Original Article

Antibacterial effects of the extracts of rosemary (Rosmarinus officinalis L.) Leaves on three multidrug resistant bacterial isolates using nanoparticle size technique

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Abstract

The present study was consisted of four simple experiments applied in the laboratories of the Department of Biology, College of Science, Salahaddin-Erbil University, and Scientific Research Center at Erbil Polytechnic University to determine the effects of methanol extract, ethyl acetate extracts and essential oils of the leaves of rosemary (Rosmarinus officinalis L.) extracts on multidrug resistant bacterial isolates such as Escherichia coli, Staphylococcus aureus and Pseudomonas aeroginosa during 2022. The results showed that the rosemary essential oil (RMEO) have antibacterial effects on E. coli and S. aureus, methanol rosemary leaf extracts (MRLE) affected on E. coli at 300 µg/ml and affected on the three bacterial isolates at conc. 300 µg/ml when used with Ag-NPs but ethyl acetate rosemary leaf extracts (EARLE) affected on S. aureus when used alone and on S. aureus and P. aeroginosa when used with Ag-NPs at conc. 50 µg/ml and affected on the three bacterial isolates at conc. 100 µg/ml. The minimal inhibition concentration (MIC) of RMEO was 300µg/ml when used alone for E. coli and 300µg/ml with Ag-NPs. The MIC of ME was 300µg/ml for E. coli alone or with Ag-NPs and 300µg/ml for the three bacterial isolates when used with Ag-NPs only, and MIC of (EARLE) was 50µg/ml for S. aureus when used without Ag-NPs, 50µg/ml for S. aureus and P. aeroginosa when used with Ag-NPs and 100µg/ml for E. coli using alone or with Ag-NPs. P. aeroginosa was resistant against Amoxicillin/clavulanic acid (AMC30).

Keywords: Rosemary, Essential Oil, GC-MS, Silver nanoparticles, Escherichia coli, Staphylococcus aureus

Introduction

Despite the recent development of several new antimicrobial drugs by the pharmaceutical industry, microorganism resistance to these medications has grown quickly. Utilizing plant extracts known to have antibacterial characteristics can be of great significance in phytotherapy. Numerous researches have been carried out recently to confirm the potency of plant extracts against bacterial infections (1; 2). Plant extracts are widely used in traditional medicine to control the growth of pathogenic bacteria and food spoilage. When the antimicrobial effects of different medicinal plants' hydro-alcoholic extracts were tested on a variety of bacteria, it was discovered that rosemary extract was the most effective extract against E. coli (3). Essential oils of rosemary plant have an antibacterial effect on E. coli, and S. aureus, which this property varies depending on the dilutions of RMEO and bacterial species (4; 5; and 6).

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Nanoparticles have shown effective antimicrobial activity against multidrug resistance bacteria, for instance Pseudomonas aeruginosa, Staphylococcus aureus and others (7). Finding plants that have antibacterial properties can aid in the development of new medications with a variety of effects (8). The nanoparticles (NPs) are efficiently being used as antibacterial agents. Some studies have been reported which displays the antibacterial nature of NPs against gram negative and gram positive bacteria, mycobacteria and fungi (9). Due to their antibacterial activity against multidrug resistant pathogens, silver nanoparticles (Ag-NPs) are very popular (10). The antibiotic resistance of bacteria is a worldwide health problem, that is continually expanding and widely distributed for several gram-negative pathogens and is recognized as a medical problem that increases morbidity and mortality rates, which implies length of hospital stays as well as cost and bad prognosis (11). Many environmental factors play a role and affect the lethality of NPS to bacteria including temperature, pH and aeration. The physicochemical properties of the particles comprising shape, size, chemical modification and coating, and mixture in various ratios with other nanoparticles and solvent used all affect greatly their antibacterial activity (12).

The objective of our study was to investigate the antibacterial activity of methanol extract, ethyl acetate extract and Essential oils of rosemary leaves with or without silver nanoparticles against multidrug-resistant pathogenic bacteria such as E. coli, S. aureus and P. aeruginosa.

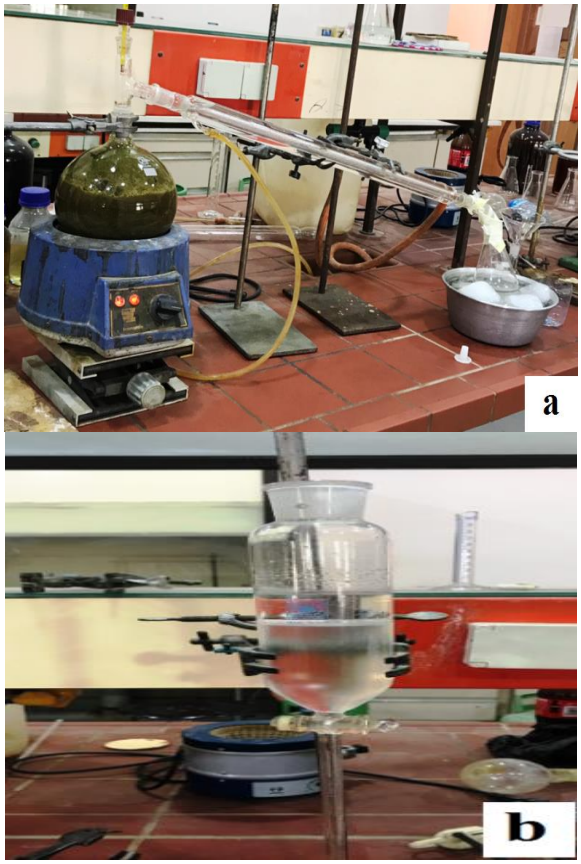


Fig.1: Hydro-distillation apparatus (a) and recovering the essential oil from distillation water using diethyl ether (b)

Material And Methods

Rosemary leaves were air-dried at room temperature for ten days with turn them up to down several times, ground to small pieces using electrical domestic grinder, then the powder was passed throw metal sieve with meshes of 2mm, and stored in plastic bottles in freezer at below -10 Co until used to extract the essential oils. To extract the essential oils from rosemary leaves (RMEO), 500gm of dried plant leaves were submitted to hydro-distillation apparatus (figure 1a), glass round bottom flask (size 2l) was filled with water (2/3 volume) and boiled using electrical heating mantle to produce the essential oil in a period of 3 hours, the cooled condensate (hydrosols) was collected in an conical receiving flask surrounded by ice (13), the thin layer of oil was recovered from hydrosol using organic solvent (diethyl ether). 30 ml of the solvent was added to 30 ml hydrosol (figure 1b), and then diethyl ether was separated from the oil using rotary vacuum evaporator. The collected RMEO was dried from water by anhydrous sodium sulfate (Na2SO4) and stored in a clean dark glass vial (tightly closed with tight lid) in freezer at -18oC till used for analysis in GC-MS apparatus.

Identification of each component in the essential oil of rosemary was done by GC-MS as described in (Adams, 2007).

Preparation of plant leaf extracts and Ag-NPs suspensions

Samples were collected from the leaves of rosemary (Rosmarinus officinalis L.), plants and transferred to the laboratory, washed with distilled water, then dried on the shade in the laboratory under sterile conditions, after that the leaves were grinded by an electric mill; the powder was kept in glass canes and stored in refrigerator at 6oC until used. Plant extracts were prepared using Absolute Methanol and Ethyl acetate as follows: (first) methanol extract: 500 ml of Absolute Methanol was added to50 g of powdered leaves and put on shaker at 100 rpm for 24 hours at room temperature, (second) ethyl acetate: 50 g of powdered leaves was taken and 250 ml of Ethyl acetate was added, left on the shaker at 100 rpm for 24 hours. The extracts were filtered, and then concentrated using rotary vacuum evaporator at 35°C, stored in dry and clean dark bottles in refrigerator until used.

A stock solution of Ag-NPs 20-30 nm was prepared. Suspended stock solutions of Ag-NPs in cooled deionized water in concentration of 20µg/ml (2mg in 100ml of sterilized

deionized water) were prepared by sonication for 60 seconds in an ultrasonic homogenizer (Telsonic power cleaning -25); particle suspensions were kept on ice for 15 seconds and then sonicated again on ice for a total of 3 minutes at a power of 400 W, Ag-NPs suspensions were shaken for 2 min, immediately before using (15).

The powder of silver nanoparticles, which purchased from Sky Spring Nanomaterials, USA the particles are dark grey spherical with purity 99.95%, 20-30nm(Figure 2), and specific surface area about 20m2/g, the density was 10.5 g/cm3, the synthesis process is wet chemistry (https://ssnano.com/i/u/10035073/h/CAT/Catalog_SSNano-2015.pdf), (https://ssnano.com/)

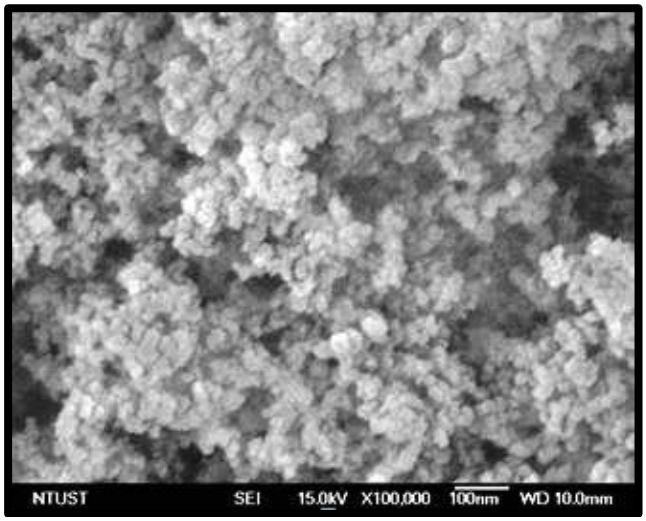


Fig.2: Scanning electron microscopy (SEM) image of silver nanoparticles

Preparation of Bacterial test isolates and activation of pure farms of microorganisms:

Three multidrug resistant bacteria were selected for the present study which was Pseudomonas aeruginosa (G-), Staphylococcus aureus (G+) and Escherichia coli (G-). The microorganisms were obtained from the laboratory of microbiology, Collage of Science, Salahaddin University-Erbil. Each strain was grown in separated test tube containing 45 ml of sterile nutrient broth at 37°C for 24h. Bacterial identification was confirmed by the laboratory of microbiology, College of Science. The isolates of the three bacterial isolates were activated 24 hours before testing of plant extracts using nutrient broth medium.

Antibiotic sensitivity test

E. coli, S. aureus, and Pseudomonas aeruginosa each were incubated at 37oC for 24 hours. The turbidity of actively growing broth culture was adjusted with sterile saline standards McFarland 0.5 ml, suspension was transferred onto the surface of nutrient agar and then spread evenly. The susceptibilities of all isolates to different antibiotics agents were tested by disc diffusion technique; the discs were applied onto the agar with a sterile forceps. The disc was pressed firmly to ensure contact with the agar, within 15 min of disc placement; places were inverted and incubated at 37oC for 24 hours.

Preparation of micro-organisms and media culture and determination of minimal inhibitory concentration (MIC)

Determination of antibacterial activities of pure RMEO, methanol, and ethyl acetate leaf extracts were carried out by agar well diffusion method (16). Sterilized Petri dishes (9 cm diameter) were used. Nutrient agar media was sterilized in the flasks using autoclave at121 oC for 20 min and cooled to 45–50 °C before poured into petidishes, the agar was distributed by pipette (15 ml) into each petri dish and the medium was distributed homogeneously. A sterile cork borer of diameter 6 mm was used to bore wells in the agar plates. Six serial dilutions (50, 100, 150, 200, 250 and 300 µl/ml) for each of RMEO and methanol extracts and four dilutions (50, 100, 150 and 200 µg/ml.) of ethyl acetate extract were prepared using dimethyl sulfoxide (DMSO) to homogenate the solutions chemical contents of rosemary leaves, then, wells (6 mm diameter) were filled with 40 µl each dilution of the RMEO and the dilutions of the extracts introduced in each well into nutrient agar plates, while the treatments with Ag-NPs, used 10 µl of the suspension of silver nanoparticle, added to the extracts on each well, ciprofloxacin 10 µg (the antibiotic disc) was used as positive control, Ag-NPs in 20µg.ml-1 (used as positive control), and DMSO 99% (used as negative control). The treated petri dishes were incubated at 37°C for 24 hours. At the end of the period, inhibition zones formed on the medium were measured with a transparent ruler in millimeters. The antimicrobial activity, indicated by an inhibition zone surrounding the well containing the extract, was recorded if the zone of inhibition was greater than 6mm (17).

The experiments were applied according to completely randomized design (CRD) with three replications.

Results And Discussion

Figure (3) shows the GC–MS profile of rosemary essential oil. GC-MS analysis of the chemical constituents (qualitative and quantitative data obtained from GC–MS), the isolated essential oil mainly revealed the presence of oxygenated monoterpene like linalool and 1,8 cineol , flavouring agent such as bornyl acetate, bicyclic sesquiterpene like caryophyllene, ketone (3-octanone) and monoterpene hydrocarbons like limonine, as shown in table (1), these results agree (18,19)

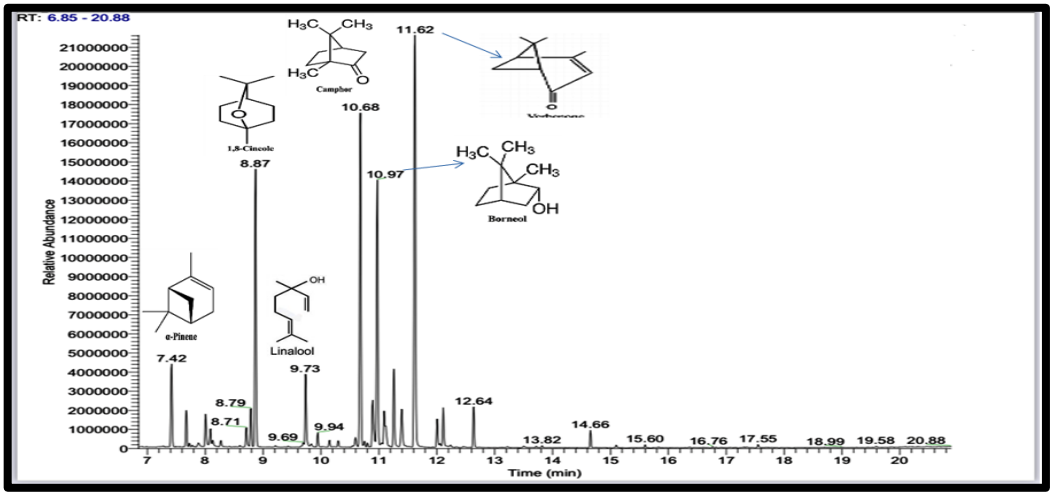


Fig.3: Analyses of rosemary essential oil (Volatile oil) from dry leaves using Gas Chromatography-Mass Spectrometry (GC-MS)

Table 1: Chemical constituents in rosemary essential oil using GC- MS

Peak No.	*Identification compound name*	*RT (min)*	*PA (%)*	*Molecular formula*
1	α-Pinene	7.42	4.13	C10H16
2	Camphene	7.67	1.99	C10H16
3	3-Octanone	8.01	1.6	C8H16O
4	Myrcene	8.09	1.34	C10H16
5	Mesitylene	8.27	0.5	C9H12
6	p-Cymene	8.71	0.92	C10H14
7	Limonene	8.79	1.86	C10H16
8	1, 8-Cineol (Eucalyptol)	8.87	13.30	C10H18O
9	Linalool	9.73	3.45	C10H18O
10	Filifolone	9.94	0.71	C10H14O
11	Fenchol	10.15	0.39	C10H18O
12	6,6-Dimethylcycloocta-2,4-dienone	10.29	0.43	C10H14O
13	Verbenol trans	10.6	0.64	C10H16O
14	Camphor	10.68	16.39	C10H16O
15	2-Norpinanone, 3,6,6-trimethyl-	10.89	2.18	C10H16O
16	Borneol	10.97	12.50	C10H18O
17	Terpene-4-ol	11.09	3.06	C10H18O
18	α-Terpineol	11.26	3.52	C10H18O
19	Borneol (isomer)	11.39	2.18	C10H18O
20	Verbenone	11.62	22.72	C10H14O
21	Myrtanol	12.01	1.1	C10H18O
22	Myrtanol (isomer)	12.11	1.78	C10H18O
23	Bornyl acetate	12.64	2.21	C12H20O2
24	Caryophyllene (E-)	14.66	0.84	C15H24
25	α-Caryophyllene	15.1	0.13	C15H24
Total identified components %			99.87	

GC-MS: Gas chromatography –mass spectrometry, RT: Retention time, PA: Peak area

Sensitivity test of bacterial isolates against antibiotics

Table showed that Ciprofloxacin10 was effective against P. aeruginosa, E. coli and S. aureus (25.6, 24.9 and 23.5mm respectively) followed by Gentamicin10 (16.6, 20.8 and 15.2mm respectively) and the results were agreed with that of (20) and (21), but P. aeruginosa was resistant completely against Amoxicillin/ clavulanic acid (no effects) followed by Azithromycin (8mm) and Tetracycline (10mm), it was sensitive to Ciprofloxacin (25.6) and Gentamicin (16.6), but results disagreed with the results of (22).

Table 2: Bacterial isolates response to different antibiotics.

Antibiotics (µg disc-1)	*The growth zone diameters (mm) of Bacterial isolates*		
	Escherichia coli G-	*Staphylococcus aureus G+*	*Pseudomonas aeruginosa G-*
Ciprofloxacin10 (CIP10)	24.9	23.5	25.6
Gentamicin10 (CN10)	20.8	15.2	16.6
Tetracycline (TE10)	18	21	10
Amoxicillin/clavulanic acid (AMC30)	7	14	N
Azithromycin (AZM15)	24	10	8

N: No effect (no inhibition zone)

Effects of Methanol extract alone or with Ag-NPs on three bacterial isolates

Table 3 showed that the minimum inhibition concentration (MIC) of methanol extract that inhibit the growth of E. coli was 300 µg.ml-1 which registered inhibition zone of 12.5mm and no effects on S. aureus and P. aeruginosa, but the MIC of ME when applied with Ag-NPs20µg/ml was 300µg.ml-1 which caused inhibition zone of 13.8 mm for E. coli, 11.1 and 15.2 for S. aureus and P. aeruginosa respectively. The most sensitive bacterial isolate against methanol extract with Ag-NPs was P. aeruginosa and the most resistant one was S. aureus (Figure 2A and 2B).

Table 3: Effects of different concentrations of methanol extracts of rosemary leaves (MERL) on three bacterial isolates using nanoparticle size solutions

MERL Conc. (µg/ml)	*Treatments*	*The growth zone diameters (mm) of Bacterial isolates*		
		Escherichia coli G-	*Staphylococcus aureus G+*	*Pseudomonas aeruginosa G-*
50	without Ag-NPs	N	N	N
	with Ag-NPs	N	N	N
100	without Ag-NPs	N	N	N
	with Ag-NPs	N	N	N
150	without Ag-NPs	N	N	N
	with Ag-NPs	N	N	N
200	without Ag-NPs	N	N	N
	with Ag-NPs	N	N	N
250	without Ag-NPs	N	N	N
	with Ag-NPs	N	N	N

300	without Ag-NPs	12.5	N	N
	with Ag-NPs	13.8	11.1	15.2
Ag-NPs (20µg/ml)		8.1	11.1	8
DMSO		N	N	N

N: No effect, Ag-NPs: Silver nanoparticles.

Antimicrobial activity of RMEO extract against some microbial pathogens

The MIC of essential oil of the leaves of rosemary (RMEO) against E. coli and S. aureus with or without Ag-NPs 20µg/ml was 100µl/ml. The same concentration of RMEO caused inhibition of the growth zone of E. coli and S. aureus to11.1mm, but no effects on S. aureus. Ag-NPs applied at 20µg/ml alone caused inhibition of the growth zone to 8.1, 11.1 and 8 mm for E. coli, S. aureus and S. aeruginosa respectively. RMEO at concentration of 300 µl/ml when applied alone registered the growth zone of 16.6 for E. coli and S. aureus but when applied with Ag-NPs at 20µg/ml as nanoparticle solution, decreased the growth zone of E. coli and S. aureus to 15.2 and 9.7mm respectively, as shown in (Table 4) and (Figure 2C), this may be due to aggregation between the two solutions throughout combination between them (23).

Table 4: Effects of different concentrations of rosemary essential oils RMEO alone or with Ag-NPs on three bacterial isolates

RMEO Conc. (µl/ml)	***Treatments***	***The growth zone diameters (mm) of bacterial isolates***		
		Escherichia coli G-	***Staphylococcus aureus G+***	***Pseudomonas aeruginosa G-***
50	without Ag-NPs	N	N	N
	with Ag-NPs	N	N	N
100	without Ag-NPs	11.1	11.1	N
	with Ag-NPs	11.1	11.1	N
150	without Ag-NPs	12.5	13.8	N
	with Ag-NPs	12.5	13.8	N
200	without Ag-NPs	11.3	16.6	N
	with Ag-NPs	11.3	16.6	N
250	without Ag-NPs	12.5	11.1	N
	with Ag-NPs	13.8	12.5	N
300	without Ag-NPs	16.6	16.6	N
	with Ag-NPs	15.2	9.7	N
Ag-NPs (20µg/ml)		8.1	11.1	8
DMSO		N	N	N

N: No effect, RMEO: Rosemary essential oil, Ag-NPs: Silver nanoparticles.

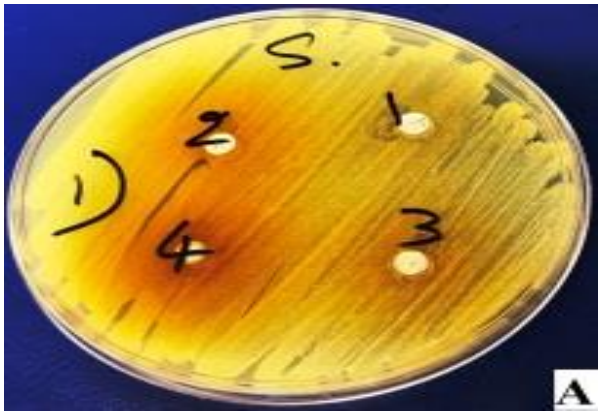
Antimicrobial activity of ethyl acetate of rosemary leaves extraction (EARLE) against some microbial pathogens

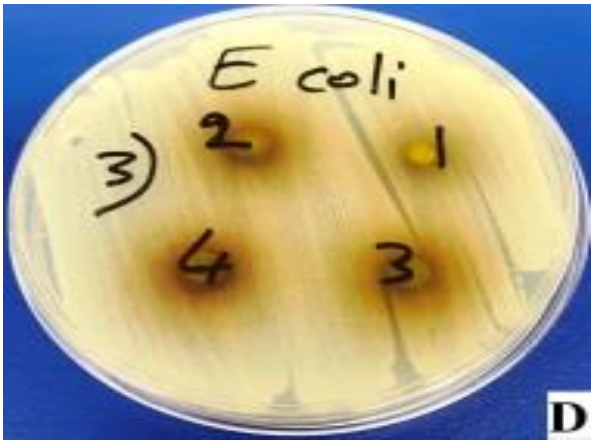
Table 5 showed that the MIC of EARLE was 50µg/ml alone or without Ag-NPs at concentration of 20µg/ml, the MIC of EARLE for E. coli and P. aeruginosa was 100µg/ml, but the MIC of EARLE applied with Ag-NPs at concentration of 20µg/ml was 50µg/ml. Ag-NPs at concentration of 20µg/ml was effective and inhibit the growth of the three bacterial isolates (Figure 2D, 2E, 2F and 2G)

Table 5: Effects of different concentrations of ethyl acetate extracts of rosemary leaves (EARLE) alone or with Ag-NPs on three bacterial isolates

EARLE Conc. (µg/ml)	***Treatments***	***The growth zone diameters (mm) of Bacterial isolates***		
		Escherichia coli G-	***Staphylococcus aureus G+***	***Pseudomonas aeruginosa G-***
50	without Ag-NPs	N	12.5	N
	with Ag-NPs	N	15.2	9.7
100	without Ag-NPs	12.5	13.8	10
	with Ag-NPs	15.2	16.6	11.8
150	without Ag-NPs	11.1	12.5	10.5
	with Ag-NPs	16.6	15.2	12.5
200	without Ag-NPs	13.8	13.8	11.1
	with Ag-NPs	16.6	13.8	15.2
Ag-NPs (20µg/ml)		8.1	11.1	8
DMSO		N	N	N

N: No effect, Ag-NPs: Silver nanoparticles.





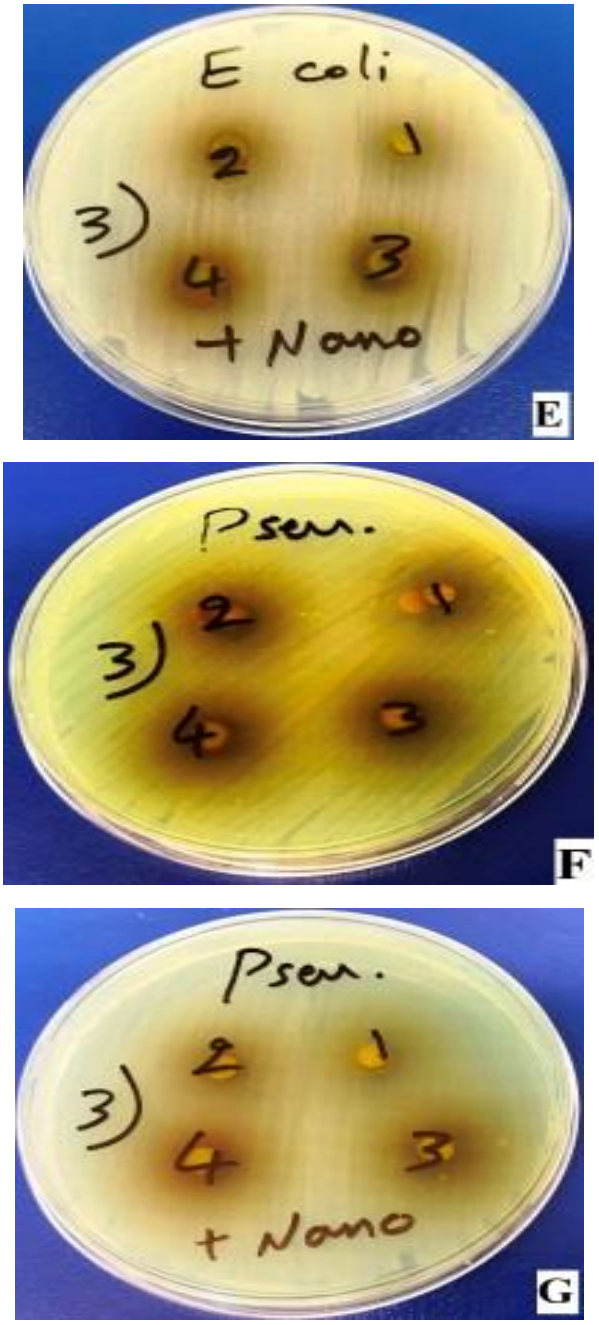


Fig.2: Effects of different concentrations of RMEO, methanol extracts and ethyl acetate extracts, alone or with Ag-NPs on three bacterial isolates (A, B): methanol leaves extract, (C): RMEO, (D-G): ethyl acetate leaves extract

CONCLUSION

MIC of methanol extract that inhibit the growth of E. coli was 300 µg.ml-1 and no effects on S. aureus and P. aeruginosa. The most sensitive bacterial isolate against methanol extract with Ag-NPs was P. aeruginosa and the most resistant one was S. aureus. MIC of essential oil of the leaves of rosemary (RMEO) against E. coli and S. aureus with or without Ag-NPs 20µg/ml was 100µl/ml. MIC of ethyl acetate rosemary leaves extract was 50µg/ml alone or with Ag-NPs.

MIC of ethyl acetate extract for E. coli and P. aeruginosa was 100µg/ml, but the MIC of EARLE applied with Ag-NPs was 50µg/ml. Ag-NPs at concentration of 20µg/ml (alone or combined with extracts and RMEO) was effective and inhibit the growth of the three bacterial isolates.

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