Original article:

Antibacterial Effect of Some Palestinian Plant Extracts against Clinical Multidrug-Resistant Gram-Negative Bacteria: A possible synergism with antibiotics

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Abstract:

Objectives: The present study was designed to screen the antibacterial and synergistic effects of *Allium sativum*, *Ecballiumelaterium*, *Pelargoniumgraveolens*, *Rosmarinusofficinalis*, *Phagnalonrupestre* and *Rutagraveolens* plant extracts and essential oils against the followingclinical multidrug-resistant(MDR) Gram negative pathogenic isolates: *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* ***Methods:*** All extracts and essential oils were screened for their antibacterial activity and synergistic effect in combination with known antibiotics.The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of the plant extracts were assessed. ***Results:*** Our results revealed that, the average diameter of inhibition zones ranged from: 7-14 mm, 7-17 mm, 8-17 mm and 7-13 mm for water, ethanol, methanol and EOs extracts, respectively. The MICs and MBCs were determined for extracts which showed antibacterial activity. The average MICs values ranged from 1.6-100 mg/ml, 1.6-50 mg/ml, 0.39 -50 mg/ml and 3.13-100 µl/ml for water, ethanol, methanol and EOs extracts, respectively. While MBCs values ranged from 25->200 mg/ml, 25-200 mg/ml and 50->200 µl/ml for water, ethanol, methanol and EOs extracts, respectively. The antibacterial activity of the afore mentioned plant extracts combined with Ciprofloxacin (CIP), Ampicillin (AM), Cefotaxime (CTX), Nalidixic acid (NA), Norofloxacin (NOR), Cefuroxime (CXM), Cefaclor (CF), Ofloxacin (OFX), Cefalexin (CL), Tetracycline (TE), Rifampicin (RIF), Amoxyclav (AMC) and Amikacin (AK) had different degrees of synergism against the selectedbacteria.Essential oils (Eos) of screened plants had the best synergism with antibiotics than the plant extracts against the tested bacteria. The best synergism was noticed in the Eos of *A. sativum, P. graveolens* and *R. officinalis*. ***Conclusion:*** It was concluded that the synergistic effect of antibiotics and plant extracts is promising approach fortreating infectious diseases caused byMDR Gram negative bacteria.

Keywords: Antibacterial; synergism; multidrug-resistance;Gram-negative; Palestinian plants.

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Introduction

Medicinal plants have been used as sources of medicine in virtually all cultures. During the last decade, the use of traditional medicine (TM) has expanded globally and is gaining popularity. People use herbal remedies due to their efficacy, tradition and their low cost[1, 53]. Medicinal plants are important elements of indigenous medical systems in Palestine as well as in other developing countries. Complementary and alternative medicine utilization in Palestine are common elsewhere, whereas other types were unique to this area[2, 55].

There has been an increasing incidence of multiple antibiotic resistances in human pathogenic

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microorganisms in recent years, largely due to indiscriminate use of commercial antibacterial drugs commonly employed in the treatment of infectious diseases, which have led to the emergence of new bacterial strains that are multi-drug resistant which have resulted in increase in morbidity, mortality and creates enormous health problems. However, the situation is alarming in developing as well as developed countries due to indiscriminate use of antibiotics[3-5, 54]. The development of drug resistance as well as the appearance of side effects of certain antibiotics has led to the search of new antimicrobial agents mainly among plant extracts with the goal to discover new chemical structures which overcome the above disadvantages [6-9].

In the present study, we have selected 6 medicinal plants A: Allium sativum (A. sativum), B: Ecballiumelaterium (E.elaterium), C: Pelargonium graveolens (P. graveolens), D: Rosmarinusofficinalis (R.officinalis), E: Phagnalonrupestre (P. rupestre) and F: Rutagraveolens (R. graveolens) to assess their antibacterial and synergistic effect with antibiotics against multi-drug resistant Gram negative pathogenic bacterial isolates of Escherichiacoli, Klebsiellapneumoniae and Pseudomonasaeruginosa.

Materials and Methods

All chemicals and culture media used in this research were of high grade trade mark.

Plant materials

The six medicinal plants investigated in this study (*A. sativum* bulbs, aerial parts of *E. elaterium* (fruits), *P. graveolens*, *R. officinalis*, *P. rupestre* (shoots) and leaves of *R. graveolens*)were collected from Gaza Strip, Khanyunescity- Abasan Alkaberah area.

Bacterial test isolates

Clinical MDR bacterial isolates of *E. coli, K. pneumoniae* and *P. aeruginosa* from nosocomial and community acquired infections were obtained from the microbiology department at Al-Shifa hospital, Gaza, Palestine. Confirmatory identification and characterization were done by conventional biochemical methods.

Preparation of plant extracts

Dried plant Martials were pulverized or grounded to coarse powder. For water extract, the powder was mixed with distilled water for 8 h in a Soxhletapparatus then the extracts were concentrated under reduced pressure (using rotary evaporator)[10]. Methanolic extract was done by placing the powder in porous bag made of muslin cloth, which was loaded into the Soxhlet. The extraction was carried out with methanol as extraction solvent in 1:10 ratio of powder to solvent at temperature 65 °C for 8 hours, and then the extract was filtered and allowed to evaporate in open air [11-12]. Meanwhile for ethanoic extract, the plant powder was extracted with ethanol solvent for 8 hours using Soxhlet. The solvent was oven evaporated at 37ºC for three days [13].Essential oils were extracted from 500 g of fresh plants by steam distillationmethod[14].

For experiments, one gram of each water, methanolic and methanolic extracts and 1ml of essential oils was carefully taken, and then each extract was made up to 5 mL by adding Dimethylsulfoxide (DMSO) and stored at -20°C until use. This formed the stock solution of 200 mg/ml [15].

Standardization of Inoculum

The optical density of each active culture was adjusted to 0.1 at 625 nm using fresh nutrient broth to give a standard inoculum of 10^6 colony forming units (cfu) per ml [16].

The Antibiotic Sensitivity Assay

The antibiotic sensitivity was determined using the disc diffusion method based on the Clinical and Laboratory Standards Institute (CLSI). Thirteen antibiotics were used in this study including: Ciprofloxacin (CIP), Ampicillin (AM), Cefotaxime (CTX), Nalidixic acid (NA), Norofloxacin (NOR), Cefuroxime (CXM), Cefaclor (CF), Ofloxacin (OFX), Cefalexin (CL), Tetracycline (TE), Rifampicin (RIF), Amoxyclav (AMC) and Amikacin (AK).

Antibacterial Activity Assays

Disc Diffusion Method

Standardized inoculums of *E. coli, K. pneumoniae* and *P. aeruginosa* were spreaded onto the entire surface of sterile Muller-Hinton agar (MHA) plates by sterile cotton swab. After a few minutes, filter paper discs of 6 mm diameter impregnated with 50 µL of known concentration of extracts (200 mg /ml) for aquatic, ethanolic, methanolic extracts and essential oils were placed on the surface of inoculated plates. Sterile

paper discs containing Dimethyl sulfoxide alone was served as control. The plates were placed at 4ºC for 2 h and then subsequently incubated at 37º C for 24 h. After incubation, the growth inhibition was quantified by measuring the diameter of the zone of inhibition in mm. For each test solution, three replicates were performed[17-18].

Determination of MIC and MBC by Microdilution Method:

The plant extracts were dissolved in DMSO. Two-fold dilution series were prepared to achieve a decreasing concentration ranging from 200 to 0.390 mg/ml of each extract which was prepared in a 96-well microtiter plate. Overnight broth cultures of the tested bacteria were prepared, the final concentration in each positive well was adjusted to 10^6 CFU/ml and the plates were incubated at 37 ºC for 24 h. The MIC was defined as the lowest concentration of the plant extract at which the bacteria does not show visible growth. To determine MBC, broth was taken from each well and inoculated in MHA at 37 ºC for 24 h. The MBC is defined as the lowest concentration of the plant extract at which inoculated bacteria was totally killed. Amikacin and 10% DMSO solution served as positive and negative controls, respectively [19].

Evaluation of the synergistic effect

The antibiotic disks were placed on the surface of inoculated and labeled MHA plates and impregnated with 50 µL of known concentration of extracts (200 mg /ml for aquatic, ethanolic, methanolic extracts and 200 µl/ml for essential oils). The plates were incubated at 37 ºC for 24 h. The diameters of cleared zones were measured and compared with that of the antibiotics[20].

All the experiments were done in triplicate. Normal saline was run as negative control along with sample analysis.

Statistical Analysis

One-way analysis of variance (SAS, 1990; ANOVA procedure) followed by Duncan's test was performed to compare means and to test the significance of differences between means obtained from the antimicrobial tests results at $p \leq 0.05$ level of significance using SPSS 18 software.

Results

1. Evaluation of antibiotics activity

The results of antibacterial susceptibility testing are represented in Tables (1-3). *Escherichia coli* was intermediate sensitive to Tetracycline, Rifampicin and sensitive to Amikacin with inhibition zones of 9, 12 and 15 respectively and *K.pneumoniae* was intermediate sensitive to Tetracycline, Rifampicin and sensitive to Amikacin with inhibition zones of 14, 12 and 16 respectively while *P.aeruginosa* was intermediate sensitive to Tetracycline, Rifampicin, Amoxyclav and sensitive to Amikacin with zone of inhibition of 12,12,11 and 15 respectively. The three tested isolates were resistant to the rest of tested antibiotics.

2. Evaluation of antibacterial activity of plant extracts

The results of antibacterial activity of Aquatic extracts, Ethanolextracts, Methanol extracts and Essential oils of all the six plants when tested individually for their antibacterial activity against the three pathogenic bacterial isolates are shown in Tables(1-3). The zones of inhibition of the four plants extracts and EOs against *E. coli, K. pneumoniae* and *P. aeruginosa* ranger from 7 to 11, 7 to 12 and 7 to 11 respectively.

3. Synergistic antibacterial activities

The diameter of inhibition zone of different combinations of plant extracts is represented in Tables (1-3). Combinations of the extracts and antibioticsin several cases demonstrated synergistic, additive or antagonistic effects on microorganisms. Enlargement of inhibition zones indicates a positive interaction (synergism) which is mentioned in tables as bold numbers.

EOs of screened plants had the best synergism with antibiotics than the plant extracts against the tested bacteria. The best synergism were noticed in the Eos of *A. sativum, P. graveolens* and *R. officinalis*.

Table 1: Antibiotics sensitivity, Antibacterial activity of different plant extracts and Synergistic activity of different plant extracts with different antibiotics against E. coli

			CIP	AM	CTX	NA	NOR	CXM	CF	OFX	CL	TE	RIF	AMC	AK
	Antibiotics		6	6	6	6	6	6	6	6	6	9	12	6	15
	Extract alone														
Aquatic	A	7	15	6	6	6	6	6	6	6	6	17	6	6	14
	B	8	13	14	6	6	6	15	14	6	6	16	15	16	14
	C	10	6	6	6	6	6	12	6	6	14	16	12	6	10
	D	9	13	17	16	18	17	18	17	18	18	6	6	6	17
	E	9	6	6	6	6	6	6	6	6	6	12	12	14	13
	F	9	6	6	6	6	6	6	6	6	6	6	6	6	6
Ethanolic	A	10	14	6	6	6	17	17	13	17	6	14	14	17	14
	B	11	14	18	18	18	14	14	16	6	14	16	13	12	15
	C	12	17	19	16	16	17	16	16	18	16	18	14	16	17
	D	10	14	12	14	16	14	16	11	13	14	12	14	14	12
	E	11	14	12	14	6	16	16	6	19	6	12	16	16	16
	F	10	14	12	12	14	18	15	15	15	12	18	14	14	14
Methanolic	A	10	17	17	18	18	18	15	17	17	18	16	17	18	18
	B	9	14	15	14	14	15	14	16	14	14	14	14	16	18
	C	11	6	6	6	6	6	6	6	6	6	18	6	18	15
	D	10	14	16	18	14	16	16	14	16	16	16	14	17	16
	E	10	13	16	14	13	16	16	14	14	14	15	17	17	16
	F	11	18	16	19	18	17	17	18	17	19	17	16	18	18
Essential Oils	A	10	21	20	20	19	20	18	19	21	20	18	18	19	18
	B	8	6	11	6	6	6	6	6	6	11	11	12	14	16
	C	9	22	20	21	21	21	21	21	21	19	20	18	19	17
	D	11	20	20	19	19	20	17	19	22	18	19	19	19	18
	E	10	12	13	12	12	12	13	12	12	12	12	15	17	17
	F	7	12	6	11	12	12	15	6	14	14	14	14	14	16

(A*: A. sativum,* B*: E. elaterium,* C*: P. graveolens,* D*: R. officinalis,* E*: P. rupestre and* F*: R. graveolens).* Diameters of inhibition zone of various plants and essential oil extracts including 6 mm disk diameters.

Table 2: Antibiotics sensitivity, Antibacterial activity of different plant extracts and Synergistic activity of different plant extracts with different antibiotics against K. pneumoniae

			CIP	AM	CTX	NA	NOR	CXM	CF	OFX	CL	TE	RIF	AMC	AK
	Antibiotics		6	6	6	6	6	6	6	6	6	14	12	6	16
	Extract alone														
Aquatic	A	8	15	16	18	16	14	14	14	15	14	13	14	14	15
	B	8	6	13	6	16	17	16	11	6	6	22	15	18	13
	C	11	18	17	17	18	18	17	18	19	16	17	16	18	12
	D	9	19	19	20	18	19	18	17	20	21	17	19	19	17
	E	8	6	6	6	6	6	6	6	12	14	6	6	14	13
	F	8	16	16	15	17	15	16	15	16	16	16	17	16	11
Ethanolic	A	10	14	13	14	16	15	17	15	16	14	14	14	14	16
	B	10	18	17	17	16	17	15	15	17	14	21	16	16	17
	C	12	18	19	19	17	19	19	17	16	17	18	18	17	18
	D	10	15	17	17	18	17	13	16	17	17	17	12	17	14
	E	7	14	14	13	13	13	14	14	13	15	11	6	13	15
	F	9	15	18	16	16	16	17	15	16	15	17	17	17	15
Methanolic	A	10	15	14	14	14	17	13	16	17	15	16	14	14	19
	B	10	19	19	19	18	19	17	16	18	18	17	15	18	17
	C	12	15	15	16	16	16	16	16	18	15	15	16	18	18
	D	11	18	18	17	16	18	17	19	19	18	18	17	19	17
	E	11	12	11	12	12	6	13	14	12	16	16	15	14	17
	F	11	17	15	18	16	19	18	16	16	16	15	17	18	19
Essential Oils	A	10	18	16	12	18	18	17	17	18	19	18	16	17	17
	B	8	6	11	6	6	13	12	6	11	12	14	13	17	15
	C	11	21	23	23	23	22	20	21	22	22	22	21	22	17
	D	12	20	19	17	17	20	18	20	19	18	19	19	18	19
	E	10	13	6	11	12	11	13	12	6	6	13	13	17	16
	F	10	14	13	13	13	15	14	14	14	13	14	4	18	17

(A*: A. sativum,* B*: E. elaterium,* C*: P. graveolens,* D*: R. officinalis,* E*: P. rupestre and* F*: R. graveolens).*Diameters of inhibition zone of various plants and essential oil extracts including 6 mm disk diameters.

Table 3: Antibiotics sensitivity, Antibacterial activity of different plant extracts and Synergistic activity of different plant extracts with different antibiotics against P. aeruginosa

			CIP	AM	CTX	NA	NOR	CXM	CF	OFX	CL	TE	RIF	AMC	AK
	Antibiotics		6	6	6	6	6	6	6	6	6	12	12	11	15
	Extract alone														
Aquatic	A	7	14	13	6	6	6	3	6	6	6	14	13	13	14
	B	9	6	6	6	6	6	6	6	6	6	14	13	6	17
	C	12	12	11	14	14	17	17	17	20	19	16	16	19	11
	D	10	13	11	6	12	11	16	12	6	6	12	13	12	17
	E	7	11	11	12	12	6	3	13	11	12	11	6	6	14
	F	11	18	17	16	16	16	14	14	17	18	15	16	18	9
Ethanolic	A	10	18	20	21	19	19	16	17	18	18	18	19	18	17
	B	10	17	15	15	15	15	15	16	16	15	17	15	15	15
	C	11	18	20	21	20	21	19	20	22	21	22	21	21	18
	D	9	15	17	17	17	16	14	14	16	17	14	17	19	13
	E	9	14	13	14	14	14	17	16	14	13	15	14	16	15
	F	8	12	12	14	12	14	14	12	14	14	12	15	17	13
Methanolic	A	10	17	15	13	14	16	17	16	16	15	16	16	18	16
	B	11	16	15	15	14	16	15	15	11	13	15	13	12	17
	C	11	19	20	22	22	24	14	21	20	21	21	21	22	16
	D	8	17	18	18	18	18	19	16	17	17	15	17	18	15
	E	9	16	11	16	12	12	16	14	14	13	14	15	15	15
	F	9	13	13	13	13	13	14	13	13	13	13	13	13	17
Essential Oils	A	11	20	20	19	21	21	20	20	20	20	17	18	21	19
	B	10	6	6	6	6	6	6	12	6	6	13	13	6	14
	C	11	19	19	20	19	20	19	18	18	19	16	19	19	18
	D	10	20	20	19	18	18	19	16	19	20	16	18	20	18
	E	9	14	14	12	12	12	12	12	11	11	12	13	12	16
	F	10	14	12	11	11	11	12	13	13	13	15	15	12	17

(A*: A. sativum,* B*: E. elaterium,* C*: P. graveolens,* D*: R. officinalis,* E*: P. rupestre*&F*: R. graveolens).*Diameters of inhibition zone of various plants and essential oil extracts including 6 mm disk diameters.

4: Determination of(MIC) & (MBC):

Extracts were tested against the isolates for their inhibitory activity, using a common broth microdilution method in 96 multi-well microtiter plates in two-fold dilution series of these extracts was prepared: 200, 100, 50, 25, 12.5. 6.25, 3.125, 1.562, 0.781 and 0.390 (mg/ml for the aquatic, ethanolic & methanolic extracts and µl/ml for the essential oils), in triplicateand the average of the obtained minimum inhibitory concentrations (MICs) & minimum bactericidal concentrations (MBCs) are listed in Table (4).

Table 4: The MICs & MBCs of the plants extracts against isolated bacteria

Scientific name of the plant used		E. coli		K. pneumoniae		P. aeruginosa	
		MIC	MBC	MIC	MBC	MIC	MBC
A. sativum	W	25	100	50	100	100	> 200
	E	25	100	50	100	50	100
	M	25	100	50	200	25	200
	EO	3.125	100	50	100	50	100
E. elaterium	W	50	100	25	100	25	200
	E	25	50	25	100	25	200
	M	25	50	25	200	250000	200
	EO	50	100	50	200	25	> 200
P. graveolens	W	3.125	25	1.562	25	6.25	200
	E	1.562	100	1.562	100	1.562	100
	M	0.390	200	1.562	100	1.562	100
	EO	50	100	25	200	25	200

Scientific name of the plant used		E. coli		K. pneumoniae		P. aeruginosa	
		MIC	MBC	MIC	MBC	MIC	MBC
R. officinalis	W	6.25	25	6.25	100	6.25	100
	E	12.5	25	6.25	100	1.562	25
	M	3.125	25	3.125	25	1.562	100
	EO	50	100	100	200	25	200
P. rupestre	W	50	100	6.25	100	50	100
	E	12.5	50	3.125	200	12.5	25
	M	12.5	50	1.562	200	12.5	50
	EO	25	50	25	200	25	200
R. graveolens	W	12.5	100	25	50	25	200
	E	1.562	200	1.562	25	3.125	> 200
	M	1.562	50	1.562	25	3.125	100
	EO	25	200	50	100	12.5	> 200

W: aquatic, E: ethanolic, M; methanolic and EO: essential oil. W, E & M expressed as mg/ml, while EO expressed as µl/ml. > 200:the MBC values were beyond the concentration of the stock solution of extracts.

The results of all the six plants when tested individually to determine their MIC and MBC against the three tested bacterial isolates are shown in Table IIII. For *A. sativum*, the aquatic extract shows the highest MIC and MBC values against the three tested bacterial isolates. Meanwhile, the essential oils of *E. elaterium*, *P. graveolens*, *R.officinalis, P. rupestre* and *R. graveolens* have the highest MIC and MBC values against the tested bacterial isolates except in the case of *P. rupestre*when the aquatic extract has the highest MIC and MBC values against *E. coli*.

Discussion

The usage of medicinal plants for primary health care needs by millions of people in developing world is still occupying a prominent position. The folk remedies are considered readily available, cheap and time tested [21,51,52].

Antibacterial activity: In this study, different extracts of A. sativum, E. elaterium, P. graveolens, R. officinalis, P. rupestre and R.graveolens showed significant antibacterial activity against (MDR) gram negative (E. coli, K. pneumoniae & P. aeruginosa) bacteria isolates as assessed by the diameter of zone of inhibition of the extracts. Although, the low values recorded for some plant extracts may be attributed to the fact that the extracts being in crude form, contain very small amounts of bioactive compounds. At the same time, several workers have reported bioactivity of crude extracts of medicinal plants within such range of diameter zone of inhibition [22].

Allium sativum:

The aqueous extract of *A. sativum* possesses antibacterial activity against*E. coli*, *K. pneumonia* & *P. aeruginosa***.**[23]obtained similar results, where they showed a strong antimicrobial activity of *A. sativum* aquatic extract *E. coli*, *K. pneumonia* & *P. aeruginosa* isolated from human urine. In agreement with [24], the ethanolic extracts of *A. sativum* had a higher inhibitory activity against the tested organisms than that of the water extract and this could be because of better extraction with ethanol solvents. The methanolic extract of *A. sativum* bulb was also effective against all the three MDR bacteria under the study which is in agreement with [25].

In general, the inhibitory activity of essential oils was greater than that of the aquatic & ethanolic extracts. The EOs of *A. sativum* bulbs was also effective against the tested bacteria which is in agreement with[17]who attributed the activity to the presence of diallyl sulfides (Disulfide, Trisulfide & Tetrasulfide) and the antimicrobial activity increased according to the number of sulfur atoms. Allicin, one of the active principles of garlic, was found to exhibit an antibacterial activity against a wide range of Gram-negative including multidrug-resistant enterotoxicogenic strains of *E. coli.* The main antimicrobial effect of allicin is due to its chemical reaction with thiol groups of various enzymes, e.g. alcohol dehydrogenase and RNA polymerase, which can affect RNA production and lipid synthesis [26].

Ecballiumelaterium:

The aquatic extract of *E. elaterium* shown a good

inhibitory activity against *E. coli*, *K. pneumoniae*&*P. aeruginosa* in contrast with[27], which stated that the antibacterial activity of the water extract of *E. elaterium* was not active against *E. coli, K. pneumoniae, P. aeruginosa* at concentration of 0.001mg/ml. Although the reason for this variation is not clear, it could be assumed to be as a result of low concentration of the aquatic extract of *E. elaterium* and difference in the extraction method and genetic differences between the plant and microbial strains used in this study. On the other hand, the ethanolic extract of *E. elaterium* fruits showed a good antibacterial activity against the tested isolates which is in agreement with[28-29].The methanolic extract also showed a good antibacterial activity. Our results are in agreement with the result obtained by[30].

Pelargonium graveolens:

The aquatic extract of *P. graveolens* in general showed a high inhibitory activity against *E. coli*, *K. pneumoniae* & *P. aeruginosa*, our results were in agreement with the results of an earlier study[31-32], in which the activity of *P. graveolens*m ethanolic extract and EO exhibited bactericidal effects against *P. aeruginosa* and *E. coli*.

Rosmarinus officinalis:

The ethanolic extract showed antibacterial activity against all tested bacteria. According to[33],Palestinian medicinal plant *R. officinalis* ethanolic extract exhibited significant antimicrobial activity against MDR *E. coli* and *P. aeruginosa* at concentration of 12.5 mg ml^{-1}, while *K. pneumonia* was sensitive at concentration of 25 mg/ml, this results were compatible with our results. Also, the results revealed that the methanolic extract exhibited varying degree of antibacterial activities against all tested bacterial strains in agreement with[34.] *Rosmarinus officinalis* EO exhibited moderate antibacterial activities against all tested bacterial isolates which is in agreement with [35].

Rutagraveolens:

The ethanolic extract showed good antibacterial activity against all the testedbacteria. In another study[36] the *R. Graveolens* ethanolic extract showed antibacterial activities towards *P. aeruginosa*, while *E. coli* was not sensitive at concentration of 12.5 & 25 mg/ml. The methanolic extract had moderate antibacterial activities against *E. coli & K. pneumoniae*whilehad a low activityagainst *P. aeruginosa.* Previous study has reported the high antibacterial activity of *R. graveolens*m ethanolic extracts on different microorganisms [37].*Rutagraveolens* EO exhibited moderate antibacterial activities against *E. coli & K. pneumoniae* while exhibited a low antibacterial activity against *P. aeruginosa*. These results were inagreement with many other studies[38-39].

Phagnalonrupestre:

Our results showed that*P. Rupestre* aquatic extract had high antibacterial activities against the tested bacteria in agreementwith[40] who stated that the*P. Rupestre* aquatic extract showed antibacterial activities towards the Gram- negative bacteria. The ethanolic extract showed good antibacterial activity against all the tested isolates. Our finding is compatible with results of study of [40], in which*P. Rupestre* ethanolic extract had a good antimicrobial activity against *K. pneumoniae,* while our findingsare incompatible with the results of this study regarding the *E. coli & P. aeruginosa* which it was resistant to the*P. Rupestre* ethanolic extract at concentration 200 mg/ml. It is thought that the observed dissimilar results may be attributed to differences in techniques of extraction and to genetic differences between the plant and microbial isolates used. In general, it was interesting to note that the antibiotic-resistant bacterial isolates showed more sensitivity to the investigated extracts. This has clearly indicated that antibiotic resistancemay does not interfere with the antimicrobial action of plant extracts, and these extracts might have different modes of action against the tested microorganisms.

Synergistic effects of plant extracts and antibiotics against Escherichia coli, K. pneumoniae and P. aeruginosa

Drug synergism between known antibiotics and bioactive plant extracts is a novel concept, and could be beneficial. Despite the abundant literature about the antimicrobial properties of plant extracts, none of plant-derived chemicals has successfully been used for clinical use as antibiotics [41]. In our experiments, despite that, some plant extracts showed weak antibacterial effect using agar disk diffusion method, the interactions between antibiotics and plant extracts were some time additive or synergistic against the three tested bacteria. The results obtained by combining the antibiotic with the extracts of *P. graveolens* EO,*R. officinalis* aquatic extracts & EO and *R. graveolens* methanolic extract which had the most synergistic inhibitory effect against *E. coli* which presented synergism with most drugs, indicated that these extracts contain chemical compounds that can modulate the activity of antibiotics against bacteria

expressing MDR phenotypes[42]study the effect of combination of plant extracts with antimicrobial agents against *E. col i*and other bacteria and evaluated the in vitro synergistic effect of curcumin in combination with third generation cephalosporins against bacteria associated with infectious diarrhea (*E. coli, P. aeruginosa & V. cholerae*).

Significant synergistic effects were noted with both *P.graveolens*(EO), *R. officinalis, E. elaterium & R. graveolens* extracts when they were associated with most tested antibiotics had the most favorable synergistic pattern.Such effects might be due either to the action of the active compounds or possible inhibition of one or more mode of bacterial resistance mechanisms by other compounds of the extracts. The plants, *P.graveolens*(EO) and *R. officinalis* followed by *R. graveolens* extracts alone or in combination, are promising in the development of phytomedicine, which may be used, alone or in combination with the antibiotics against *K. pneumoniae* infections. The results are compatible with results of [43] in which *R. officinalis /* ciprofloxacin combination against *K. pneumoniae* displayed the most favorable synergistic pattern.Such effects might be due either to the action of the active compounds or possible inhibition of one or more mode of bacterial resistance mechanisms by other compound of these extracts. The plants *P. graveolens* (aquatic, ethanolic, methanolic extracts & EO) followed by *R. officinalis*(methanolic extracts & EO) alone or in combination, are promising in the development of phytomedicine, which may be used, alone or in combination with the antibiotics against *P. aeruginosa* infections.

Minimal inhibitory concentration & Minimal bactericidal concentration of plant extracts

The results of MIC and MBC values showed that the tested plants had potential inhibitory activity against the tested bacterial isolates. This activity could be attributed to the components present in plant extracts, which might be involved in some type of antibacterial synergism. In general, our results showed that all the studied plants are potentially rich source of antimicrobial agents. The main target of the hydrophobic essential oil compounds is the cell membrane. They lead to a cell membrane damage causing increased membrane permeability, ions leakage, and inhibition of different enzymes and proteins. Gram-negative (*E. coli*) cells treated with *A. sativum* EO revealed significantly severe damaging effect on the cell morphology of the tested bacterial pathogens, showing disruption of cell membrane and swelling of the cells. The resistance of gram-negative bacteria toward antibacterial substances is related to the hydrophilic surface of their outer membrane, which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antimicrobial molecules, and is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside [44.]

The bactericidal activity of the essential oil of *P.graveolens* could be explained by the presence of high concentrations of oxygenated relatively stable monoterpenic primary alcohols. β-Citronellol and geraniol, the two major monoterpenes component of Geranium oil, have reported antimicrobial activit[10]. On the other hand, the methanol and water extracts of flowers and leaves did show antimicrobial activity, with the flower extracts being overall more active than the leafextracts. This activity is most likely associated with the phenolic compounds in these extracts that can effect cellular membranes altering their permeability and release of intracellular constituents (e.g. ribose and Na glutamate), but also interfere with membrane functions (electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity) [10,52].

The relative bactericidal effectiveness of *R. officinalis* EO may come from the synergic effect of α-pinene and camphor highly represented in these oils [45]. Also [46]described the chemotypes which correspond eucalyptol-α-pinene-camphor, eucalyptol-camphor-α-pinene, camphor-eucalyptol-α-pinene, camphor-α-pinene-eucalyptol and α-pinene-eucalyptol-camphor. All the chemotypes showed strong antibacterial activity against *E. coli.* The levels of antimicrobial activity in Tunisian *R. officinalis* oils were similar to those reported for *R. officinalis* from Iran, exhibiting a high antimicrobial activity against *E. coli* due mainly to the dominance of borneol followed by camphor and verbenone, respectively. The Turkish *R. officinalis* oil possesses a moderate antibacterial activity attributed to the high content of 1.8-cineole, the low content of camphor and verbenone, respectively. A weak activity was reported for samples from Sardinia dominated by α-pinene, camphene, verbenone, bornyl-acetate, camphor and borneol tested against *E. coli, P. aeruginosa*[45]. Also [47]&[48]attributed the antimicrobial property of the essential oil of *R. officinalis* to the presence of α-pinene, β-pinene, 1, 8-cineole & borneol as the major components. These compounds possess strong antibacterial and

antimicrobial activities, and camphor and verbenone being the most potent. These chemical components exert their antimicrobial activity on bacteria through the disruption of bacterial membrane integrity. Another important characteristic of essential oils is their hydrophobicity which enables them to penetrate lipid components of bacterial cell membrane and mitochondria, disrupting the cell structure and rendering them more permeable resulting in leakages of critical molecules from within the cell and eventual death of the bacteria cells[48, 49, 50]

Conclusion & Recommendations

The overall results of the present work provide baseline information for the possible use of the studied plant extracts in the treatment of bacterial infections involving MDR phenotypes. In addition to these antibacterial activities, the data reported herein indicated that possible combinations of the extracts of *A. sativum, E. elaterium, P. graveolens, R. officinalis, P. rupestre*and *R. Graveolens* plants with several antibiotics could be used in the control of bacterial infections involving MDR phenotypes. Our results support the use of these plants extract and its essential oils in traditional medicine and suggest that some of the plant extracts possess compounds with good antibacterial properties that can be used as antimicrobial agents in the search for new drugs. The tested crude extract from *A. sativum, E. elaterium, P. graveolens, R. officinalis, P. rupestre*and*R. graveolens* have proved to be promising treating agents against the tested Gram negative rods, but it needs to be concentrated and furthermore evaluated. Hence, more studies pertaining to the use of medicinal plants as therapeutic agents should be emphasized, especially those related to the control of antibiotic resistant microbes.

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Author's contribution:

Data gathering and idea owner of this study: A,B

Study design: B

Writing and submitting manuscript: A, C

Data gathering : A, B, D

Editing and approval of final draft: A, B, C

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