Extraction of the Volatile Oil of Stachys lavandulifolia and Study the Activity of Antibacterial and Antioxidant

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Abstract:

The volatile oil of Stachys lavandulifolia was extracted from the aerial parts of the plant, with colorless and has aromatic odor. The yield were represented in the weight percentage of extracted oil was obtained (0.195% w/w) at best condition. Effects of experimental parameters such as temperature at (40 - 100) C^{o} and the time (1 - 5) hrs on the efficiency of the extraction were studied.

The experiments were designed by using Box-Wilson method, a relationship between the above two experimental parameters and weight percentage of extracted oil were obtained. The experimental data were fitted to second order polynomial methods, and the optimum conditions for the extraction process were obtained.

The chemical composition of extracted volatile oil was analyzed by GC/MS showed the presence of fifty fife components representing (96.4)% of the volatile oil. The main components were α-Thujene (3.8)%, α-Pinene (7.8)%, β-Pinene (8.2)%, Myrcene (11.4)%, β-Phellandrene (14.9)%, Z-β-Ocimene (5.0)%, germacrene-D (16.1)%.

The volatile oil has limited inhibition and show activity against gram positive comparative with gram negative. Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae were more resistance of volatile oil, which showed good antioxidant activity.

Keywords: Extraction of the Volatile Oil, Stachys lavandulifolia, Antibacterial, Antioxidant.

Introduction:

Starchy species which belong Lamiaceae family are grown and endemic in many parts of north of Iraq and used in the treatment of various disorders in folk and traditional medicines. Extracts obtained from aerial parts of *Stachys inflata* have been used in folk medicine in treatment of infection, rheumatic and other inflammatory disorders and histological examination showed a marked reduction in tissue injury and inhibition in neutrophil infiltration in rats treated with the extract (200 mg/kg) [1].

The ethanol extracts obtained from *Stachys* spruneri, exhibited antioxidants in preventing oxygen radical and hydrogen peroxide induced cytotoxicity and tissue damage in various human diseases [2].

The aqueous extract of *Stachys riederi* inhibited systemic allergic and histamine release and these results provide evidences that may be beneficial in the treatment of acute and chronic allergic diseases [3].

Anti-inflammatory effects of acetone and methanolic extracts of aerial parts of Stachys byzanthina were investigated in the inhibition of pain and inflammatory processes [4].

The aim of the present work is the extraction of volatile oil from the aerial parts of the *Stachys* lavandulifolia. The effects of temperature and time on the extraction efficiency were studied. The Activity of Antibacterial and Antioxidant to the volatile oil were studied also.

Experiments and material:

Plant material:

The plant material of Stachys lavandulifolia was collected in May 2010 from Shaglawa, Erbil north of Iraq and identified with according to confirmed by the herbarium in Department of Biology, College of Science, University of Baghdad, Iraq.

Extraction Unit:

The extraction was carried out in a three – neck, 3-L Pyrex glass flask to which a condenser (vertical position), a thermometer and a mechanical stirrer is made of stainless steel and driven by a (1.25 kW) / (220 – 240 v) motor were fitted. The flask was placed in a water bath (Memmert WB22 - Germany). The overall set up of the extraction unit is shows in Fig.(1).

The volatile oil was extracted by soaked (10) gm of aerial parts of the plant in (500) ml of the distilled water at temperature (40 - 100) ^{o}C and time (1 - 5) hrs with slow stirring (250) r.p.m. by using stirrer. The produced mixture were filtered and (200) ml of diethyl ether was added. The mixture was shacked and placed in the Separating funnel to separating a volatile oil from water. The extracted volatile oil was dried over anhydrous sodium sulfate, and then disposed of the solvent

by using a rotor evaporator and keep oil output to conduct the tests.

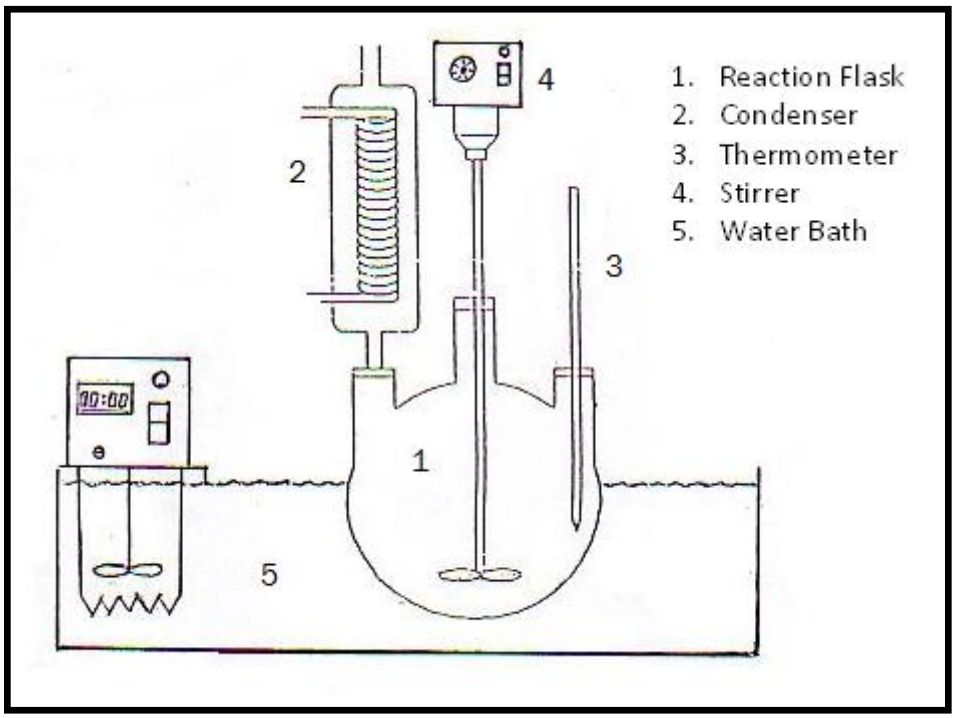

Figure (1): Extraction Unit**.**

Analysis the Constituents of the Extracted Oil By GC/MS:

The analytical Gas Chromatography (GC) method was carried out using the apparatus Shimadzu GC/MS-QP 5050 A. Software Class 5000.Column: DBI, 30m x . 35 mm i.d, 1.5 μm film thickens was used with Helium as carrier gas at a flow rate (l ml/min). Ionization mode: E.I. at (70 ev). Initially temperature program is 30 ^{o}C (static for 2 min) then gradually increasing (at a rate of 2 ^{o}C /min) up to (250) ^{o}C (static for 5 min). Detector (FID) temperature (280) ^{o}C, and injector temperature (280) ^{o}C.

Qualitative identification of the volatile constituents was achieved by library searched data base Willey 229 LIB., and by comparing their mass fragmentation patterns with those of the available published data [5, 6]. The quantitative estimation of the volatile constituents was determined by computerized peak area measurements using internal normalization method.

Antimicrobial activities:

The antibacterial effect of the volatile oil on the growth of twelfth types of the pathogenic bacteria gram positive (Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Streptococcus pyogenes, Bacillus subtilis, Bacillus cereus) and gram negative (Proteus mirabilis, Escherichia coli, Salmonella typhimuium, Salmonella enteritidis, Pseudomonas aeruginosa, Klebsiella pneumoniae) using disc diffusion assay in which the paper discs were saturated with the volatile oil directly [7]. All bacteria were supplied from Department of microbiology, College of medicine, University of Babylon, Iraq.

Four mm discs of filter paper (Whitman No. 3) were impregnated in volatile oil solution in diethyl ether (0.1) % which were placed on the surface of nutrient agar seeded with tested micro–organisms. The plates incubated at (37) ^{o}C for (24) hrs and at (25) ^{o}C for (72) hrs to investigate the antibacterial (Chloramphenicol 5 μg/ discs) is use as the control.

Antioxidant activities:
Chromatographic analysis of TLC:

$$W\% = W_V / W_T \times 100\% \quad \ldots\ldots(1)$$

TLC was carried out on (10 × 20) cm silica gel plates (Merck, Germany), (5) µL of volatile oil was applied to (1) cm of the base of plat and developed with benzene/ethyl acetate (75:25 v/v).

TLC plates were used to detect antioxidant activity based on spraying the plates with oxidizing reagents.

The separated compounds on TLC plates were spraying with ß-carotene/ linoleic acid reagent as described by [8] to located and detect antioxidant active compounds. The protecting against the bleaching ß-carotene gave orange spots were taken as positive results .The (R_f) values of pigments were measured.

Free radical scavenging:

The free radical scavenging activity of volatile oil of Stachys lavandulifolia was determined by the DPPH assay described by [9]. In its radical form, DPPH absorbs at (517) nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, (0.1) mM solution of DPPH in methanol was prepared and 4 mL of this solution was added to (1) mL of sample solutions in methanol at different concentrations. Thirty minutes later, the absorbance was measured at (517) nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated.

Results and Discussion:

Analysis and optimization of Experimental Results:

The response of experimental work conducted according to Box-Wilson [10], is yield were represented by the weight percentage of extracted oil (W %) :

Where:

W_V: weight of extracted volatile oil (mg).
W_T: weight of the aerial parts of the plant material (mg).

A second order polynomial equation is employed in the range of the independent two variables (X_1= extraction temperature and X_2= extraction time) were considered. The general form of a second order polynomial can be represented in the following equation:

$$W\% = B_o+B_1X_1+B_2X_2+B_4X_1^2 +B_5X_2^2 +B_6 X_1X_2 \quad \text{.....(2)}$$

The real data of Table (1) were fitted to equation (2), so that the regression analysis of central composite design can be applied to the approximation model to obtain the optimum conditions.

Table (1): Coded and Real Values with yield were represented by weight percentage of extracted oil using Central Composite Routable Method**.**

Exp. No.	Coded variables		Real variables		W %
	X_1	X_2	X_1	X_2	
1	1	1	91.21	4.414	0.194
2	-1	1	48.78	4.414	0.102
3	1	-1	91.21	1.585	0.033
4	-1	-1	48.78	1.585	0.098
5	1.414	0	100.00	3.000	0.056
6	0	1.414	70.000	5.000	0.191
7	-1.414	0	40.000	3.000	0.051
8	0	-1.414	70.000	1.000	0.080
9	0	0	70.000	3.000	0.156
10	0	0	70.000	3.000	0.156
11	0	0	70.000	3.000	0.156
12	0	0	70.000	3.000	0.156

The coefficients of equation (2) are determined by using available STATISTICA software ver. 6. Thus, the form of the equation representing the weight percentage of extracted oil can be written as follows:

Correlation coefficient (R) =0.9657.

Statistical analysis was made by using the above software according (T-test) and least significant differences (L.S.D) at P-Value equal or less than (0.05). Table (2) shows that all coefficients of equation (3) were significant.

$$W\% = -0.2460 +0.0116 X_1 - 0.0347 X_2 - 0.0001 X_1^2 -0.0039 X_2^2+0.0012 X_1X_2 \quad \text{.....(3)}$$

Table (2): Statistical analysis according (T-test) and least significant differences (L.S.D).

Predictor	Coef.	SE.	T-Test	P-Value
Constant	-0.2460	0.034640	-7.10	0.000
X_1	0.0116	0.000779	14.94	0.001
X_2	-0.0347	0.009803	-3.54	0.012
X_1^2	-0.0001	0.000005	-21.18	0.011
X_2^2	-0.0039	0.001152	-3.39	0.015
$X_1 X_2$	0.0012	0.000097	12.61	0.000
Significance equal or lower than (0.05). (P≤ 0.05)				

The optimization procedure was applied to equation (3) to find the optimum operating conditions (X_1 and X_2) by :

a. Differentiating equation (3) for two times, once with respect to X_1 and X_2.
b. Setting the resulting equations to zero.
c. Solving these equations simultaneously to find the optimum values of variables ($X_1$and X_2).
d. Conducting a second differentiation to test for the sufficient conditions to ascertain that the optimum point is indeed a maximum point.

The results of optimization indicate that the optimum conditions are: X_1 = 92 ^oC; X_2 = 4.5 hr.

The yield were represented in the weight percentage of extracted oil was obtained (0.195% w/w) at above best condition.

Effect of Operating Variables on Extraction process:

Effect of temperature:

The weight percentage of extracted oil increased with increasing temperature until it reached (0.194) % at (91.2) ^oC after (4.414) hrs and the yield then decreased to (0.181) % with temperature increase to (100) ^oC and that shown in Fig. (2). on the other hand, after an extraction time of (1) hrs, the yield increased with increasing temperature and maximum of (0.120) % at (91.2) ^oC. Further increase of temperature up to (91.2) ^oC was caused a decrease in the weight percentage of extracted oil, and that occurred because the volatile oil, which is exposed to high temperatures during the extraction process, perhaps makes this oil loses part of its quality and biological properties, thus reducing the efficiency through production different patterns in oxidative and hydrolytic degradation of oil, as confirmed by published results [11].

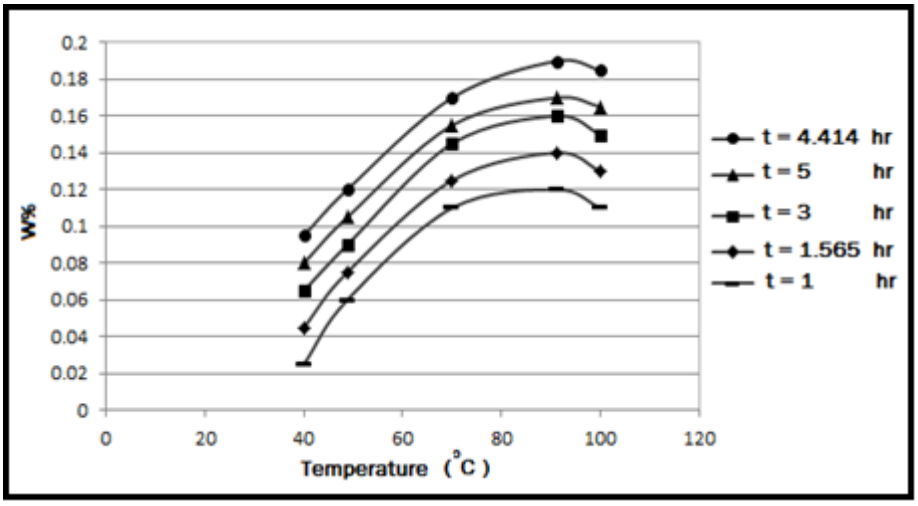

Figure (2): Effect of temperature on the weight percentage of extracted oil at different times

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Effect of time:

The effect of extraction time on the weight percentage of extracted oil at various temperatures is typically shown in Fig. (3). at the yield increased with time up to a maximum value of (0.193) % after (4.5) hrs of extraction process and continues at the same percentage after that. Further times caused an increase in the yield up to (0.192) %, and not increase in yield with increase the time, this is due in our opinion that the all amount of volatile oil was found in plant material were extracted.

The weight percentage of extracted oil obtained from Stachys lavandulifoila was (0.195) % colorless and has an aromatic odor. In similar study on other species, in similar manner [12] estimated the weight percentages of the oils extracted from other species were (0.18) % from Stachys chrysantha, and (0.12) % from Stachys candida.

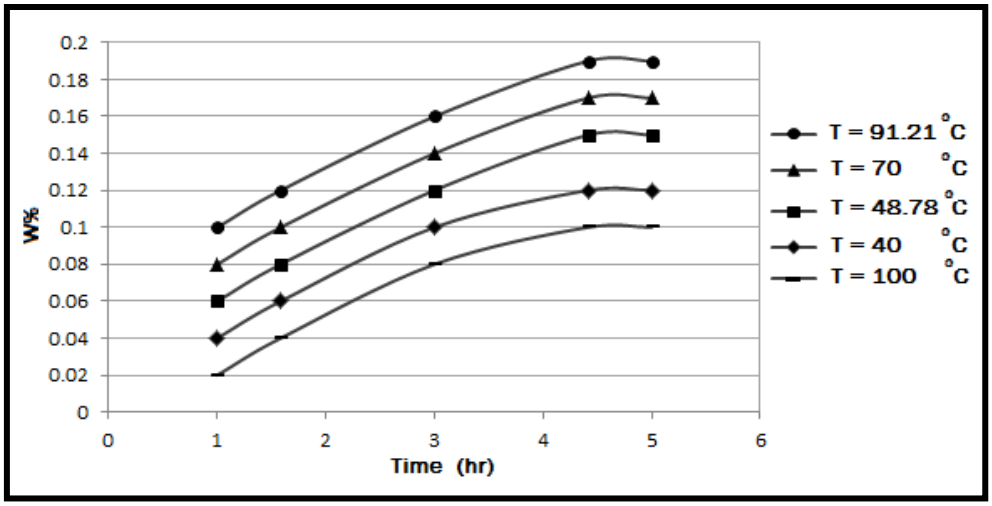

Figure (3): Effect of time on the weight percentage of extracted oil at different temperature.

Composition of the extracted oil:

In the present study a sample of Stachys lavandulifolia with different chemical composition has been reported. The volatile oil of Stachys lavandulifolia showed the presence of fifty fife components and represent 96.4% of the volatile oil, seven of them were found in high percent especially germacrene-D and β-Phellandrene, Myrcene and α-Pinene Table (3). While in reverse result in the same species, [13] demonstrated the spathulenol and caryophyllene oxide as the main constituents of Stachys lavandulifolia.

In contrast to our results,another compounds in high percentage of the oils obtained by extraction of the aerial parts of other species of Stachys, such as Stachys persica and Stachys byzantina were analyzed by GC/MS in study of [14], where the first was characterized by a high amount of non-terpenoid components of which methyllinoleate (27.7)%, hexadecanoic acid (9.8)% and 6,10,14-trimethyl-2-pentadecanone (9.2)% were the major constituents, and the second was rich in sesquiterpenes like a-copaene (16.6)%, spathulenol (16.1)% and beta-caryophyllene (14.3)%.

In addition to [12] recognized another components differenced of our major component the in his study of the aerial parts of Stachys candida and Stachys chrysantha which analysed by GC and GC/MS, and found that the alpha-cadinol, manoyl oxide, caryophyllene oxide, epi-alpha-muurolol and (E)-caryophyllene were the major component.

Table (3): Composition of the volatile oil of Stachys lavandulifolia**.**

Peak no.	Compound name	RI	% in oil
1.	Heptanal	910	t
2.	α-Thujene	920	3.8
3.	α-Pinene	935	7.8
4.	Camphene	956	0.7
5.	β-Pine e	970	8.2
6.	Myrcene	990	11.4
7.	α-Phellandrene	1000	t
8.	α-Terpinene	1022	0.4
9.	β-Phellandrene	1030	14.9
10.	Z-β-Ocimene	1038	5.0
11.	E-β-Ocimene	1045	1.4
12.	γ-Terpinene	1055	1.0
13.	Terpinolene	1069	t
14.	Linalool	1088	0.9
15.	Nonanal	1100	1.8
16.	allo-ocimene	1122	0.
17.	E-Pinocarveol	1132	0.2
18.	E-verbenol	1140	t
19.	borneol	1152	0.7
20.	terpinen-4-ol	1160	0.3
21.	methyl salicylate	1172	0.4
22.	nerol	1220	t
23.	cuminal	1227	0.1
24.	geraniol	1254	t
25.	E-anethole	1265	2.0
26.	*p*-cymen-7-ol	1290	t
27.	δ-elemene	1299	1.1
28.	eugenol	1342	t
29.	closativene	1365	1.2
30.	β -bourbonene	1370	0.2
31.	β-elemene	1394	1.7
32.	α-gurjunene	1400	1.2
33.	β-caryophyllene	1419	0.3
34.	Z-β-Farnesene	1428	1.5
35.	α-humulene	1440	t
36.	germacrene-D	1465	16.1
37.	bicyclogermacrene	1470	2.0
38.	β-bisabolene	1500	1.8
39.	γ-cadinene	1 1	0.5
40.	cadina-1,4-diene	1520	0.8
41.	α-cadinene	1558	2.1
42.	β-calacorene	1566	0.1
43.	Spathulenol	1577	0.8
44.	*T*-cadinol	1640	t
45.	α-muurolol	1655	0.3
46.	α-bisabolol	1666	1.0
47.	heptadecane	1690	0.8
48.	benzyl benzoate	1760	t
49.	palmitic acid	1856	0.5
50.	trans-phytol	102	t
51.	docosane	2144	t
52.	tricosane	2190	0.5
53.	tetracosane	2480	0.1
54.	heptacosane	2680	0.2
55.	octacosane	2785	0.3
t=trace : (t<0.05%): (t) Will be neglected			**96.4%**

Activity of Antibacterial:

The volatile oil has limited inhibition against the gram positive and negative bacteria and Table (4) show activity against gram positive comparative with gram negative without any activity against Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae. in a similar manner to our study [15] tested the essential oils of eight Stachys species including Stachys alopecuros, Stachys. scardica, Stachys cretica, Stachys. germanica, Stachys. recta, Stachys spinulosa, Stachys euboica and Stachys menthifolia and his results showed better activity against bacterial species than against fungi and Pseudomonas aeruginosa was the most resistant strain, as none of the essential oils was active against this strain also the essential oil of Stachys scardica has been proven most active against both bacteria and fungi.

From Table (3) it could be expected that compounds such as myrcene, thujene and pinene are responsible for antimicrobial activities of Stachys lavandulifoila volatile constituents. This is consistent with another study [16] that shown activity these compounds when the essential oil of Coridothymus capitatus with beta-myrcene (3.0) % and alpha-thujene (1.3)% showed strong activity against S. aureus.

The effectiveness of the volatile oil obtained in our study may be due to the effect the pinene compounds through the study of [17] when he referred that the beta-pinene (8.8) % in the essential oil of Callicarpa Americana was selectively toxic toward the cyanobacterium and Oscillatoria perornata with complete growth inhibition at (28.5) µg /ml or due to the role of thujone volatile oil of salvia fruticosa as antimicrobial, cytotoxic and antiviral according to [18].

Table (4):Antimicrobial activity of the volatile oil of Stachys lavandulifolia

	Bacteria spp.	Inhibition values mm	
		*A	**B
Gram-positive	Staphylococcus aureus	2.2	8.9
	Staphylococcus epidermidis	2.8	7.6
	Streptococcus mutans	1.4	6.5
	Streptococcus pyogenes	2.0	7.8
	Bacillus subtilis	0.8	1.8
	Bacillus cereus	1.0	2.7
Gram-negative	Escherichia coli	0	6.6
	Proteus mirabilis	1.0	1.1
	Salmonella typhimuium	1.2	8.2
	Salmonella enteritidis	1.5	8.3
	Pseudomonas aeruginosa	0	5.8
	Klebsiella pneumoniae	0	7.8

***A:** volatile oil ** **B:** Antibacterial (control, Chloramphenicol)

Activity of Antioxidant:

As shown in Fig. (4), bands at R_f = 0.15 and 0.53 showed a good antioxidant properties. DPPH radical scavenging activity tests were carried out at four different concentrations, however, inhibition percentages were very low in concentration (10) and (25) µg. As seen in fig. (5), relatively better inhibitions were obtained in concentrations (50) and (100) µg. Our results agreed with [9], of the role of antioxidants in preventing oxygen radical and hydrogen peroxide induced cytotoxicity and tissue damage in various human diseases is increasingly recognized of the ethanol extracts obtained from (21) aromatic plants belonging to the Lamiaceae family (Salvia ringens, Salvia pomifera, Stachys spruneri, Origanum dictamnus, Phlomis lanata, Ballota pseudodictamnus, Ballota acetabulosa, Teucrium polium, Calamintha glandulosa and Micromeria graeca).

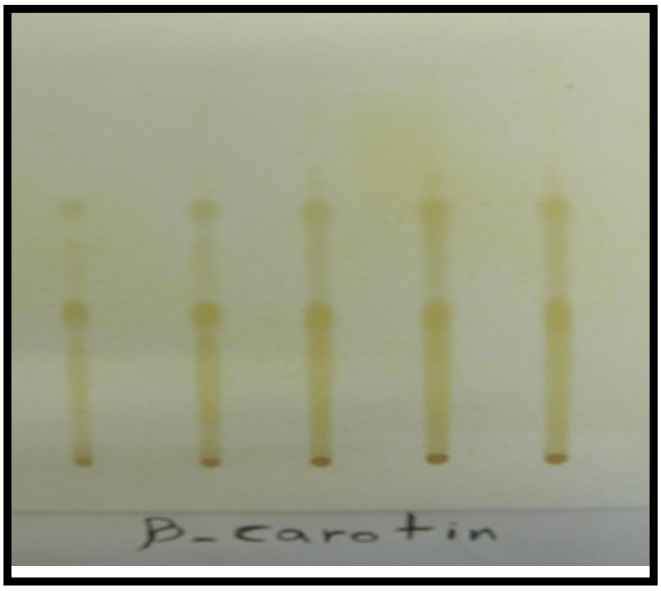

Figure (4): Antioxidant activity of the volatile oil of Stachys lavandulifolia by spraying with ß-carotene**.**

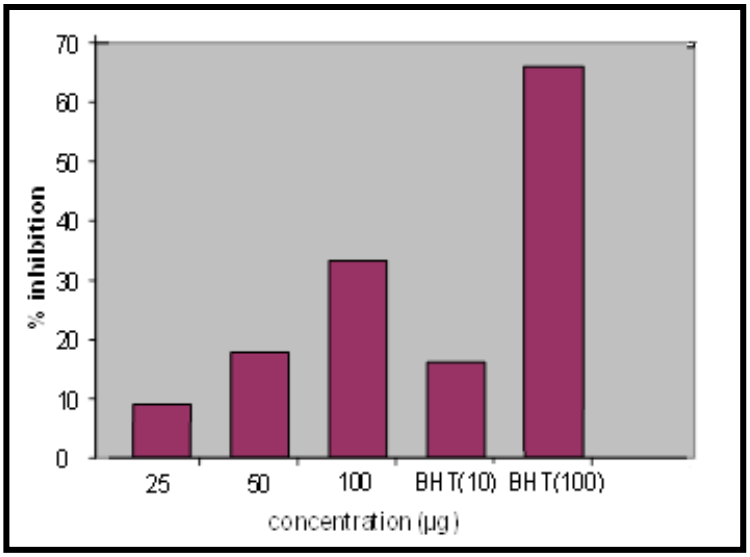

Figure (5): DPPH radical scavenging activity (% inhibition) of volatile oil of Stachys lavandulifolia , BHT as standard

Conclusions:

The volatile oil of Stachys lavandulifolia was extracted from the aerial parts of the plant in a good efficient. Effects of the experimental parameters such as (temperature and time) are very important in the extraction process. The relationship between the yields was represented in the weight percentage of extracted oil and above parameters is shown in equation (3).The highest extraction efficiency of volatile oil was obtained (0.195) %, at best operating conditions as follows: - Temperature (92) C^{o} at time (5) hr.

The analyzed of chemical composition of extracted volatile oil by GC/MS showed the presence of fifty fife components representing (96.4) % of the volatile oil. The main components were α-Thujene (3.8)%, α-Pinene (7.8)%, β-Pinene (8.2)%, Myrcene (11.4)%, β-Phellandrene (14.9)%, Z-β-Ocimene (5.0%), germacrene-D (16.1)%.

The activity of the volatile oil has limited inhibition against gram positive comparative with gram negative, which showed good antioxidant activity.

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استخلاص الزيت الطيار لنبات البيتوني (Stachys lavandulifolia) ودراسة فعالية المضادة للبكتريا والأكسدة

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الخلاصة:-

تم استخلاص الزيت الطيار لنبات البيتوني Stachys lavandulifolia من الأجزاء الهوائية وكان ذو رائحة اروماتية وعديم اللون. بلغت الإنتاجية كنسبة وزنيه للزيت المستخلص 0,195% وزن / وزن في الظروف التشغيلية المثلى, تم دراسة تأثير بعض المتغيرات التجريبية على كفاءة عملية الاستخلاص مثل درجة الحرارة بين (40 - 100) درجة مئوية والزمن بين (1- 5) ساعات.

تم تصميم التجارب باستخدم طريقة (Box-Wilson), وتم ايجاد علاقة رياضية تربط المتغيرين التجريبيين والنسبة الوزنية للزيت المستخلص. تمت مطابقة النتائج العملية التي أمكن الحصول عليها بهذه الطريقة مع معادلة رياضية من الدرجة الثانية, وتم ايجاد الظروف التشغيلية المثلى للاستخلاص.

كشف التحليل الكيميائي للزيت بواسطة كروموتوجرافيا الغاز المدمج بمطياف الكتلة (GC/MS) عن وجود خمساً وخمسون مركباً تشكل 96,4% من مكوناته مع نسبة عالية لسبع منهم تمثلت بـ α-Thujene (3,8%)، α-Pinene (7,8%)، β-Pinene (8,2%)، Myrcene (11,4%)، β-Phellandrene (14,9%)، Z-β-Ocimene (5,0%) وgermacrene-D (16,1%) .

سجل الزيت فعالية محدودة نحو العزلات البكترية الموجبة والسالبة لصبغة كرام, كما أظهرت البكتريا الموجبة لصبغة جرام تحسس أكثر تجاه الزيت مقارنة بالسالبة, والتي سجلت ثلاث أنواع (Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa) منها مقاومة عالية تجاه الزيت الطيار, و اظهر الزيت الطيار فعالية جيدة مضادة للاكسدة.