

**Evaluation of antimicrobial and anticancer activity of Rosemary
Rosmarinus officinalis leaves extracts on pathogenic
bacteria and cancer cell lines**

تقييم الفعالية التثبيطية الجرثومية والسرطانية لمستخلص اوراق اكليل الجبل
Rosmarinus officinalis في عزلات بكتيرية مرضية وخطوط خلوية سرطانية

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Abstract

The present study was carried out to detect the active constitutions extracted from *Rosmarinus officinalis* leaves by 70% methanol, the dried weight of aqueous extract (F1) were 12%(w:w) contain polyphenols represented by phenols, flavonoids and coumarins as a major compounds , while the dried weight of organic extract, [chloroform(F2)] was 2.8%(w:w) represented as terpenoids, tannins and coumarins detected by qualitative chemical analysis. Antimicrobial activity of Polyphenols and terpenoids were tested against four pathogenic bacteria isolates including: *Escherichia coli*; *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus cereus* ,which grown on Mueller Hinton agar (MHA) plates and then treated with different concentrations of rosemary extract (F1) and (F2) ranged from 0.15-1.2mg/ml, the results revealed that the higher inhibition zone obtained by F1 were 35mm in *S. aureus*, 24mm in *B. cereus* and lowest inhibition were 16mm in *E. coli* and *P. aeruginosa* at concentration 1.2mg/ml, and the inhibition were depends on extract concentrations. The MIC value of F1 was 600 µg/ml against *S. aureus*, 1200 µg/ml against *B. cereus* and 2400 µg/ml against *E. coli* and *P. aeruginosa*, while the MIC_s values of F2 were 2400 µg/ml against *S. aureus* and *P. aeruginosa*, and 4800 µg/ml against *E. coli* and *B. cereus*. Furthermore calculating index fractional inhibitory concentration (Σ FIC) of rosemary extracts (F1 or/and F2), the Σ FIC_A ranged from synergistic to additional on *E. coli* and *P. aeruginosa* respectively, antagonistic on *S. aueras* and *B. cereus*, while the Σ FIC_B index showed synergistic effects on all tested organisms except the additional effect on *P. aeurginosa* on the basis of resultant zone of inhibition. The anticancer effects of (F1) were used on three cell lines, human larynx epidermoid carcinoma (Hep-2), mammary adenocarcinoma (AMN-3), rhabdomyosarcoma (RD) and one normal cell line, Rat embryo fibroblast (REF) at different concentrations ranged from 62.5-1000 µg/ml. The highest inhibition rate (IR) was 82.41%, 85% on RD, 52.62%, 75.48% on Hep2, 39.14% and 49.75% on AMN-3 at concentrations 500 and 1000µg/ml after 72h exposure time respectively, while IR of Ref cell line exhibit low inhibition rate ranging from 5.8 to 15.7% after 72h in the same conditions.

Key words: antimicrobial , anticancer , *Rosmarinus officinalis* , extract , pathogenic

المستخلص

اجريت الدراسة للكشف عن المركبات الفعالة المستخلصة من اوراق اكليل الجبل *Rosmarinus officinalis* بالكحول المثلي 70%، حيث احتوى الوزن الجاف للمستخلص المائي (F1) 12% (وزن:وزن) على الفينولات المتعددة، تمثل الجزء الاكبر منها بالفينولات والفلافينويدات والكومارينات، في حين احتوى الجزء العضوي والمستحصل عليه بعد فصل الجزء المائي ومعاملته بالكلوروفورم (F2) على 2.8% (وزن:وزن) من المواد الفعالة التي تمثل الجزء الاكبر منها بالتربينات والثانينات والكومارينات. اختبرت الفعالية ضد الجرثومية للمستخلص المائي (F1) والكحولي (F2) في اربع عزلات بكتيرية مرضية تمثلت *Escherichia coli*، *Bacillus cereus*، *pseudomonas*، *aeruginosa*، *Staphylococcus aureus*، اطباق تحتوي وسط (MHA)، ثم عوملت بتركيزات مختلفة من المستخلص F1 و F2 تراوحت من 0.15-1.2 ملغم/مل، اظهرت النتائج ظهور اكبر حالة تثبيط للمستخلص F1 بقطر 35 ملغم لبكتريا *S. aureus* و 24 ملغم لبكتريا *B. cereus*، 16 ملغم لبكتريا *E. coli*، *p. aeruginosa* عند تركيز 1.2 ملغم/مل وقد اعتمد التثبيط على تركيز المستخلص. كما قيس التركيز المثبط الادنى (MIC) بطريقة التخفيف لـ F1 وبلغت قيم 600 ملغم/مل *S. aureus* 1200 ملغم/مل ضد *B. cereus* و 2400 ملغم/مل ضد *p. aeruginosa* و *E. coli*، بينما بلغ MIC لـ F2 2400 ملغم/مل ضد *S. aureus* و *p. aeruginosa* و 2400 ملغم/مل ضد *E. coli* و *B. cereus* فضلا عن حساب قيم معامل التركيز التثبيطي الجزئي (FIC) (Fractional inhibitory concentration) لمستخلصي اكليل الجبل F1 و F2 حيث تراوح $\sum FIC_A$ بين تأزري الى اضافي لبكتريا *E. coli* و *p. aeruginosa* على التوالي وتأثيرا تضاديا على بكتريا *S. aureus* و *B. cereus*. بينما كان $\sum FIC_B$ تأثيرا تأزريا على ثلاثة اجناس من البكتيريا المدروسة ماعدا تأثيرا اضافيا في بكتريا *p. aeruginosa* اعتمادا على قطر منطقة التثبيط الناتجة. كما تم استخدام المركبات الفينولية F1 لدراسة الفعالية السمية في ثلاثة خطوط خلوية سرطانية تضمنت خلايا سرطان الحنجرة البشري Hep-2 وخلايا سرطان الغدة اللبنية للفار 3-AMN وخلايا سرطان العضلة للانسان RD، وخط الخلايا الطبيعية لجنين الجرذ Ref بتركيز تراوحت بين 62.5-1000 مايكروكرام/مل، بلغت اعلى نسبة للتثبيط (IR) (Inhibitory rate) 85%، 82.41% في RD، و 52.62%، 75.48% في Hep-2 و 39.14% و 49.75% في 3-AMN عند التركيزين 500 و 1000 مايكروكرام/مل بعد 72 ساعة من وقت التعرض على التوالي. بينما كانت نسبة التثبيط لخط الخلايا الطبيعية واطنة تراوحت بين 5.8% الى 11.15.7% بعد 72 ساعة من وقت التعرض في الظروف نفسها.

Introduction:

Rosemary (*Rosmarinus officinalis* Linn.) is a common household plant grown in many parts of the world. Rosemary is commonly used as a spice, a flavoring agent in food processing and a beverage drink as well as in cosmetics. The distribution of six compounds with three different polyphenol skeletons have been studied in rosemary: phenolic diterpenes (carnosic acid, carnosol, and 12-O-methylcarnosic acid), caffeoyl derivatives (rosmarinic acid), and flavones (isoscuteallarein 7-O-glucoside and genkwanin), each showing a characteristic behavior and distribution during the vegetative cycle. Only in leaves were all six compounds present. Rosmarinic acid showed the highest concentrations of all the polyphenols in all organs [1].

The most important constituents are carnosol, carnosic acid, caffeic acid and its derivatives such as rosmarinic acid [2]. These compounds have powerful antioxidant activity that Carnosic acid, the polyphenolic diterpene derived from rosemary, is a strong dietary antioxidant that exhibits antimutagenic properties in bacteria and anticarcinogenic activity in various cell and animal models. The inhibition capacities; of carnosol, rosmarinol, and epirosmarinol, which are phenolic diterpenes from rosemary, to oxidized low-density lipoprotein (LDL) formation in human blood and detect their scavenging activities to lipid free radical and superoxide anion *in vitro* [3].

Rosemary and its constituents have a therapeutic potential in bronchial asthma, peptic ulcer, prostate disorders, inflammatory diseases, liver toxicity, atherosclerosis, stroke, ischemic heart disease, cataract, leukemia, cancer and poor sperm motility. In folk medicine it is used as an antispasmodic in renal colic and dysmenorrhea, and in relieve respiratory disorders, as well as its extract relaxes smooth muscles of trachea and intestine, and has choleric, hepatoprotective and antitumorigenic activity [4]. Furthermore, there have been few reports on the volatile components of rosemary. These data suggest that the volatile constituents of a rosemary extract had antioxidative properties [5].

Our study aimed to extract the active constituents of rosemary and evaluate their antimicrobial activity against some pathogenic bacteria isolates and its synergistic effects with standard antimicrobial compounds, furthermore the cytotoxicity of the rosemary extract on some cancer cell lines.

Materials and methods:

Plant collection: The dried leaves of rosemary were purchased from traditional and folk medicine store in Baghdad then ground to obtain the powder and store at dry place.

1-extraction of active compounds:

Active constituents were extracted according to [6] by mixing 100g of rosemary leaves powder with 400ml of 70% methanol by magnetic stirrer for 18h at room temperature, then the mixture was filtered through Whatman filter paper No.1 by vacuum pump, the filtrate was kept in clean flask, while the precipitate was remixed with 70% methanol (1:2w/v) at the same conditions, the obtained filtrate accumulated with the first filtrate. Second step involved evaporation of alcohol from the extract (filtrate), and partially purified with chloroform at ratio 1:5 (v/v) with separatory funnel. Two layers were formed, upper layer (aqueous phase) (F1) represent polyphenols and the lower layer (organic phase) (F2) represent terpenoids, were collected separately, and dried by lyophilizer. The qualitative chemical tests of the active compounds in extract solutions (F1&F2) such as phenols were detected according to [7]. Alkaloids, flavonoids and glycosides were detected according to [8, 9]. Tannins and terpenes according to [10, 11].

High performance liquid chromatography (HPLC) was accomplished in reverse phase by injection 10µl of rosmarinic acid at a concentration 10µg/ml (as standard solutions), and 10µl of five times diluted solution (F1) before lyophilization using ODS-C18 column, the mobile phase was 1% of phosphate buffer: methanol (40: 60) at flow rate 0.5 ml/min by detector Ach at 254 nm.

2-tested microorganisms:

The cultures of bacteria were obtained from department of biotechnology, College of Science, University of Baghdad. Two isolates of gram positive bacteria are. *S. aureus*, *B. cereus* and two isolates of gram negative bacteria *P. aeruginosa*, *E. coli* were used to evaluate the antimicrobial activity of aqueous (F1) and alcoholic (F2) extracts. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

3-Antimicrobial assay:

Screening of rosemary extracts for antimicrobial activity was done by the disk diffusion method [12], which is normally used as preliminary check. It was performed using an 18hr culture at 37°C in 10ml of Mueller Hinton broth (MHB) the culture was adjusted approximately 10^5 cell forming unit/ml (CFU) with sterile saline solution. Five hundred microliters of suspension were spread over the plates containing Mueller Hinton agar (MHA) using a sterile spreader in order to get a uniform microbial growth on both control and test plates.

The rosemary extracts powder (polyphenols and terpenoids) each one was dissolved in 10% aqueous dimethylsulfoxide (DMSO) then the solubility was complete with phosphate buffer saline (PBS) to prepare stock solutions, later sterilized by filtration through 0.45µm membrane filter. Under aseptic conditions, empty sterilized disks (Whatman No.5, 6mm diameter) were impregnated with 50µl of different concentrations (0.15, 0.3, 0.6, 1.2)gm/ml of respective extract and placed on inoculated culture plate separately, and the plates were left for 30 min at room temperature to allow the diffusion of extracts, as well as standard disk containing Streptomycin (25µg/disc), Ampicillin (10µg/disc) and Tetracycline (30µg/disc) were used as reference control, and then the plates were incubated at 37°C for 48h under aerobic conditions [13].

4-Minimum inhibitory concentrations (MIC):

Four genera of bacterial isolates were re-activated in 10ml MHB. The agar dilution method recommended by clinical laboratory standard (CLSI) [14] was used with some modifications. A series of two fold dilutions of each extract ranging from 0.15 to 4.8mg/ml were prepared in MHA at 48°C. plates were dried at room temperature for 30min prior to spot inoculation with 10µl aliquots of culture containing approximately 10^5 cfu/ml of each organism. Inoculated plates were incubated at 37°C for 18h, and the MIC determined. Experiments were carried out in triplicate.

Incubation of bacterial growth in plates containing tested extracts was judged by comparison with growth in blank control plates. The MICs were determined as the lowest concentration of extract inhibiting visible growth of each organism on the agar plate [15].

5- Combinations test (E test):

An inoculum equal to 0.5 McFarland turbidity standards was prepared from each organism, and 10 µl of suspension was inoculated onto MHA plate. E test strip of ampicillin, tetracycline and streptomycin (Sigma chemical co.) were stored at -20 until used. The E test strips were applied to the inoculated culture plates separately by using template as recommended by manufacturer and the plates were incubated at 35 °C for 48h under aerobic conditions.

For testing, the combinations of E test of standard antimicrobial and extracts (F1 or F2) solutions, strips were placed on the same culture medium in across formation, with a 90° angle at the intersection between the scales at the respective MICs for organisms, and the plates were incubated at 35 °C for 48h.

Determining MICs by E test were performed in duplicates, according to the recommendations of manufacturers, and the MICs were interpreted at the point of intersection between the inhibition zone and E test strip [16].

The fractional inhibitory concentration index (ΣFIC) was calculated on the basis of the resultant zone of inhibition as follows: $\Sigma FIC = FIC_A + FIC_B$, where FIC_A is the MIC of combination or mixed extract (F1+F2) /MIC of drug A alone (or one extract, F1), and FIC_B is the MIC of combination or mixed extract (F1+F2) /MIC of drug B alone (or one extract F2) [16].

6- Cell line growth and cytotoxicity assay:

Human larynx epidermoid carcinoma (Hep-2), mammary adenocarcinoma (AMN-3), Rhabdomyosarcoma (RD) and Rat embryo fibroblast (REF) were obtained from Iraqi center of cancer and medical genetic research, Al Mustansiria University. The cells were grown in RPMI-1640 medium containing 10% fetal calf serum to form confluent monolayer.

The attached cells washed firstly with PBS and harvested from the flask by treatment with trypsin-versine solution. The cells were counted by trypan-blue (about 95% viability), then cell suspension used for determination of cytotoxicity, 200 μ l of cell suspension were seeded in 96-well microtiter plate at density 1×10^5 cells/ml and incubated in CO₂ incubator for 24h at 37°C, then 200 μ l of different concentration of rosemary extract (F1) (1000, 500, 250, 125, 62.5) μ g/ml were prepared in serum free media (SFM) and added to cells, then re-incubated the plates for additional 48h and 72h [17].

Five replicate wells were used for each concentration and negative control wells were treated with SFM. At the end of exposure periods, the cells were stained by 100 μ l of crystal violet solution and incubated at 37 °C for 30 min [18]. The optical density of each well was read by micro-ELISA reader at 492nm. The percentage of inhibition was calculated according to [19].

Inhibition rate (IR) = (optical density of control wells-optical density of test wells)/optical density of control wells $\times$ 100

Statistical analysis: the experiments data were analyzed using statistical software SPSS version 16, significant differences between control and sample means were assessed using student's T-test and P values ≤ 0.05 were considered significant.

Results and discussion:

Qualitative and quantitative tests of some active compounds in rosemary leave extract:

Results obtained by qualitative chemical detection indicated the presence of phenols, flavonoids, coumarins, alkaloids and weak indication for tannins and the absence of terpenes, glycoside and saponines in aqueous extract (F1) and the yield of partially purified extract was 12%(w/w), while the qualitative tests for organic extract (F2) showed the presence of terpenes, tannins and coumarins as a major compounds and flavonoids, alkaloids and saponine in lowest contents, and the yield of this fraction was 2.8%.

HPLC analysis was accomplished to determine the most important compound in rosemary leave extract (F1) using a rosmarinic acid standard as reference. The result showed in Figure (1) indicates the retention time of standard rosmarinic acid was 6.258 min, while it was 6.624 min for partially purified polyphenols (rosmarinic acid) in aqueous extract F1. So the concentration of rosmarinic acid content was 59% of aqueous extract according to total peak area (TPA).

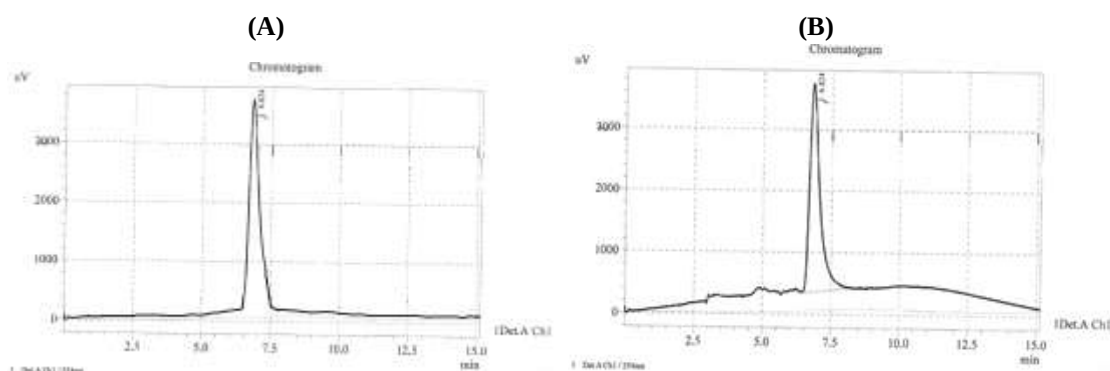


Fig.(1): chromatographic resolution by HPLC for standard rosmarinic acid (A), and fraction components of rosemary leaf extracts (F1) (B) in reverse phase of column ODS-C18, mobile phase methanol: phosphate buffer (60:40) at flow rate 0.5 ml/min reading by detector Ach 1 in 254 nm.

The distribution of compound in aqueous extract (F1) showed the major peak is rosmarinic acid and there are many minor peaks of other compounds that may be carnosic acid, carnosol and other phenolic compounds such as flavonoids, coumarins and phenols. Many researchers revealed many compounds were detected in rosemary such as [1] recorded the distribution of six compounds with three different polyphenol skeletons have been studied in *Rosemarinus officinalis*: phenolic diterpenes (carnosic acid, carnosol, and 12-O-methylcarnosic acid), caffeoyl derivatives (rosmarinic acid), and flavones showing a characteristic behavior and distribution during the vegetative cycle. Only in leaves were all six compounds present, and all the highest accumulation rate was related with the young stages of development. Furthermore the 12 different rosemary accessions were obtained dependent on variety polarity of solvent used in extraction [13]. As well as Rosmarinic acid, carnosic acid, and seven of their terpen type metabolites, and seven flavonoids were identified from liquid chromatography-mass spectrophotometric analysis of rosemary, and during the distillation treatment used with plant material, strongly affected the content of the two compounds of higher antioxidant activity, rosmarinic acid and carnosic acid [20].

Our results came in agreements with that obtained by [21], water extract of rosemary containing only 12% of rosmarinic acid, while the methanol extract containing 30% of carnosic acid, 16% carnosol and 5% rosmarinic acid. In addition to, our extract were obtained by 70% methanol might accumulate that compounds.

Antimicrobial activity:

The antimicrobial activity of rosemary extracts is summarized in Table (1); the results revealed that rosemary extract (F1) showed antibacterial activity with varying magnitudes. The zone of inhibition of resistance depends on that zone diameter limited to control strains of (*E. coli* ATCC25922, *P. aeruginosa* ATCC27853 and *S. aureus* ATCC25923) for NCCLS [22]. Generally most of the tested organisms were sensitive to the polyphenols (F1); the results exhibited a graduated increasing on inhibition depending on increasing the concentration of extracts from 0.15 to 1.2 mg/ml. Significant inhibitory effects of F1 were shown against *S. aureus* (35mm), *B. cereus* (24mm), and insignificant effects revealed against *E. coli* and *P. aeruginosa* (16mm) depending on extract

concentrations. Less differences and insignificant in resistance were found between different concentrations of rosemary terpenes (F2) on tested organisms, and the results showed no inhibition of growth with the negative control (10% DMSO). The effects of three standard antibiotic disc (Amp, Tetr, Strep) were high susceptibility against local isolates including *E. coli*, *S. aureus* and *P. aeruginosa* while *B. cereus* exhibit resistance on Amp and Tetr, and intermediate susceptibility on Strep, our results compared according to [12].

Table (1): Antimicrobial activity of rosemary leave extracts against growth of tested organisms using disc diffusion method

organisms Conc. (mg/ml)	Diameter of zone of inhibition (mm) caused by								Inhibition zone(mm)for		
	F1(mg/ml)				F2 (mg/ml)				standard antibiotic		
	Aqueous extract				Organic extract				disk		
	0.15	0.3	0.6	1.2	0.15	0.3	0.6	1.2	Amp	tetr	strept
<i>E. coli</i>	13	10	15	16	12	12	12	12	35	25	28
<i>S. aureus</i>	15	30	32	35	8	12	14	16	32	43	30
<i>B. cereus</i>	12	17	22	24	10	10	10	12	10	11	15
<i>P. aeruginosa</i>	14	16	15	16	14	14	15	17	35	28	22

Antimicrobial rosemary extract efficacy was associated with specific phenolic compounds. Carnosic acid and rosmarinic acid may be the main bioactive antimicrobial compounds present in rosemary extract. Rosemary extract may be a good candidate for functional food as well as pharmaceutical plant-based product [21]. The inhibition zones obtained by our experiments were comparable with standard inhibition zones recorded for antibiotics to standard strains [8]. The antioxidant activity of tow extracts referred to its different polyphenolic compositions that were evaluated in methanolic system that clearly suggest as expellant antioxidant [1]. To achieve effective treatment, antimicrobial that can be penetrating cell at high concentration should be determined, and the duration of therapy should be set properly [22].

Minimum inhibitory concentration (MIC):

Minimum inhibitory concentration (MIC) for testing extracts ranged from 150 to 4800 µg/ml showed in Table (2). This experiment revealed that rosemary polyphenols (F1) recorded maximum activity with MIC values ranging from 600 to 4800 µg/ml against *S. aureus* and 1200-4800 µg/ml against *B. cereus* followed by MIC values ranging from 2400-4800 µg/ml against *E. coli* and *P. aeruginosa*, whereas all concentrations of rosemary terpenes (F2) showed moderate MIC values concentration (2400µg/ml) against *S. aureus* and *P. aeruginosa* and slight MIC values against *E. coli* and *B. cereus*. While the mixed rosemary extract (F1and F2) not always revealed maximum activity with the MIC values against tested bacteria, a variety differences results in susceptibility was found as shown in Table (2).

Table(2): Minimum inhibitory concentrations (MIC) of rosemary (aqueous F1 and organic extracts F2) extracts separately and combined and standard antimicrobial solutions on tested bacteria.

organism	MIC conc.(µg/ml)			FIC index according to		MIC conc.(µg/ml)of standard		
	MICconc.	F1	F2	F1+F2	Mix(F1&F2)/F1 ΣFIC_A	Mix(F1&F2)/F2 ΣFIC_B	Antimicrobial solutions tetra. Amp. strep.	
<i>E. coli</i>		2400	4800	600	0.255	0.125	256	1024 512
<i>S. aureus</i>		600	2400	1200	2	0.5	512	128 512
<i>B. cereus</i>		1200	4800	2400	2	0.5	1024	2048 1024
<i>P.aeruginosa</i>		2400	2400	2400	1	1	256	512 1024

ΣFIC (fractional inhibitory concentration= MIC for mixed (F1&F2)/MIC for single extract (F1 or F2).

≤ 0.5 syn, $0.5 \leq 1$ additional, $1 \leq 2$ uneffected, > 2 antagonistic [23].

According to FIC_A and FIC_B index, many concentrations of F1 or F2 were tested separately on microorganisms, ΣFIC_A ranged from synergistic to additional on *E. coli* and *P. aeruginosa* respectively, antagonistic on *S. aureus* and *B. cereus* while the ΣFIC_B index showed synergistic effects on three tested microorganisms and the additional effect on *P. aeruginosa* Table (2) [23].

The MIC values of Amp against *E. coli* and *P. aeruginosa* were ≥ 256 µg/ml, while it was ≥ 512 µg/ml on *S. aureus* and was 1024 µg/ml against *B. cereus*. The lowest MIC values of tetra were recorded from 128-2048 µg/ml on *S. aureus*, and from 512-2048 µg/ml, 1.02-2048 µg/ml, and ≥ 2048 µg/ml against *P. aeruginosa*, *E. coli* and *B. cereus* respectively, while MICs values of streptomycin ranging from 512-2048 µg/ml against *E. coli*, and *S. aureus*, and from 1024-2048 µg/ml against *B. cereus* and *P. aeruginosa* Table (2).

The MIC of ethanolic extract of rosemary was 1 % (100mg/ml) for gram positive bacteria including *Lecunostoc mesnteroides*, 0.5% for *Listeria monocytogenes* and *Staphylococcus*, 0.13% for *Streptococcus mutanus*, 0.06% for *Bacillus cereus*, while up to 1% of ethanolic solution had no activity on gram negative bacteria such as *E. coli*, salmonella and *Erwinia* [24].

Methanol extract of rosemary containing 30% carnosic acid, 16% carnosol and 5% rosmarinic acid were the most effective antimicrobial compounds tested against G+ve bacteria, MIC between 2-15 µg/ml, MIC for G-ve bacteria 2-60 µg/ml, by contrast, water extract containing only 15% rosmarinic acid showed narrow activity [14], as well as the MIC values of rosemary oil were recorded on gram positive and gram negative bacteria ranged from 6.4- 12.8 mg/ml [23].

By E test, the ΣFIC_A results of the combination of respective MICs of rosemary extracts and the antimicrobial solutions, F1-amp. Yield synergy against *E. coli* and *P. aeruginosa* and yield additional against *S. aureus* and *B. epidermis*, whereas ΣFIC results of the combination of F1-tetra and F1-strep yield synergistic against *S. aureus*, additional against *E. coli* and *P. aeruginosa*, while there were antagonistic ≥ 2 against *B. cereus* Table (3). From other point the ΣFIC_B index of the combination of F2-Amp, F2-tetra and F2-strep. Yield unaffected and antagonistic against all tested organisms Table (3).

Table (3): Fractional inhibitory concentration (FIC) index of standard antimicrobial solutions and rosemary extracts against tested organisms obtained by E-test.

Tested organisms	ΣFIC_A activity			ΣFIC_B activity		
	F1-Amp	F1-tetra	F1-strep	F2-Amp	F2-tetra	F2-strep
<i>E. coli</i>	0.5	1	1	1	2	1
<i>S. aureus</i>	1	0.5	0.5	1	1	1
<i>B. cereus</i>	1	2	3	<1	>2	2
<i>P.aeruginosa</i>	0.5	1	1	1	1	2

Even through of these experiments, different conditions and end points have been used. There was frequent agreement between the results of the two methods (E-test and checkerboard) [13]. A comparable results were obtained throughout our experiments using E-test and disc diffusion, also Manoharan, *et al* 2003 [24] revealed over all agreement results of E-test and agar dilution method for some standard antimicrobial solutions (100% cefotaxime and ampicillin and least for chloramphenicol 92%). Incorporation of carnosic acid and carnosol into the growth media at 10 μ g/ml caused a 32 and 16 fold potentially of the activity of erythromycin against erythromycin-effluxing strains, respectively [25].

Further comparison of E-test technique with MICs methods for determination of synergy is warranted. E test can easily applied to antibiotic susceptibility test as it is less labor, intensive and less time consuming. The standardization of these techniques for routine laboratory testing is needed because of the common therapies against growing numbers of multi-drug resistant strain [26].

Effects of rosemary extract on growth of tumor and normal cell lines:

The *in vitro* growth inhibitory effects of rosemary extract (F1) was assayed for five concentrations (1000, 500, 250, 125, 62.5) μ g/ml against three tumor cell lines (Hep-2, AMN-3 and RD), and one normal cell line (REF). The results showed in figure (2-A) revealed a significant ($P < 0.05$) increasing inhibition rate of Hep-2 cultured cells as compared to control cultures, ranging from 5.6% to 50.81% and 12.19% to 75.48% at concentrations ranged from 62.5 to 1000 μ g/ml at 48h and 72h of exposure time respectively.

The growth inhibition rate of AMN-3 cultured cells revealed moderate effects in significant increasing caused by increasing concentrations of rosemary extract from 62.5 to 1000 μ g/ml, inhibition rate ranging from 9.62% to 36.64% and from 23.27% to 49.75% at exposure time 48h and 72h respectively figure (2-B).

The pattern of inhibition was highest significant ($P \leq 0.05$) in RD cultured cells as compared with control cultures, the rate ranging from 43.35% to 89.41% and from 70.07% to 85.03% at 48h and 27h of incubation in concentrations from 62.5 to 1000 μ g/ml respectively figure (2-C). The inhibition rate was concentration dependent, as well as time dependent on three tested tumor cell lines, except the inhibition rate on RD cells at concentrations 62.5, 125 and 250 μ g/ml after 72h which showed insignificant differences depending on these concentrations.

The inhibition rate was also concentration and time dependent on normal cell (REF); since mild effects of inhibition less than 10% were revealed at concentrations, 500 and 1000 μ g/ml after 48h and at concentrations (62.5, 125, 250) μ g/ml while slight effects

were detected at concentrations 500 and 1000 $\mu\text{g/ml}$ after 48h and 72h of exposure time figure (2-D).

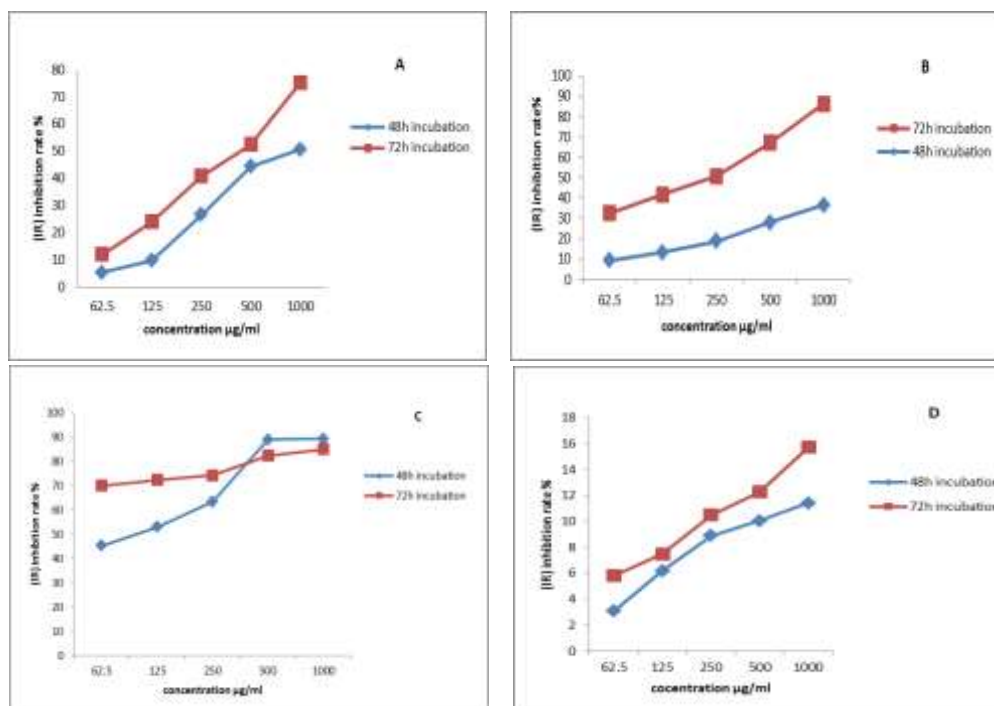


Fig. (2): Effects of *rosmarinus officinalis* leaves extract (F1) at different concentrations on percentage of inhibition rate (IR) of tumor cell lines [Hep-2(A), AMN-3 (B), RD (C)] and normal cell line (REF) (D) after 48h and 72h of exposure time.

Cytotoxicity screening models reveals important preliminary data to help in selection plant extracts with potential antineoplastic properties [27]. However the present study showed that rosemary extract (polyphenol, F1) inhibit significantly the proliferation of human tumor cell lines $\text{RD} > \text{Hep2} > \text{AMN-3}$ and its activity is concentration and time dependent.

These effects can be referred to the nature and chemical constituents of rosemary extract (polyphenol), mainly rosmarinic acid (α -o-caffeoyl-3,4dihydroxy phenl lactic acid) which has been observed to possess and anti-cancer properties[28] and antioxidant activities, these effects may be due to (carnosic acid, carnosol, and 12-O-methylcarnosic acid to induce the enzymes such as cyclooxygenase (COX) aids the defense mechanisms in the body to detoxify carcinogens and xenobiotic, as well as reduce oxidative stress[29]. And induce the proapoptotic genes (BAX) and reduce antiapoptotic genes [30].

These results are in agreement with the result of [31] (Ros A) rosmarinic acids inhibit Jurkat cell proliferation by altering the expression of cyclins and cyclin-dependent kinases and induce apoptosis [30] Similar results indicated that rosemary extract have anti-proliferative activities and growth inhibition on various cancer cell lines including colon and breast cancer cell lines and in nonmalignant mammary epithelial cells. Researchers have had promising results in studies of its efficacy against breast cancer, prostate cancer, colon cancer, leukemia and skin cancer [31]. The anticancer effect has been suggested to be mediated through induction of p53 expression which causes cell cycle arrest and apoptosis [32]. Furthermore, the antineoplastic effect of rosemary extract

(carnosic acid) based on increase intercellular accumulation of common used chemotherapeutic agents including doxorubicin and vinblastine in cancer cell [33].

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