Phytochemical and anti bacterial studies of leaves of Rosmarinus officinalis cultivated in Karbala, Iraq.

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Abstract:

Rosmarinus officinalis is one of the important bioactive medicinal plants belongs to labiatae family. The aim is to identifying the essential oil and rosmarinic acid extracted from rosemary leaves and to evaluate its anti bacterial activity. Gas Chromatography-Mass analysis was performed for essential oil of rosemary and HPTLC analysis was done for 90% methanolic extract of rosemary leaves. Rosmarinic acid was detected and The essential oil content was 1.5% and characterized with high amount of 1,8 cineol (53.63%) . The zone of inhibition of some bacteria was estimated. Our findings showed that rosemary leaves has Good percentage of rosemary phytochemicals (essential oil and rosmarinic acid) and has significant antibacterial activity against different species of bacteria.

Keyword: Rosmarinus officinalis L., essential oil, GC/MS, rosmarinic acid.

دراسة كيميائية النبات لاوراق نبات اكليل الجبل المستوع في محافظة كربلاء في العراق ومعرفة فعاليتها كمضاد حيوي.

الخلاصة:

نبات اكليل الجبل هو احد النباتات الطبية المهمة في الفعاليات الحيوية.التي تعود لعائلة labiatea. استهدفت الدراسة معرفة الزيوت الطيارة و استخلاص مادة الروزمارنك اسد المستخلصة من اوراق اكليل الجبل وتقييم فعاليتها الحيوية . تم تحليلGCMS للزيوت الطيارة وتم استخدام تحليل HPTLC للمستخلص الكحولي 90% لاوراق اكليل الجبل. تم تحديد مادة حامض الروزمارنك و قياس كمية الزيت الطيار 1.5% الذي تميز باحتوائه على كمية عالية من مادة اليوكاليبتول (53.63%) وكذلك تم تقدير منطقة تثبيط البكتريا. يستنتج من هذه الدراسة انه اوراق الروزماري لها نبسة جيدة من المواد الكيمونباتية (روزمارنك اسد والزيوت الطيارة) ولها فعالية مضادة لمختلف انواع البكتريا.

الكلمات المفتاحية: اكليل الجبل، الزيوت الطيارة، GCMS،حامض الروزمارنك.

Introduction :

Medicinal plants are an important and intense source of bioactive natural compounds or bionutrients; that have vital role in enhancing health and preventing different diseases [1]. *R. officinalis* is one of important bioactive medicinal plants and belongs to labiatae family and to subfamily Nepetoideae [2]. This plant is charac-terized by its aromatic odor with needle-like leaves. It is native to the Mediterranean and grows to a height of 1 to 2 m [3]. *Rosmarinus officinalis* varieties (cultivars) can be distinguished in relation to morphological descriptors of rosemary (like calyx, corolla, and dimension of leaf, inflorescence and the presence of glandular trichomes) [4]. The nutrient compositions of rosemary show a good amount of vitamins and minerals. The most important bioactive compounds are essential oil, rosmarinic acid, carnosic acid, carnosol, caffeic acid and its derivative[5], triterpenoid (betulinic acid) and flavanoids[6],[7].Chemically, they are composed of highly volatile lipophilic compounds with terpene skeleton that is monoterpenes (two isoprene units) and sesquiterpenes (three isoprene units) [8]. Furthermore, these compounds are divided into major subgroups cyclic (mono-,di-,tri-cyclic), acyclic and oxygen derivatives [9]. Rosmarinic acid, is an important naturally occurring phenolic bioactive compound. Chemicallly, it is 3,4-di-hydroxyphenyl lactic acid and an ester of caffeic acid [10],as shown in Figure-1.

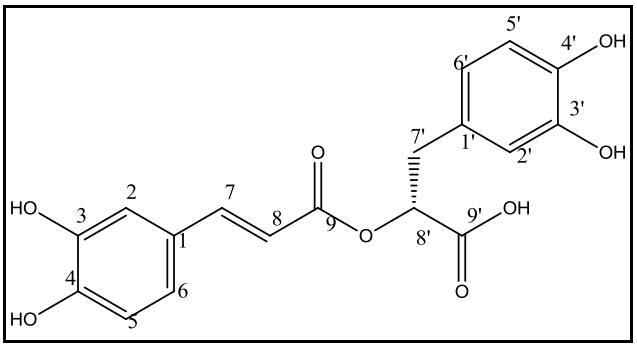


Figure- 1: Basic structure of rosmarinic cid.

Currently, Antimicrobial agents are really important in reducing the global burden of various infectious diseases. In spite of this, the resistance of pathogens to antimicrobial agent increased and spread, also the efficiency of the antibiotic drugs is diminished; all these may give rise to affect on human health. Using rosemary plant for traditional-alternative medicine as a source to treat infectious diseases has been accomplished since the origin of mankind [11], it is used as anti-inflammatory, analgesic,antispasmodic, abdominal pain, carminative, arthritis, gout problems, also for wound healing (antiseptic), diuretic, antirheumatic and antide-pressant [12],[13]. The aim of this study was qualitative-quantitative analysis of essential oil and detection of romarinic acid.

November, 2016. The plant was authenticated by National Iraqi Herbarium, Botany Directorate at Abu-Ghraib. The plant leaves were shade-dried in the air at room temperature during twelve days.

Analysis of essential oil components by Gas Chromatography-Mass:

Extraction of essential oil

A shade- dried rosemary leaves (200 g) hydrodistilled with two litters of water for three hours by Clevenger-apparatus. Pale yellow oil was isolated and dried

over anhydrous sodium sulphate, then after kept at 4 °C in air-tight container until analysis by GC/MS.

(GC/MS) conditions

Gas Chromatography-Mass analysis was carried out with SHIMADZU GCMS-QP-2010 ULTRA at College of science-chemistry department-Almustansiriyah University. The capillary column ($30\,m \times 0.25$mm Internal Diameter, film thickness 0.25µm) at a flow rate 1.53 ml/min, helium carrier gas was used. Injection mode was split and injection temperature was 240°C, the oven temperature was programmed at 70 °C for 3 min, then raised to 150 °C with hold time 2 min and raised to 240° C, the ionization mode was electronic impact mode (SEI) at 70e.

Analysis of rosmarinic acid by High Performance Thin Layer Chromato-graphy (HPTLC):

Extraction of rosmarinic acid from rosemary leaves:

Extraction of rosemary leaves was prepared by mixing 1 g of powdered plant with 10 ml of 90% methanol and boiled in a water bath under reflex condenser for 30 minutes. Then used for determination of rosmarinic acid in rosemary leaves in comparison with standard rosmarinic acid which is prepared by dissolving 1mg/10ml of methanol solvent (0.1mg/ml) , then stored at -15 ^{0}C.

HPTLC conditions:

HPTLC finger print analysis was conducted to detect the presence of rosmarinic acid in two specimens. The HPTLC analysis was performed using Pre-coated silica gel 60 F 254 plates (10x20 cm) with layer thickness of 0.5 mm were used as a stationary phase. The standard rosmarinic acid 2 µL and 3 µL from crude extract were applied automatically on the plate by CAMAG Linomat 5. The plate was automatically submerged into automatic developing chamber (ADC2 CAMAG) using solvent system (Hexane, ethyl acetate, formic acid, 20: 19: 1, v/v/v)[14]. The plate was scanned under UV 366 nm using CAMAG TLC scanner 4. The data were processed using win CATS software.

Collection of test organism and preparation of stock culture:

The antimicrobial activity of the rosemary leaves extract was done in biology department /college of science / Mustansiriyah University. A preliminary antibacterial activity was carried out according to Well Diffusion Method: The rosemary extract has been studied for their antimicrobial activity *in vitro* against four tested bacteria.

Microorganisms used in the experiments:

Four species of bacteria were used to assay the antibacterial activity of crude extract in this study, two of them are gram positive (*Staphylococcus aureus* & Streptococcus pyougenes) and the gram

negative were (Klebsiella pneumoniae & Escherichia coli).

Estimation of antibacterial activity:

The stock solutions 100mg/mL were prepared by dissolving 100 mg from methanolic extract of rosemary leaves from each region in 1mL of dimethyl-sulfoxide (DMSO). The dilution serials of 50.0, 25.0, and 12.5 mL were prepared for determination of antibacterial assay by agar well diffusion assay and carried out by using pure culture for all species of bacteria, inoculum of bacteria was first sub-cultured in brain heart infusion broth & incubated at 37°C for 18-24 hour. After incubation a loopful of each species transferred to tube containing 3 mL normal saline and vortex well. The concentration of (1.5×10^8 CFU/mL) was obtained by using McFarland turbidity standard of each bacteria

inoculated by using glass spreader on the surface of Mueller Hinton Agar (MHA) plates previously prepared. The plate was allowed to dry and punched wells (five) in diameter of 6 mm into agar. Subsequently, in each agar plate of tested bacteria five wells were made and (100µl) of dilutions of the extracts (100, 50.0, 25.0, and 12.5 mg/ mL) introduced into wells on MHA plate. The DMSO used as the negative controller. The plates were kept at 37 °C for 24 hours and the antimicrobial action was estimated by determining the diameter of the inhibition zone. Evaluation of antibacterial action was based on extent of the diameter of inhibition zone formed all over the place of the well.

Results and Discussion:

Isolation and identification of rosemary oil:

We have carried out an investigation on the chemical composition of dried R.officinalis leaves cultivated in Karbala/ Iraq as shown in figure 2. The quantity of essential oil isolated by hydrodistillation method was found 1.5% (ml/100g), revealed a good percentage in comparisons with the percentage in the European monograph (2011:1846) that provides the following definition: whole, dried leaf of Rosmarinus officinalis L. contain minimum 12 ml/kg (1.2%) of essential oil obtained by steam distillation [15].

The GCMS analysis of the components of the oil was performed as shown in Figure3. The chromatogram reveals thirteen peaks; only six volatile compounds were identified from the separated components in comparison with National Institute of Standard and Technology (NISTA08) library data base; representing 96.05% as oxygenated monoterpenes from the total oil, as seen in Table 1,while unidentified compound represent the remaining contents.

Figure-2: Roaemary plant cultivated in Karbala, Iraq.

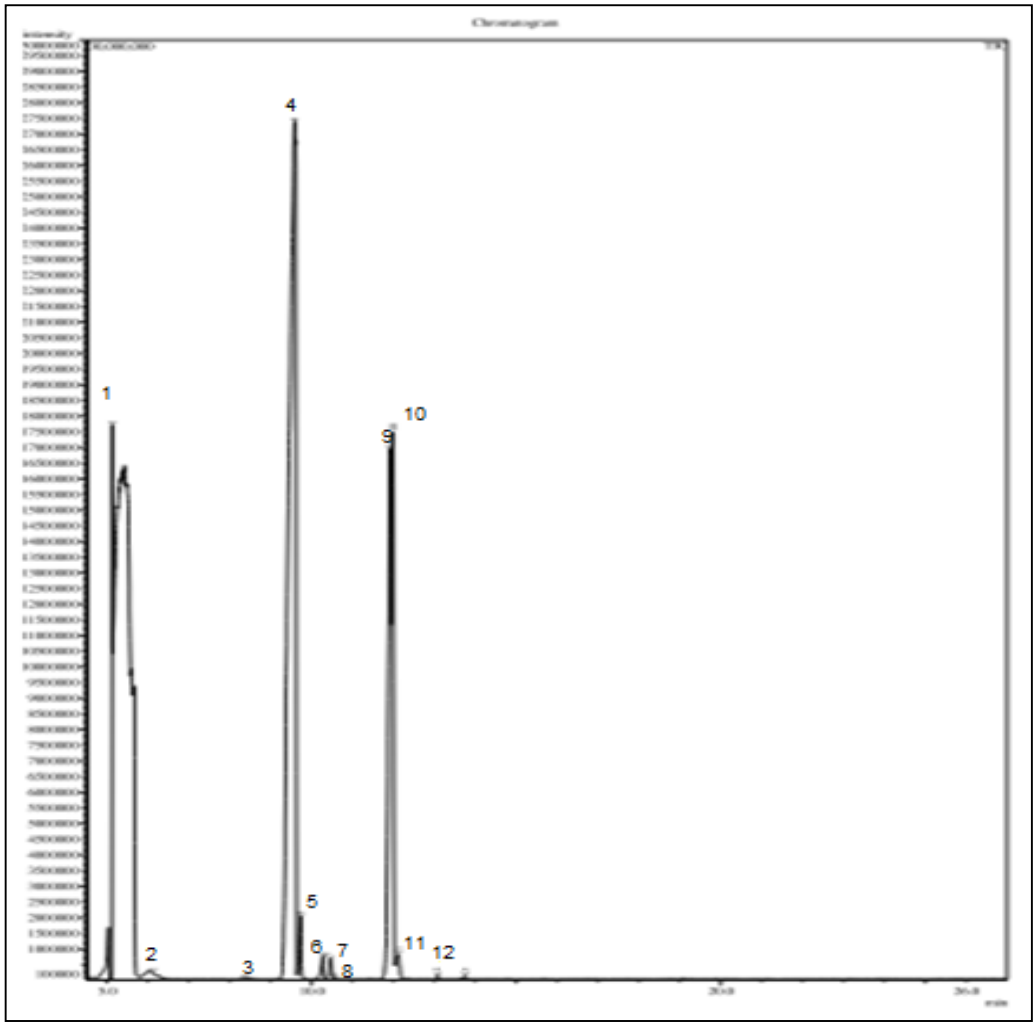


Figure-3: Identification of essential oil contents in rosemary plant from Karbala by GCMS.

Table-1: The chemical compositions of rosemary essential oil from leaves of Karbala plant by GC/MS analysis.

Peak no.	R.T	Area%	Mass Peak (m/z)	Name of compound
1	5.12	53.63	400	(1,8 cineol)
4	9.58	37.32	411	Camphor
5	9.72	0.84	358	β –Linalool
7	10.46	0.23	330	β- terpineol
10	11.99	3.66	418	Boraneol
11	12.12	0.37	361	Verbenone
Total identified compounds: 96.05 %				

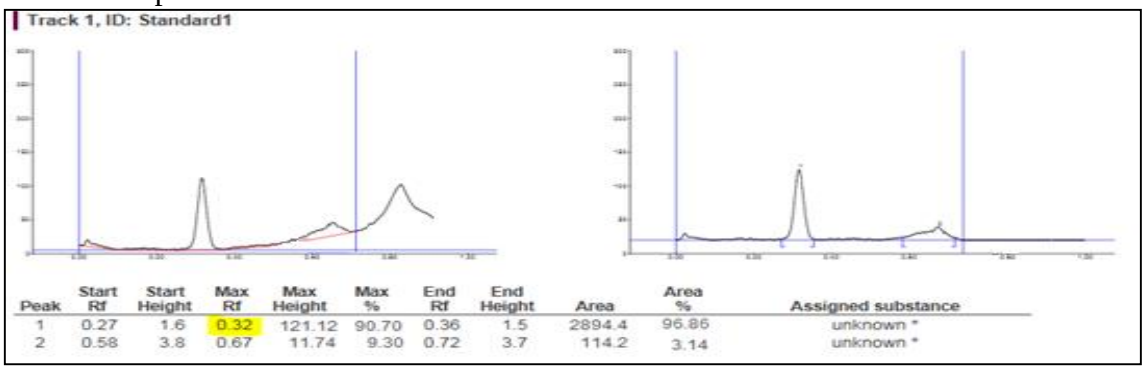
On other hand, the results showed that major constituents are 1, 8 cineol (53.63%) and camphor (37.32%), followed by minor components as boraneol (3.66%), linalool (0.84%), verbenone (0.37%) and β-terpineol (0.23%). So, the chemotypes of Karbala plant are: 1, 8 cin-eol /camphor/ boraneol.

The volatile oil of rosemary leaves grown in Karbala had particularly high levels of 1,8-cineole (53.63%) and sometimes higher than literature values from previous studies such as: The Lebanese essential oils of rosemary collected from three loca-tions were determined by GC/MS. The three oil samples were revealed to be rich in a-pinene (18.8-38.5%) and 1, 8-cineole (19.1-25.1%). Also, a high quantity of α-terpineol(2.9-11.2%) and geraniol(1.8-9.3%)[16]. In Graz, Austria the mass analy-sis detected the essential oil constituents of the dried leaves were 1,8-cineole (41.6%), α-terpineol (4.9%) , α-pinene (9.9%), borneol (4.8%) and camphor (17.0%)[17] . The oil samples of R.officinalis L. native to India subjected to GC and GC-MS detection showed the presence of α-pinene (6.7-15.6%), camphor (23.1-35.8%) and 1,8-cineole (21.4-31.6%) as major constituents in the oils[18].

The major components in Spanish oils are: α-pinene (19.4 to 24.7) Eucalyptol (19.0 to 21.8%) and camphor (16.3 to 18.9%), in addi-tion to the higher percentages in French oils are: α-pinene (19.9-35.1%) , eucalyptol (5.3-24.8%) and bornyl acetate (1.2-14.3%)[19].

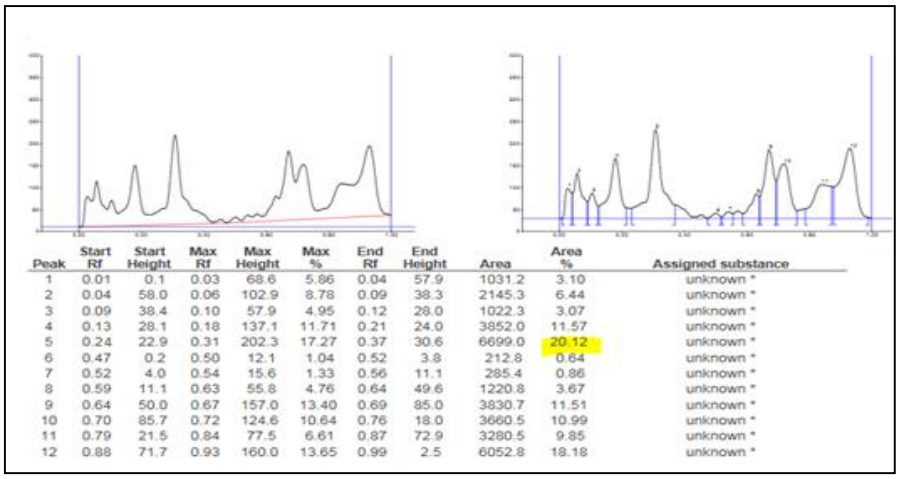
Detection of rosmarinic acid in rosemary leaves:

The extracts were subjected to HPTLC along with the standard rosmarinic acid. The HPTLC analysis showed that standard rosma-rinic acid has maximum Rf value of 0.32, as shown in Figure- 4. Figure -5 shows clear peak number 5 at max. Rf value =0.31 that represents 20.12% of the total extract composition of rosemary extract with reference to Rf value of standard rosma-rinic acid, the observed peak detected as ros-marinic acid. So, the results showed that ros-marinic acid is the major compound in 90% alcoholic extract of rosemary leaves.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.27	1.6	0.32	121.12	90.70	0.36	1.5	2894.4	96.86	unknown *
2	0.58	3.8	0.67	11.74	9.30	0.72	3.7	114.2	3.14	unknown *

Figure-4: HPTLC chromatogram of standard rosmarinic acid.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	0.1	0.03	68.6	5.86	0.04	57.9	1031.2	3.10	unknown *
2	0.04	58.0	0.06	102.9	8.78	0.09	38.3	2145.3	6.44	unknown *
3	0.09	38.4	0.10	57.9	4.95	0.12	28.0	1022.3	3.07	unknown *
4	0.13	28.1	0.18	137.1	11.71	0.21	24.0	3852.0	11.57	unknown *
5	0.24	22.9	0.31	202.3	17.27	0.37	30.6	6699.0	20.12	unknown *
6	0.47	0.2	0.50	12.1	1.04	0.52	3.8	212.8	0.64	unknown *
7	0.52	4.0	0.54	15.6	1.33	0.56	11.1	285.4	0.86	unknown *
8	0.59	11.1	0.63	55.8	4.76	0.64	49.6	1220.8	3.67	unknown *
9	0.64	50.0	0.67	157.0	13.40	0.69	85.0	3830.7	11.51	unknown *
10	0.70	85.7	0.72	124.6	10.64	0.76	18.0	3660.5	10.99	unknown *
11	0.79	21.5	0.84	77.5	6.61	0.87	72.9	3280.5	9.85	unknown *
12	0.88	71.7	0.93	160.0	13.65	0.99	2.5	6052.8	18.18	unknown *

Figure- 5: HPTLC chromatogram of extract from leaves of R.officinalis cultivated in Karbala

Preliminary anti bacterial assay:

The rosemary extract was screened for its antibacterial activity and DMSO used as a control against two gram negative bac-teria (Acenitobacter baumannii and Pro-teus mirabilis) and two gram positive bac-teria (Enterococcus faecalis and Staphy-lococcus saprophyticus) at concentrations of (12.5, 25.0, 50.0 and 100mg/mL) ,while the control was used in a pure state. Table 2 illus-trates the inhibition zone in (mm) for each concentration of the extract. The results of antibacterial activity of rosemary ex-tracts under study against shows good activity of crude extract.In this study crude extract of rosemary showed an ef-ficient dose dependent -antibacterial ac-tivity against different species of bacte-ria. The most sensitive bacteria was Acenito.baumannii with inhibition zone 33 mm at high concentration.

Table-2: Antibacterial activity of *R.officinalis* extracts with different bacterial species measured in millimeter.

Bacterial species	Concentrations of extracts mg/mL			
	100	50	20	12.5
	Inhibition zone (mm)			
Proteus mirabilis	15	15	13	14
Acenito.baumannii	33	31	30	27
Staph.saprophyticus	23	16	15	14
E.Faecalis	25	20	18	23

Similar to our results, studies showed that extract of rosemary plant had a good antibacterial action and as a source of natural antibiotics against different species of pathogenic bacteria, this antibacterial effect belongs to the presence of diterpeneoids and phenolic acid compounds[20],[21].

Conclusions:

From this study we conclude the leaves of rosemary plant cultivated in Karbala has good content of essential oil (1.5%). Also, high amount of 1,8 cineol (53.63%) as an oxide-monoterpene in comparison with some previous literatures. Rosmarinic acid was detected as a major component in aqueous–methanolic extract of rosemary leaves. Biologically, rosemary plant has a good anti-bacterial activity against some of gram negative and gram positive bacteria.

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