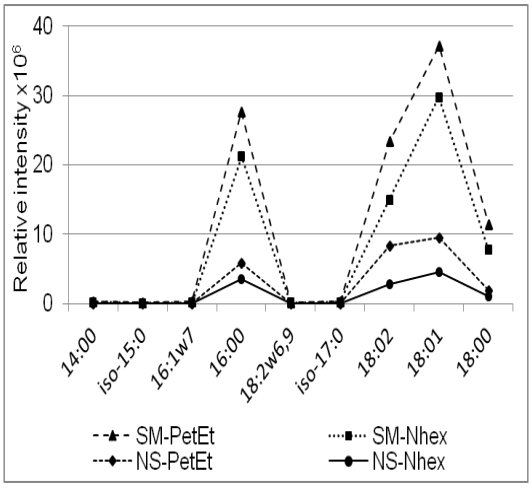


Figure 1: Diagram showing the relative amount of the fatty acid compound found in extracts of the *Silybum marianum* and *Nigella sativa* seeds using petroleum ether. The data was estimated by measuring the peak area of the GC analysis.

The gas chromatogram of the extracts shows that the level of fatty acid content in *Silybum marianum* extracts is significantly higher than the extracts of *Nigella sativa* (Figure 2).

In addition, the use of petroleum ether in extraction of both seeds of *Silybum marianum* & *Nigella sativa* obtaining resulted in extraction of higher amount of fatty acids than extraction with *n*-hexane (table 1).

This might be related to the differences in polarity of the solvents [23]. In comparison to the saturated fatty acid contents of the seeds extracts, low amounts of each branched (iso-15:0 and iso-17:0) and unsaturated omega fatty acids were identified. Although differences in the fatty acid content were observed, but the percentage of the compounds found in the plants extracts showed a high similarity (Table 2).

The only differences in fatty acid patterns is that of *Nigella sativa*, in which no unsaturated omega fatty acid, 18:2ω6,9 was investigated. The ratio of unsaturated fatty acids to the saturated in *Silybum marianum* seed oil extract were compared and showed ratio of 1:54, which revealed significant higher content in the favour of the unsaturated omega fatty acids as shown in table 2. The percentage of unsaturated fatty acids was 60.67% comprises to the total fatty acid in the sample extract. While the percentage of omega fatty acids were 0.65% with pet-ether extract of the total unsaturated fatty acids. Such a high ratio will make this plant a good candidate for production of omega fatty acids rich oil in the region.

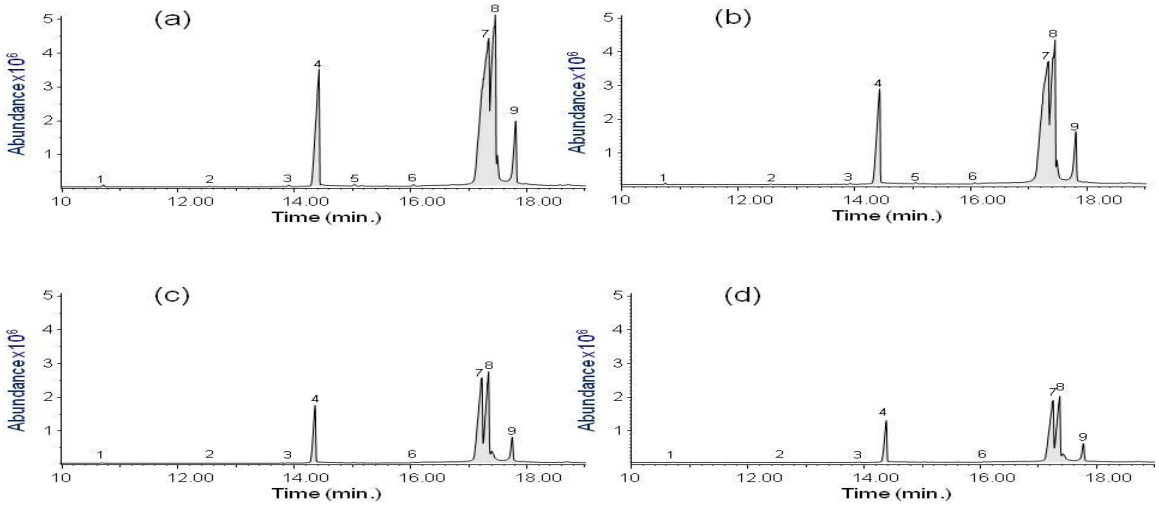
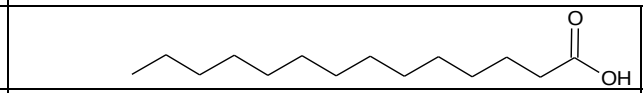
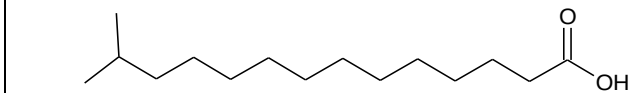
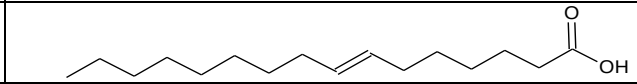
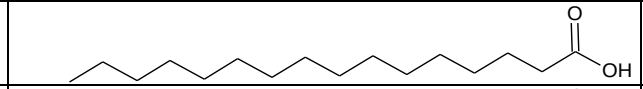
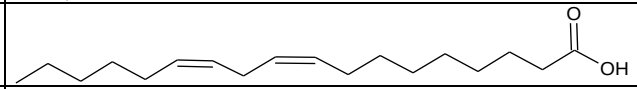
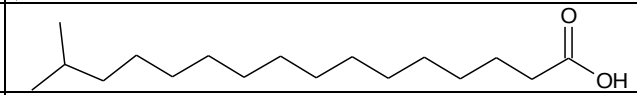
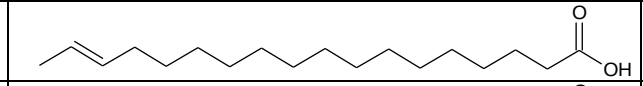
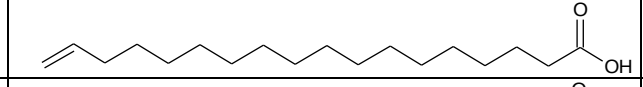
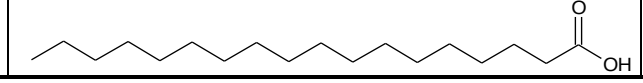


Figure 2: GC-MS/MS spectroscopy analysis of the organic extracts a: *Silybum marianum* seeds using petroleum ether and *n*-hexane, b: *Silybum marianum* seeds using *n*-hexane, c: *Nigella sativa* seeds extract using petroleum ether and *n*-hexane, d: *Nigella sativa* seeds extract using *n*-hexane. The peaks 1= 14:00, 2= iso-15:0, 3= 16:1ω7, 4= 16:00, 5= 18:2ω6,9, 6= iso-17:0, 7= 18:02, 8= 18:01, and 9= 18:00 fatty acids).

Table 2: Percentage of the fatty acid content of both *Silybum marianum* & *Nigella sativa* plant seed extracts using petroleum ether and *n*-hexane.

No.	Fatty acid		Fatty acid ratio (%)			
			SM-PetEt	SM-Nhex	NS-PetEt	NS-Nhex
1		**14:00**	0.25	0.25	0.16	0.12
2		**iso-15:0**	0.07	0.08	0.04	0.05
3		**16:1ω7**	0.24	0.18	0.13	0.11
4		**16:00**	27.47	28.52	22.81	29.81
5		**18:2ω6,9**	0.16	0.18	0	0
6		**iso-17:0**	0.23	0.21	0.13	0.16
7		**18:02**	23.3	20.14	32.28	22.75
8		**18:01**	36.9	40	36.82	38.41
9		**18:00**	11.38	10.46	7.63	8.59

Conclusion

The differences in fatty acid pattern can be used to differentiate between the two plants. However genetic analysis and transcriptomic studies are required to shed more light on the absence of the fatty acid in the extracts components.

Fatty acid (FA) profiles can be used as chemotaxonomic markers to define groups of various taxonomic ranks in flowering plants, trees and other embryophytes.

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