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INVESTIGATION OF CONSTITUENTS AND BIOLOGICAL ACTIVITY AND GC-MS ANALYSIS OF OIL FROM SAUDI ARABIA SPECIES: LACTUCA SATIVA

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ABSTRACT

The present study was designed to investigate the chemical constituents of Saudi *Lactuca sativa* seed oil and to evaluate its potential antimicrobial activity. 20 components were detected by GC-MS analysis. major constituents are: 6- Octadecenoic acid, methyl ester, (Z)- (64.01%), 9,12-Octadecadienoic acid (Z,Z)-, methyl ester(13.21%), Carotol (9.92%), Hexadecanoic acid, methyl ester (6.50%), Methyl stearate (1.40%) and Daucol (1.26%). The antimicrobial activity of the oil was evaluated via cup plate agar diffusion assay against five standard human pathogens (gram negative :staphylococcus aureus, bacillus subtilis and the fungi Candida albicans, gram positive: Escherichia coli and Pseudomonas aeruginosa. *Lactuca sativa* oil showed good activity against Escherichia coli and Pseudomonas aeruginosa. However, it was partially inactive against other test organisms. Percentage radical scavenging activity by samples was good active.

INTRODUCTION

Lactuca sativa Linn, is one of the most important vegetable crops in the world. It belongs to the Asteraceae family[1], annual or biennial vegetation. It is edible, delicious, and has a crisp taste. At the same time, it can also stimulate the secretion of digestive enzymes, increase appetite, promote the peristalsis of the human intestinal wall, and prevent constipation. There are rich genetic variations among different cultivation types of lettuce, resulting in rich phenotypic differences.[2] In recent years, researches on lettuce gene modification have continued to emerge, including speeding up the seed germination process, changing the color of leaves, and improving disease resistance, insect resistance, herbicide resistance, salt tolerance, cold tolerance, storage tolerance, fruit flavor, lycopene yield, *etc.*[3]

Lactuca sativa Linn. (or lettuce) is a well known plant worldwide due to its use in the preparation of salad, soup, and vegetable curries.[4] This plant also has excellent medicinal properties.[5] Lettuce is cultivated worldwide, and is one of the most consumed green leafy vegetables in the raw form for its taste and high nutritive value. It is regarded as an important source of phytonutrients.[6] It is characterized by considerable morphological and genetic variations. This leaf vegetable was first cultivated by the Egyptians.[7] Today, it is produced all over the world and is used in forensic medicine for many ailments including pain, stomach problems and inflammation, and urinary tract infections.[8] Different studies provided the scientific evidence of its pharmacological potential including antimicrobial, antioxidant, neuroprotective, and hypnotic effects.[9] The chemical composition of the plant revealed the presence of different classes of secondary metabolites, such as terpenoids, flavonoids, and phenols which should be responsible for its biological activities. The plant also contains essential elements, such as vitamins as well as minerals and organic substances.[10]

MATERIALS AND METHODS

Plant material

Lactuca sativa Seeds were collected from Saudi Arabia. The plant was authenticated by direct comparison with a herbarium sample.

Methods

Phytochemical screening

Lactuca sativa was screened for major secondary metabolites according to the method described by harborne. Lactuca sativa was extracted with 80% aqueous methanol (soxhlet) until exhaustion. This prepared extract (PE) was used for phytochemical screening.[11]

Test for unsaturated sterols and triterpenes

10 ml of the (PE) were evaporated to dryness on a water bath and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chloroform solution was dehydrated over sodium sulphite anhydrous. 5ml portion of the solution was mixed with 0.5 ml of acetic anhydride, followed by two drops of concentrated sulphuric acid. Two separate layers (green, red) were observed.

Test for flavonoids

(20 ml) of the (PE) were evaporated to dryness on water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

-To 3 ml of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added. Red color was observed.

-To 3 ml of the filtrate few drops of aluminium chloride solution were added. A dark yellow color was formed.

-To 3 ml of the filtrate few drops of potassium hydroxide solution were added. A dark yellow color was observed.

Test for alkaloids

(10 ml) of the (PE) were evaporated to drynees on water bath and 5 ml of 0.2 N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and divided into two portions:

To one portion a few drops of Mayer reagent were added. A white precipitated appeared, to the other portion few drops of Wagner reagent were added. A brown precipitate appeared.

Test for tannins

(10 ml) of (PE) were evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n- hexane and 10 ml of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled, filtrated and the volume adjusted to 10 ml. with more saline solution. 5 ml of this solution was treated with few drops of ferric chloride solution. A dark blue color was observed.

Test for saponins

(1 g) of dried powdered plant material was placed in a test tube. 10 ml of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds, and allowed to stand. Honey comb was formed.

Extraction of oil from *lactuca sativa*

Dry powdered seeds of *lactuca sativa* (300 g) were macerated with hexane at room temperature for 48 h. The solvent was removed under reduced pressure and the oil was kept in the fridge at 4°C for further manipulation.

Esterification of oil

2ml of the sample was mixed thoroughly with 7ml of alcoholic sodium hydroxide (NaOH) that was prepared by dissolving 2 g in 100 ml methanol. 7 ml from alcoholic sulfuric acid (1ml H_2SO_4 to 100 ml methanol) was then added. The mixture was then shaked for 5 minutes. The content of the test tube was left to stand overnight. 1 ml of Super saturated sodium chloride (NaCl) was then added and the contents being shaken. 2ml of normal hexane was added and the contents were shaked thoroughly for three minutes. Then the n-hexane layer (the upper layer of the test tube) was taken using disposable syringe. 5 µl from the n-hexane extract was diluted with 5 ml of diethyl ether. Then the mixture was filtered through syringe filter 0.45 µm and dried with 1g of anhydrous sodium sulphate as drying agent and 1µl of the diluted sample was injected in the GC. MS instrument.

GC-MS analysis

The qualitative and quantitative analysis of the sample was carried out by using GM/MS technique model (GC/MS-QP2010-Ultra) from japans 'Simadzu Company, with serial number 020525101565SA and capillary column (Rtx-5ms-30m×0.25 mm×0.25µm). The sample was injected by using split mode, helium as the carrier gas passed with flow rate 1.61 ml/min, the temperature program was started from 60c with rate 10c/min to 300c as final temperature degree with 5 minutes hold time, the injection port temperature was 300c, the ion source temperature was 200c and the interface temperature was 250c. The sample was analyzed by using scan mode in the range of m/z 40-500 charges to ratio and the total run time was 29 minutes. Identification of components for the sample was achieved by comparing their retention index and mass fragmentation patents with those available in the library, the National Institute of Standards and Technology (NIST)., results were recorded.

Antimicrobial assay

Lactuca sativa oil was screened for antimicrobial activity against five standard human pathogens (Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans and Aspergillus niger) using the cup plate agar method with some minor modifications.

Preparation of bacterial suspensions

One ml, aliquots of 24 hours broth culture of the test organisms were distributed onto agar slopes and incubated at 37°C for 24 hours.

The bacterial growth was harvested and washed off with

sterile normal saline, and finally suspended in 100 ml of normal saline to produce suspension containing about 10^8-10^4 colony forming units per ml. The suspension was stored in refrigerator at 4°C until used. The average number of viable organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volume (0.2 ml) of the appropriate dilutions were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature to dry and then incubated at 37°C for 24 hours.

Preparation of fungal suspensions

Fungal cultures were maintained on potato dextrose agar incubated at 25°C for four days. The fungal growth was harvested and washed with sterile normal saline and the suspension was stored in the refrigerator until used.

Antimicrobial assay

The cup plate agar diffusion method was adopted with some minor modification, to assess the antimicrobial activity of the *lactuca sativa* oil. Two ml of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45°C in water bath.

(20ml) Aliquots of the incubated nutrient agar were distributed into sterile petri dishes and the agar was left to settle. In each of these plates which were divided into two halves, two cups in each half (10 mm in diameter) were cut using sterile cork borer (No.4). Each of the halves was designed for one of the test samples.

The agar discs were removed and cups were filled with (0.1 ml) of test sample using adjustable volume microtiter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for 24 hours. After incubation the diameters of the resultant growth inhibition zones were measured in duplicates and averaged.

Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but instead of nutrient agar potato dextrose agar was used. Samples were used here by the same concentrations mentioned above.

Antioxidant Activities

DPPH radical scavenging assay

The DPPH radical scavenging was determined according to the method of Shimada et.al. (1992). With some modification. In 96-wells plate, the test samples were allowed to react with 2.2Di (4-tert-octy1phenyl)-1-picryl-hydrazyl stable free radical.

(DPPH) for half an hour at $37C^0$. The concentration of DPPH was kept as (300µm).

The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiplate reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests and analysis were run in triplicate.[12]

RESULTS AND DISCUSSION

Phytochemical screening

In the present study, Saudi Arabia *lactuca sativa* extracts were screened for the occurrence of bioactive compounds. The results (Table:1) positively showed the presence of flavonoids, saponins, tannins and alkaloids in both aqueous and methanolic extract of *lactuca sativa.* These findings suggested that phytochemicals present in *lactuca sativa* are potentially beneficial as therapeutic and antioxidative agents in pharmaceuticals, food and other related industries.

Table 1: Phytochemical screening of *lactuca sativa.*

Species	Flavonoids	Saponins	Alkaloids	Tannins
lactuca sativa	+ve	+ve	+ve	+ve

GC-MS analysis of *lactuca sativa*

Gc-MS analysis of *lactuca sativa* oil was conducted and the identification of the constituents was initially accomplished by comparison with the MS library (NIST) and further confirmed by interpreting the observed fragmentation pattern. Comparison of the mass spectra with the database on MS library revealed about 90-95% match.

Constituents of oil

The GC-MS spectrum of the studied oil revealed the presence of 20 components (Table 2). The typical total ion chromatogram (TLC) in shown in fig.1.

Table 2: Constituents of lactuca sativa oil.

ID#	Name	Ret.Time	Area	Area%
1.	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7- methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-	8.905	437085	0.27
2.	Caryophyllene	9.463	62539	0.04
3.	.beta.-copaene	10.121	103271	0.06
4.	Methanol, [6,8,9-trimethyl-4-(1-propenyl)-3- oxabicyclo[3.3.1]non-6-en-1-yl]-	10.503	535333	0.33
5.	cis-p-Mentha-2,8-dien-1-ol	10.621	155057	0.09
6.	Carotol	11.690	16317678	9.92
7.	Daucol	12.197	2072510	1.26
8.	9-Hexadecenoic acid, methyl ester, (Z)-	14.858	1206505	0.73
9.	Hexadecanoic acid, methyl ester	15.041	10689430	6.50
10	Methyl 5,12-octadecadienoate	16.565	302084	0.18
11	6,9-Octadecadienoic acid, methyl ester	16.608	205422	0.12
12	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	16.688	21738269	13.21
13	6-Octadecenoic acid, methyl ester, (Z)-	16.784	105278753	64.01
14	Methyl stearate	16.963	2310926	1.40
15	cis-11-Eicosenoic acid, methyl ester	18.497	878809	0.53
16	Eicosanoic acid, methyl ester	18.717	207889	0.13
17	Kauran-18-oic acid, 7-oxo-, methyl ester, (4.beta.)-	19.811	456530	0.28
18	Kaur-16-en-18-oic acid, 13-hydroxy-, methyl ester, (4.alpha.)-(.+/-.)-	20.207	1149258	0.70
19	.gamma.-Sitosterol	26.500	308780	0.19
20	.beta.-Amyrin	26.947	88019	0.05

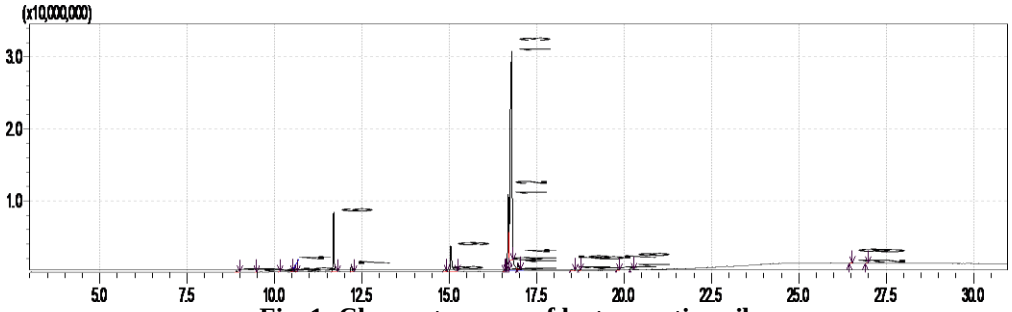

Fig. 1: Chromatograms of lactuca sativa oil.

6-Octadecenoic acid, methyl ester, (Z)- (64.01%)
9,12-Octadecadienoic acid (Z,Z)-, methyl ester(13.21%) Carotol (9.92%)
1- The major constituent are discussion (Fig 2) shows the mass spectrum of 6-Octadecenoic acid, methyl ester, (Z)-, The peak at m/z 296 which appeared at R.T. (16.678) in total ion chromatogram, corresponds to the moleculer ion M^+ $[C_{19}H_{36}O_2]^+$

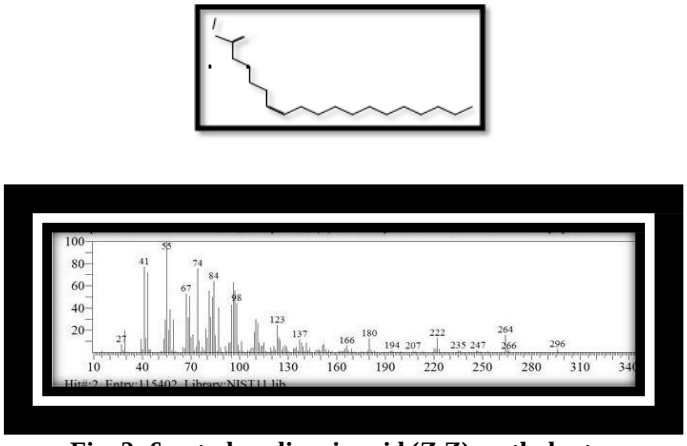

Fig. 2: 6-octadecadienoic acid (Z,Z),methyl ester.

2- The major constituent are discussion (Fig 3) shows the mass spectrum of 9,12-Octadecadienoic acid (Z,Z)-, methyl ester, The peak at m/z 294 which appeared at R.T. (16.688) in total ion chromatogram, corresponds to the moleculer ion M^+ $[C_{19}H_{34}O_2]^+$

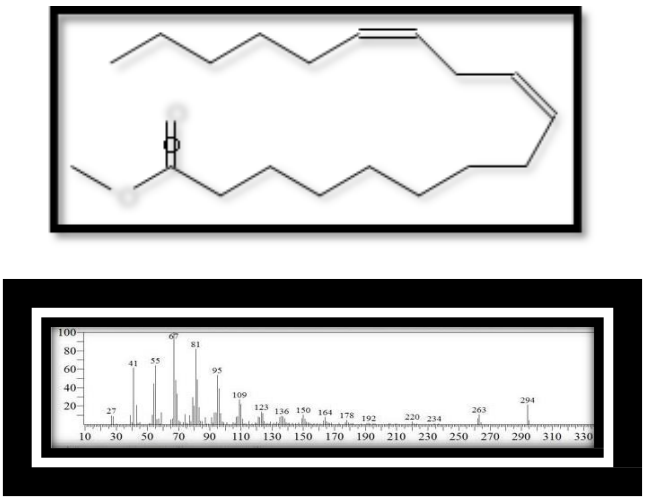

Fig. 3: 9,12-Octadecadienoic acid (Z,Z)-, methyl ester(13.21%).

3- The major constituent are discussion (Fig 4) shows the mass spectrum of Carotol, The peak at m/z 222 which appeared at R.T. (11.690) in total ion chromatogram, corresponds to the moleculer ion M^+ $[C_{15}H_{26}O]^+$

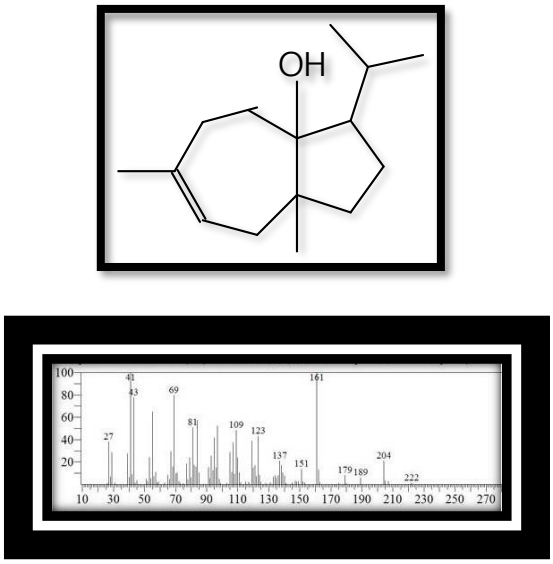

Fig. 4: Carotol (9.92%).

Antimicrobial assay

In cup plant agar diffusion bioassay, the oil was screened for antimicrobial activity against five standard human pathogens. The average of the diameters of the growth of inhibition zones are depicted in Table3. The results were expressed in terms of the diameter of the inhibition zone:< 9 mm, inactive; 9-12 mm, partially active; 13-18 mm, active ;> 18 mm, very active.

	Conc.(mg/ml)	E.C	Ps.a	S.a	B.s	C.a
Oil	100	**10-12**	**10-10**	**8-9**	**8-8**	**8-8**

E.c: Escherichia coli
Ps.a: Pseudomonas aeruginosa S.a: Staphylococcus aureus
B.s : Bacillus subtilis C.a: Candida albicans

In cup plate agar diffusion method *lactuca sativa* oil showed partially active against Escherichia coli and Pseudomonas aeruginosa, but inactive against Bacillus subtilis and Candida albicans.

Antioxidant

Percentage radical scavenging activity by samples in comparison with a DMSO (standard 90±0.01) treated control group 59± 0.07.

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