

**STUDY OF THE ANTIMICROBIAL ACTIVITY OF ROSEMARY
(*ROSMARINUS OFFICINALIS* L.) CALLUS EXTRACT AND
SELECTED TYPES OF ANTIBIOTICS AGAINST SOME BACTERIAL
SPECIES⁺**

***Rosmarinus officinalis* L. دراسة الفعالية الميكروبية لمستخلص الكالس لنبات اكليل الجبل**

وانواع منتخبة من المضادات الحيوية ضد بعض انواع البكتريا

Shayma'a J. Ahmed (PhD) *

Ranin K. Al-Azawee (Bsc) *

Khaleel I. Rashid (PhD) **

Marwa M. Abdel-Kareem (Bsc)*

Abstract:

The chemical analysis of rosemary (*Rosmarinus officinalis* L.) callus ethanol extract was carried out using gas chromatography-mass spectrometry (GC-MS). The most abundant compounds of callus ethanol extract were, quinoline alkaloid, phytol, eucalyptol, camphor, borneol, verbenone and bornyl acetate.

Escherichia coli and *Salmonella typhi* showed resistance to Rifampicin and Erythromycin, while they were sensitive to other antibiotics. On the other hand, *Staphylococcus aureus* showed moderate sensitivity to antibiotics used.

Standard compounds have an activity at range of 0.7 to 2.5 cm, Rosmarinic acid and carnosic acid were found to be the most effective antimicrobial compounds against bacterial species involved in this study.

The study indicates that higher concentrations of the extract were required to inhibit gram-negative bacteria. It was found that 320 ul/ml of callus extract was enough to inhibit *Escherichia coli*, while 640 ul/ml of extract was required to inhibit *Salmonella typhi*. Callus ethanol extract was more active against gram-positive bacteria; *Staphylococcus aureus*. Their growth was inhibited at 60 ul/ml of the extract. Thus, the callus extract having a broad spectrum activity against gram positive and gram negative bacteria.

Key words: *Rosmarinus officinalis*, essential oil, LD50, MIC, antibiotics.

المستخلص:

اجري التحليل الكيماي للمستخلص الكحولي لكالس نبات اكليل الجبل *Rosmarinus officinalis* L. باستعمال كروماتوغرافيا الغاز GC-MS. كانت المكونات الرئيسية لمستخلص الكالس الكحولي هي: quinoline alkaloid ، Phytol ، eucalyptol ، camphor ، verbenone و bornyl acetate. اظهرت بكتريا *Escherichia coli* و *Salmonella typhi* مقاومة للمضادين ريفامبيسين Rifampicin وارثرومابسين Erythromycin، بينما كانت حساسة لبقية المضادات الحيوية. من جانب آخر، اظهرت بكتريا *Staphylococcus aureus* حساسية متوسطة للمضادات الحيوية المستعملة.

⁺Received on 3/5/2010 , Accepted 5/1/2011.

^{*}Biology Department-College of Medicine-Baghdad University

^{**}College of Health and Medical Technologies-Baghdad

امتلكت المركبات القياسية فعالية للمدى 0.7-2.5 سم، وجد ان حامض الروزمارينك **Rosmarinic acid** وحامض الكارنوسك **Carnosic acid** اكثر المركبات تأثيراً مايكروبياً ضد انواع البكتريا في هذه الدراسة. اشارت البيانات الى الحاجة الى تراكيز عالية لتثبيط البكتريا السالبة لصبغة كرام. وجد ان 320 مايكروليتر/ مللتر من مستخلص الكالس كافية لتثبيط *E. coli*، بينما يحتاج الى 640 مايكروليتر / ملليتر من المستخلص لتثبيط *Salmonella typhi*. كان مستخلص الكالس الكحولي اكثر فعالية ضد البكتريا الموجبة لصبغة كرام: *Staphylococcus aureus*، حيث ثبت نموها عند 60 مايكروليتر/ مللتر من المستخلص. لهذا، فإن مستخلص الكالس يمتلك فعالية واسعة ضد البكتريا الموجبة والسالبة لصبغة كرام.

Introduction:

Rosemary (*Rosmarinus officinalis* L.) which has long been known as a spice and medicinal herb belongs to the Lamiaceae family and receives an increasing attention due to its antimicrobial, anti-inflammatory and antioxidative constituents [1].

Plant material from rosemary is of commercial interest for its essential oil content and its antioxidant compounds. Carnosic acid and carnosol are shown to be the major phenolic diterpenes in leaves of rosemary [2].

There is an increasing interest in phytochemicals as new sources of natural antioxidant and antimicrobial agents. The popular rosemary plant is a native to the Mediterranean region; however, it has been cultivated throughout world and accepted as one of the spices with highest antioxidant activity (3).

The natural compounds which have biological activities are interest to pharmaceutical industries especially for treatment of human diseases of microbial origin and for the prevention of lipid peroxidation, which has been implicated in several pathological disorders, such as coronary atherosclerosis, Alzheimers disease, cancerogenesis, as well as in ageing processes. The essential oils are volatile compounds of plants secondary metabolism and may act as phytoprotective agents [4].

Synthetic chemicals are widely used against microorganisms, unfortunately they develop resistance to many antibiotics due to the indiscriminate use of commercial antibiotics. In addition, these antibiotics sometimes cause allergic reactions and immune suppression. Therefore, the use of plant extracts is safer to the human health and environment [5, 6, 7].

The objective of this study was to determine the MIC (Minimum Inhibitory Concentration) of callus extract of rosemary and comparisons of the antimicrobial potencies of this extracts with some antibiotics and standard solutions of caffeic acid, rosmarinic acid, carnosic acid, carnosol and rosmanol.

Materials and Methods :

This study was carried out in the laboratories of College of Medicine, Biology Department, during the period from 5/1/2009 to 15/8/2009.

1- Callus extraction

A quantity of 10 g of callus powder was extracted with 50 ml of 70% ethanol by soxhlet apparatus for 6 hrs at 60°C. The solution is then evaporated to dryness using a rotary evaporator at 40 °C, then the extract was stored at 4°C in a refrigerator for future use in GC-MS analysis [8].

2- Preparation of standard solutions

Stock solutions of: Rosmanol (MW 346.4), Caffeic acid (MW 332.4), Rosmarinic acid (MW 380.3), Carnosic acid (MW 330.4) and Carnosol (MW 331.3), were prepared by dissolving 0.01 g of each standard in 50 ml of methanol 70% to obtain a final concentration of 200 µg/ml, the work solution was 20 µg/ml. The standards were supplemented by DHL post company-Germany [9].

3- Gas Chromatography Mass Spectrometry analysis (GC-MS)

Analysis of callus extract was performed using GC-MS, equipped with mass selective detector. For GC-MS detection electron ionization system with ionization energy of 70 eV is used. Samples were injected automatically in the split mode at split ratio of 1:100. All the tests were performed in triplicate. The compounds were identified by comparison of recorded mass spectra with those of a computer library or with those of authentic compounds [10].

4-Tested microorganisms

The micro-organisms which used in this study were *Escherichia coli* MM294, *Salmonella typhi* GEB111 and *Staphylococcus aureus*. They were supplemented by Biology Department / College of Medicine /University of Baghdad. The bacterial cultures were maintained on Mueller-Hinton Agar (MH). It was prepared according to the manufacturer's instructions [11,12].

5-MIC test

Sterile filter paper disks of uniform size (6 mm) were soaked with different concentrations (10, 20, 40, 80, 160, 320 and 640 µg/ml) of callus ethanol extract, placed on the surface of agar plate that previously inoculated with a standard amount (1.5×10^5) of the micro-organisms. The plates were incubated at 37°C for 24 hrs. After incubation, the plates were examined for the presence of inhibition zone of bacterial growth (clear hallows) around the disks [12].

6- Resistance to antibiotics

The bacterial strains were suspended in nutrient broth loopfull , inoculated onto the surface of each nutrient agar plate. The antibiotic discs were placed on inoculated plates at 37°C for 24 hrs [13]. Ampicillin Sodium (AMS), Rifampicin (Rif), Ofloxamine (OFX), Erythromycin (E), Amoxicillin(AM), Tetracycline (TE) and Vancomycine (VA) were used in this study.

Results:

Chemical analysis of rosemary callus extract revealed the presence of 20 active compounds and other non-identified components at different quantities as shown in table 1 and figure 1.

Table 1: Retention time and peak area (%) of the active compounds found in rosemary callus extract analyzed by GC-MS

Compound	RT (min)	Peak area%
Eucalyptol	9.36	4.44
Camphor	15.10	2.51
Borneol	16.58	1.91
Verbenone	19.04	2.30
5-oxymethylfurfurol	22.29	0.72
Bornyl acetate	23.66	1.66
Caryophyllene	31.59	0.64
Ferruginol	58.07	1.59
Isocarnosol	61.89	1.27
Stearic acid	62.35	0.71
Payarzolobis (bbn) thiolium	64.41	1.66
Phytol	64.82	9.12
Tetrahydroxy-tetramethyl-dihydrophenalenol-furan-3-one	65.33	14.45
Tetramethylbenzaldehyde 2,4-dinitrophenylhydrazone	65.68	17.78
Quinoline alkaloid	67.11	16.78
Dihydromorphinone	67.47	3.73
Sclareol	67.78	0.71
Lidoflazine	71.68	1.85
Non-Identified compounds		16.37

Data showed that the most abundant compounds were quinoline alkaloid, phytol, eucalyptol, camphor and verbenone. Other compounds were found at lower levels such as, borneol, bornyl acetate, isocarnosol, ferruginol, sclareol and lidoflazine.

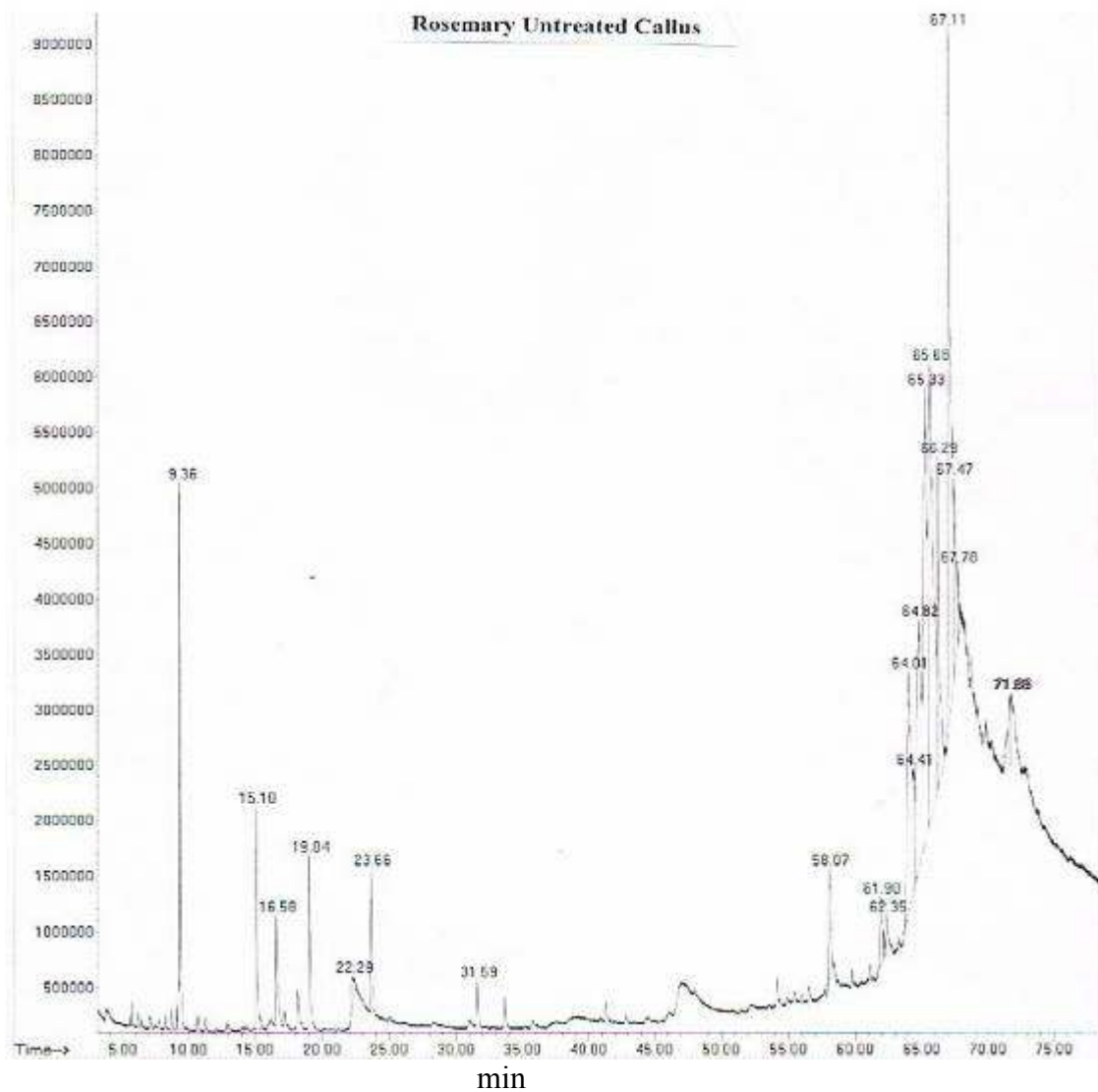


Figure 1: Chromatogram showing the chemical composition of rosemary callus ethanol extract analyzed by GC-MS

The antimicrobial activities of different antibiotics were carried out on two types of pathogenic microorganisms; gram-positive bacteria (*Staphylococcus aureus*) and gram-negative (*Escherichia coli* and *Salmonella typhi*) as shown in Table 2 and figure 2.

Table 2: Antimicrobial activity (inhibition zone diameter cm) of some antibiotics against tested microorganisms

Bacteria	AMs*	Rif	OfX	E	AM	TE	VA
<i>Staphylococcus aureus</i>	2.1**	2.5	2.2	2.6	1.6	2.8	1.8
<i>Escherichia coli</i>	4(S)	0(R)	3(S)	0(R)	5(S)	1.3	1.5
<i>Salmonella typhi</i>	3(S)	0(R)	5(S)	0(R)	6(S)	3(S)	2

*Ampicillin Sodium (AMS), Rifampicin (Rif), Ofloxacin (OFX), Erythromycin (E), Amoxicillin(AM), Tetracycline (TE) and Vancomycin (VA).

** Inhibition zone diameter (cm), MIC (20µg/ml), R=Resistance, S=Sensitive.

The antimicrobial activities of the standard compounds on the two types of pathogenic microorganisms were shown in table 3 and figure 3.

Table 3: Antimicrobial activities of standard compounds against tested microorganisms represented as inhibition zone diameter(cm) .

Standard compounds	MW	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Salmonella typhi</i>
Rosemanol	346.4	1.2	1.5	1.5
Caffic acid	332.4	1.7	1.6	1.5
Rosmarinic acid	380.3	2.5	1.6	1.5
Carnosic acid	330.4	2.3	1.8	1.9
Carnosol	331.3	1.2	0.7	0.8

The antimicrobial activity of callus ethanol extract against tested microorganisms was shown in table 4 and figure 4.

Table 4: Effect of different concentrations of rosemary callus ethanol extract on bacterial growth represented as inhibition zones (cm).

Bacterial species	Concentration (ug/ml)							
	10	20	40	80	160	320	640	MIC
<i>Staphylococcus aureus</i>	0.0	0.0	0.0	0.0	1.7	1.9	2.0	160
<i>Escherishia coli</i>	0.0	0.0	0.0	0.0	0.0	1.5	1.7	320
<i>Salmonella typhi</i>	0.0	0.0	0.0	0.0	0.0	0.0	1.6	640

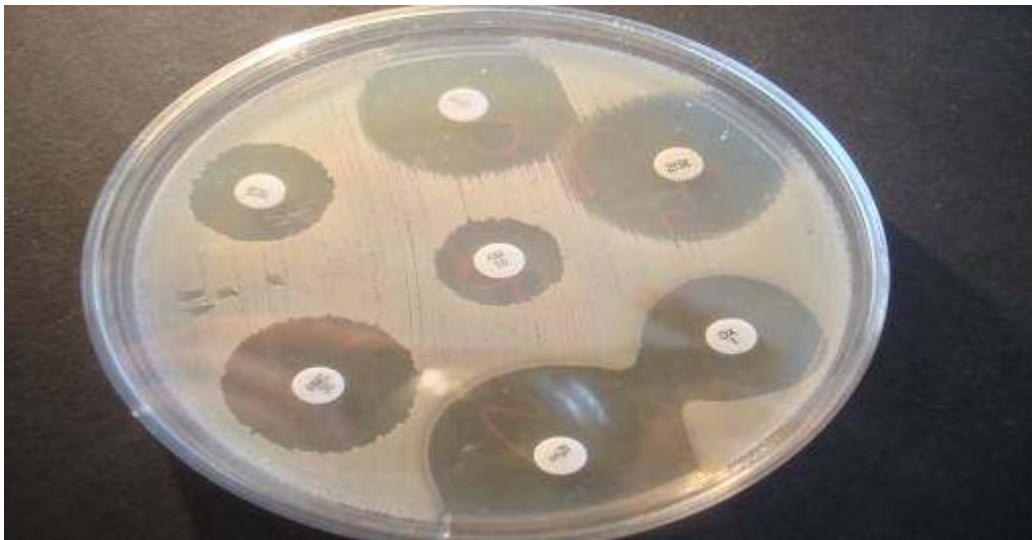
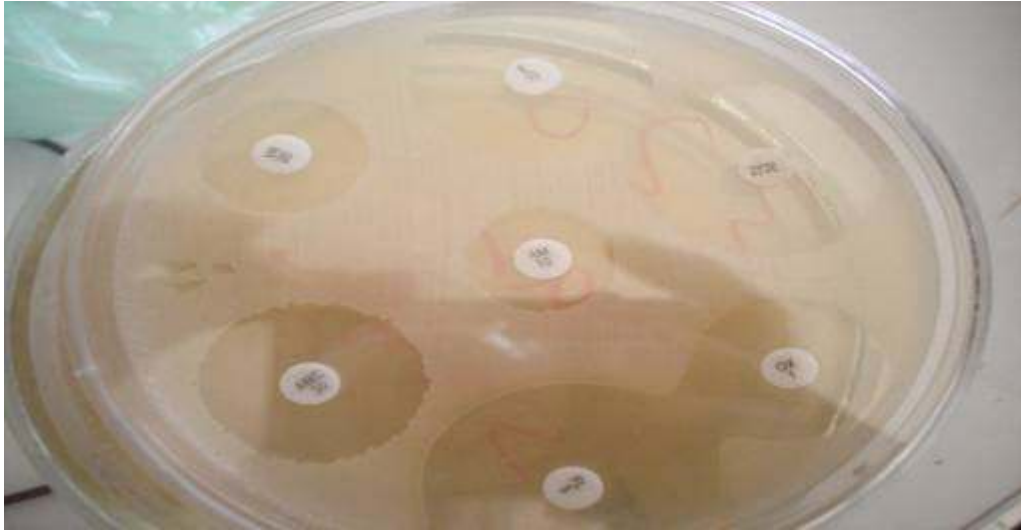


Figure 2: Inhibition zones (cm) of antibiotics against 1- *E. coli*, 2- *Salmonella typhi* and 3- *Staphylococcus aureus* respectively

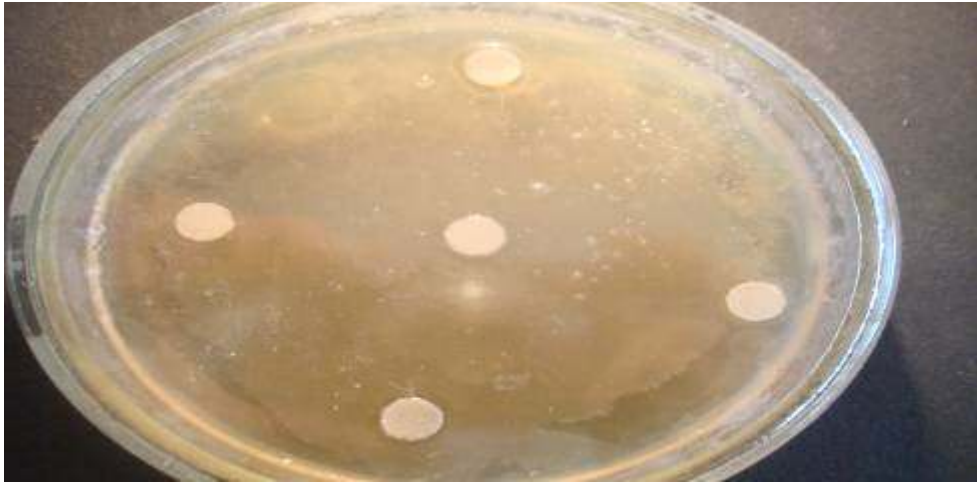


Figure 3: Inhibition zones (cm) of standard compounds against 1- *E. coli*, 2- *Salmonella typhi* and 3- *Staphylococcus aureus* respectively



Figure 4: Inhibition zones (cm) of rosemary callus ethanolic extract against 1- *E. coli*, 2- *Salmonella typhi* and 3- *Staphylococcus aureus* respectively

Discussion:

Results showed that the more available constituents of rosemary callus extract were phenolic compounds and substituted benzene hydrocarbons. Analysis of rosemary callus by GC-MS led to the identification of more than 20 compounds by comparison of recorded mass spectra with those of a computer library, but the most active compounds were, eucalyptol, camphor, borneol, bornyl acetate and verbenone. Quinoline alkaloid which was found in callus extract has a great therapeutic value, the antimicrobial activity of this alkaloid has been documented besides their pharmaceutical uses, they are used frequently in the food and soft drink industry. Phytol was obtained at a highest percent which was considered as antiseptic agent that used as mouth wash. Caryophyllene was recorded the lowest percent (0.64%) followed by stearic acid and sclareol (0.71%). These results are in harmony with [13] who found that rosemary extracts contain camphor, borneol, bornyl acetate and eucalyptol but at higher concentrations. The differences may be due to seasonal variation, plant status and the extraction method. .

Escherichia coli and *Salmonella typhi* showed a resistance to Rifampicin and Erythromycin, while they were sensitive to other antibiotics. On the other hand, *Staphylococcus aureus* showed moderate sensitivity to other antibiotics. These results are in agreement with [14] who stated that *E. coli* strain that is resistant to several antibiotics showed susceptible to the essential oils of *R. officinalis*.

Standard compounds have an activity at range of 0.7 to 2.5 cm as shown in table 3. Rosmarinic acid and carnosic acid were found to be the most effective antimicrobial compounds against bacterial species in this study. The antimicrobial efficacy may be associated with their specific action as antioxidants. It was found by [15] that ethanol plant extracts displayed broad spectrum activity against gram-positive and gram-negative bacteria.

Callus extracts activity against pathogenic micro-organisms, gram-positive bacteria (*Staphylococcus aureus*) and gram-negative (*Escherichia coli* and *Salmonella typhi*) was shown in table 4. The MIC of rosemary extract was considered as the lowest concentrations which inhibit growth of microbial cells. Data indicated that higher concentrations of the extract were required to inhibit G-negative bacteria. It was found that 320 ul/ml of extract was enough to inhibit *E. coli*, while 640 ul/ml was required to inhibit *Salmonella typhi*. Callus ethanol extract was more effective against gram-positive bacteria; *Staphylococcus aureus*. Their growth was inhibited at 160 ul/ml of extract. These results are in an agreement with [16] who stated that gram-positive bacteria were the most sensitive bacteria as compared with other bacterial species. Higher activity of the extract as well as its components against bacterial species was reported by [17], whom found that the differences in the antimicrobial activity might be due to the difference in bacterial cell wall constituents.

In conclusion, rosemary essential oil can be used in pharmaceutical industries for the production of new synthetic agents to treat the infectious diseases caused by these bacterial species. Food pathogens, such as, *E. coli* and others which might cause food spoilage and subsequently human diseases. The use of plants to heal diseases including infectious ones, has been extensively applied by people.

References:

- 1-Eva, S. B.; Maria, H. T.; Attila, H.; Csilla, R.; and Szollosi, V. "Antioxidant effect of various rosemary (*Rosmarinus officinalis* L.) clones". Acta Biol., 47(1-4): 111-113,2003.
- 2-Sergi, M. B.; Leonor, A. and Karin, S. "The formation of phenolic diterpenes in *Rosmarinus officinalis* L. under Mediterranean climate". Eur. Food Res. Technol., 210: 263-267, 2000.
- 3-Genema, A. K.; Hense, H.; Junior, A. S. and Souza, S. M. "Rosemary (*Rosmarinus officinalis*) a study of the composition, antioxidant and antimicrobial activities of extracts obtained with supercritical carbon dioxide."Ciência Tecnológica de Alimentos. 28(2):463-469, 2008.
- 4-Oluwatuyi, M.; Kaatz, G. W. and Gibbons, S. "Antibacterial and resistance modifying activity of *Rosmarinus officinalis*." Phytochemistry-London /Detroit, 65(24):3249-3254, 2007.
- 5-Misra, G. and Pavlov, S. (1997). Biodegradation kinetics of monoterpenes in liquid soil-slurry systems". Appl. Microbial., Biotechnol., (7): 562-577,2000.
- 6-Mukherjee, P.; Saritha, G. and Suresh, B. "Antimicrobial potential of two different *Hypericum* species available in India". Phytother. Res., (16): 692-695, 2002.
- 7-Obame, L.; Koudou, J.; Kumulungui, B.; Bassole, I.; Edou, P.; Quattara, A. and Traore, A. Antioxidant and antimicrobial activities of *canarium schweinfurthii* engl". Essential oil from Centrafrican Republic, African J. of Biotechnol., 6(20): 2319-2323,2007.
- 8-Harborne, J. B. "Phytochemical methods. A guide to modern technique of plant analysis". Chapman Hall, London, 1984.
- 9-Masa, I. R.; Darinka, B.V.; Valter, D. and Eenes, V. "Determination of major phenolic acids, phenolic diterpenes and triterpenes in rosemary (*Rosmarinus officinalis* L.) by gas chromatography and mass spectrometry". Acta Chem. Slov., 54: 60-67, 2007.
- 10-Adams, R. P. "Identification of essential oil compounds by Gas Chromatography / Mass Spectroscopy". Allured, Carol Stream, Illinois,: 1-15, 2002.
- 11-Ahmed, S. J. "The transfer of genetic and immune-determinant and genetic improvement of *Salmonella typhi* strain used as oral vaccine" (M.Sc. thesis) College of Science. Baghdad University, 1997.
- 12-Bauer, A. W.; Kirby, W. M.; Sherris, J. C. and Turck, M. "Antibiotic susceptibility testing by a standardized single disc method". AM. J. Clin. Pathol., 45: 149-158, 1996.
- 13-Martos, M. V.; Navajas, Y. R.; Fernandez-Lopez, J. and Perez Alvarez, J. A. "Chemical composition of the essential oils obtained from spices widely used in Mediterranean region". Acta Chim. Slov., 54: 921-926, 2007.
- 14-Oyedemi, S. O.; Pirochenva, G.; Mabinya, L.V.; Bradley, G. and Afolayan, A. J. "Composition and comparisons of antimicrobial potencies of some essential oils and antibiotics against selected bacteria." African J. Biotech. 7(22): 4140-4146, 2008.
- 15-Santoyo, S.; Cavero, S.; Jaime, L.; Ibanez, E.; Senorans, F. and Reglero, G. "Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. obtained via supercritical fluid extraction". J. Food Port., 68(4): 790-795, 2005.
- 16-Grosvenor, P.; Supriona, A. and Gray, D. "Medicinal Plants from Riau Province, Sumatra, Indonesia. Part 2: Antibacterial and antifungal activity". J. of Ethnopharm., 45: 97-111, 1995.
- 17-Rico, M. C. and Rios, J. L. "A review of some antibacterial compounds isolated from medicinal plants". Phytother. Res., 3: 116-117, 1989.