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Antibacterial Response of Combination Between Antibiotics and Some Plant Extracts Against Multidrug Resistant Bacteria

Wagih Abd El-Fattah El-Shouny, Nanis Gamal Allam, Maha Abd El Fattah Khalil and Afwat Mohamed Hegazy

Department of Botany, Microbiology Section, Faculty of Science, Tanta University, Egypt

Abstract: The aim of the study was to estimate antibacterial activity of some plant extracts against resistant nosocomial bacterial isolates. Seventy bacterial isolates were obtained from patients at Tanta University Hospital, Egypt. The activity of methanol extract of *Rosmarinus officinalis* was the most efficient one against seventy bacterial isolates. Antibiotic resistant pattern revealed presence of 18 multidrug resistant (MDR) Gram negative bacteria among seventy isolates. The results revealed that resistance of MDR bacteria to antibiotic or *Rosmarinus officinalis* extract was less than combination between them. In conclusion, the present work recommended using methanol *Rosmarinus officinalis* extract or its combination with some antibiotics against human pathogenic bacteria as cheap and alternative agents to some ineffective antibiotics.

Key words: Plant extracts · Antibacterial activity · Medicinal plants

INTRODUCTION

Pathogenic bacteria are bacteria that cause infectious disease for human. The 2002 survey report by Nosocomial Infection National Surveillance Service [1] indicates that the incidence of hospital acquired infection (HAI) was 10%. These infections complicate illness, anxiety; increase patient discomfort and can lead to death. The appearance of multidrug resistant bacteria leads to searching for new substances with antibacterial activities. The dramatic increase in nosocomial infections caused by resistant or multi-resistant bacteria is one of the most serious public-health problems and it remains cause of illness and death in developed countries [2]**.** Herbal plants are frequently used in popular medicine as remedies for many infectious diseases. Using medical plants have less side effects and are economically cost effective, have been recently taken into consideration because of the side effects, cost and difficulty of therapeutic-chemical materials production [3]**.** Therefore, there is requirement for new classes of antibacterial compounds, nature has provided a source of medicinal agents for thousands of years and number of modern drugs has been isolated from natural sources [4]. Since time man has used various parts of plants, especially that have been of historic importance for centuries, in the treatment and prevention of various diseases [5, 6].

Traditional herbal medicine provides several remedies for strengthening the body's resistance to illness through effects on immune system components. The discovery of some drugs from plants was recommended for the treatment of some diseases and can save the patient troubles. Medicinal plants are known to contain several compounds with antimicrobial properties and the uses of these types of compounds are being increasingly reported from different parts of the world**.** Subsequently, a major strategy that could be employed in the treatment of new emerging infectious diseases and prevention of the development of resistant isolates is the combination of herbal remedies with the first-line antimicrobial agents to which most of them have become resistant [7]. While Kamatou *et al.* [8] and Aiyegoro *et al.* [9] showed that combination of antimicrobial agents had expressed significant interactions and two or more compounds interact to produce mutual enhancement, amplification of each other's effects when combined. These combinations could enhance the efficacy of the other antimicrobial agents and acted as alternative to treating infections caused by multidrug-resistant microorganisms having no effective therapy [10, 11].

The study aimed to estimate the antibacterial activity of some plant extracts or their combination with antibiotics against clinically isolated multidrug resistant bacteria as an alternative treatment.

Corresponding Author: Nanis Gamal Allam, Department of Botany, Microbiology Section, Faculty of Science, Tanta University.

MATERIAL AND METHODS

Plant Materials: The plants used in this study included *Ocimum basilicum* (Family Lamiaceae), *Vinca differmis* (F. Apocynaceae), *Psidium guajav*a (F Myrtaceae*), Foeniculum vulgare* (F Apiaceae**)** and *Rosmarinus officinalis* (F Lamiaceae**)**. These plants were collected from local market of Tanta, Egypt.

Plant Extracts Preparation: The leaves of herbal plants *Ocimum basilicum* (basil), *Vinca differmis* (venca), *Psidium guajav*a (guava)**,** *Rosmarinus officinalis* (rosemary) and seeds of *Foeniculum vulgare* (fennel) were purchased from local market of Tanta, Egypt. Fresh plants (500 g) were dried in the shade at room temperature then grinded into powder. About 30 g of dried powdered leaves were extracted with 400 ml petroleum ether then 400 ml methanol for 48 h at room temperature. The resultant mixture was filtered then the clear supernatant was evaporated to dryness in a rotatory evaporator at 35°C the weigh extract was stored at 18°C to avoid decomposition. The prepared extracts were dissolved in dimethyl sulfoxide and subjected to filtration with 0.45 µl Millipore filter [12].

Antimicrobial Susceptibility Test

Bacteria: Seventy different bacterial swabs were obtained from patients (between November 2011 and January 2012) at the university Hospital, Tanta, immediately placed in nutrient broth transport medium and then transferred to laboratory of bacteriology in Botany Department, Faculty of Science, Tanta University. Gram staining was performed to differentiate isolated bacteria according to Gram reaction.

Swabs of 66 Gram negative pathogenic bacteria were streaked into two different media; MacConkey's agar and Cetrimide agar as well as two tests including methyl red and Voges Proskauer. Gram positive pathogenic bacteria (4) were streaked into two different; media Blood agar and Mannitol salt agar plates and then incubated overnight at 37°C.

Characterization of Gram Negative Isolated Bacteria: Standard biochemical tests of the selected bacteria including triple sugar iron agar (TSI) and EMB media as well as two tests including indole and citrate were used for characterization [13].

Assay of Antibacterial Activity of Different Plant Extracts: Susceptibility of seventy bacterial isolates to 12 different antibiotics using the disk diffusion method was studied [14]. The tested isolates were classified as sensitive or resistant according to the published break points of fastidious microorganisms of National Committee for Clinical Laboratory Standard [15]. Seventy tested bacterial isolates were screened for their susceptibility to different plant extracts using the agar well diffusion method to determine the inhibition zone diameter described as follows, an inoculum of 100 µl of each bacterial isolate at 10^6 CFU/ml was spread on the surface of nutrient agar plate and then left to dry for 15 min. Wells of 6 mm in diameter were made in agar surface using sterile cork borer, 50 µl of each extract at concentration 50 mg/ml were pipetted to the wells made in the plates. The plates were incubated at 37°C for 24 h and the zones of inhibition were measured. DMSO served as negative control [16].

Antibacterial Activity of Combined Form Between Antibiotic and Plant Extract Against Multidrug Resistant Bacteria: An inoculum of 100 µl of the MDR bacterial isolate at 10^6 CFU/ml was spread on nutrient agar plates and left to dry at 37°C for 15 min. The antibiotic disks were loaded in 10 µl of 50 mg/ml methanol *Rosmarinus officinalis* extract (dissolved in DMSO) dried at room temperature. The disks impregnated with plant extract were placed on bacterial inoculated plates at 37 °C for 24 h and the zones of inhibition were measured. Disks prepared with DMSO served as negative control [17].

RESULTS

Seventy bacterial isolates were grown on different media (MacConkey and Cetrimide for Gram negative bacilli or Blood and Mannitol salt agar for Gram positive cocci. The results revealed that 66 isolates were Gram-negative bacilli and four isolates were Gram-positive cocci (Table 1a and b). The Gram negative bacterial isolates were characterized to 53 *E. coli*, 6 *Klebsiella aerogenes,* 2 *Citrobacter freundii* and *5 Salmonella typhi*.

Table 2 showed screening of the sensitivity of the seventy tested bacterial isolates to the 12 tested antibiotics. For penicillin's group, the resistance of all bacterial isolates to oxacillin and ampicillin was 94 and 97% while; the lowest resistance was recorded for amikacin (16%). The resistances of all tested bacterial isolates for cephalosporin's groups was (81%) for cephalexin, while the resistances of the tested bacterial isolates to the aminoglycoside drugs, which included gentamicin and tobramycin were 69 and 54% respectively. On the other hand, the seventy bacterial isolates showed resistance to chloramphenicol (64%). Finally the tested

Table 1a: Characterization of the Gram negative bacterial isolates

Bacterial isolates	Source of isolation	Number	Lactose fermentation		Methyl red	V.P
CF	stool	1	Lactose	2	+ve 2	-ve 2
	Urine	1	non lactose	-		
EC	stool	16	Lactose	52	+ve 53	-ve 53
	Urine	26	non lactose	1		
	Pus	8				
	Ear	2				
	Eye	1				
KA	stool	2	Lactose	6	-ve 6	+ve 6
	Urine	3	non lactose	-		
	Eye	1				
ST	stool	2	Lactose	-	+ve 5	-ve 5
	Urine	3	non lactose	5		

CF= *Citrobacter freundii*, EC =*E. coli*, KA= *Klebsiella aerogenes*, ST=*Salmonella typhi*, V.P =Voges Proskaüer

Table 1b: Characterization of the Gram positive bacterial isolates from urine

Bacterial isolates	Number	Mannitol salt	Haemolysis	Number
SF	2	-ve	α-haemolysis	-
			non-haemolysis	2
			β-haemolysis	-
SA	2	Golden yellow	α-haemolysis	2
			non-haemolysis	-
			β-haemolysis	-

SF =*Streptococcus faecalis*, SA=*Staphylococcus aureus*

Table 2: Incidence of antibiotic resistance among different bacterial isolates

Antibiotic Class	Antibiotics	Concentration µg / disk	Resistant%
Penicillin	Ampicillin (AMP)	10	97
	Oxacillin (Oxa)	1	94
Cephalosporins	Cephalexin (CL)	30	81
Aminoglycosides	Gentamicin (Gen)	10	69
	Tobramycin (Tob)	10	54
	Amikacin (AK)	30	16
Chloramphenicol	Chloramphenicol (Chl)	30	64
A macrolide	Erythromycin (Ery)	15	100
Quinolones	Ciprofloxacin (Cip)	10	60
Glycopeptide	Vancomycin (VA)	30	83
Polyketid	Tetracycline (Te)	30	91
Lincosamides	Clindamycin (DA)	2	91

Tested isolates number = 70

bacterial isolates resist some antibiotics like macrolide antibiotic (erythromycin), quinolone antibiotic (ciprofloxacin), polyketid antibiotic tetracycline, glycopeptide antibiotic vancomycin by 100, 60, 91 and 83% respectively.

Standard biochemical tests for identifying 18 multidrug antibiotic resistant bacteria including two different media; TSI and EMB and two tests; indole and citrate were carried out. Results in Table 3 illustrated that multidrug resistant bacteria including *E. coli*, *Klebsiella aerogenes* and *Citrobacter freundii* were characterized. *E. coli* No. 13 and 17 were highly resistant to antibiotics and sensitive to tested plant extracts.

The petroleum ether extracts of *Ocimum basilicum*, *Vinca difformis* (F Apocynaceae*)*, *Psidium guajav*a*, Foeniculum vulgare* and *Rosmarinus officinalis* were evaluated for their antibacterial activity against the seventy tested bacterial isolates. The growth inhibition of the tested bacterial isolates was detected in presence of the petroleum ether of *Ocimum basilicum* and *Vinca difformis* with values of 23 and 39%. On the other hand, the growth inhibition of tested bacterial isolates in case of *Foeniculum vulgare*, *Rosmarinus officinalis* and *Psidium guajav*a extracts obtained by petroleum ether was 65, 61 and 60%, respectively.

Table 3: Biochemical tests for characterization of multidrug resistant bacterial isolates

Bacterial isolate	Antibiotic resistance R%	Indole	TSI Surface	butt	Citrate	EMB
1-KA	83	-ve	Yellow	Yellow	+ve	Green
2- KA	83	-ve	Yellow	Yellow	+ve	Green
3-EC	92	+ve	Yellow	Yellow	-ve	Green metallic
4- EC	92					
5-EC	75					
6-CF	75	-ve	Red	Yellow	+ve	Pink
7- EC	92	+ve	Yellow	Yellow	-ve	Green metalic
8- EC	75	+ve				
9- KC	67	-ve	Yellow	Yellow	+ve	Green
10- EC	83	+ve	Yellow	Yellow	-ve	Green metalic
11- EC	75					
12- EC	92					
13- EC	100					
14- EC	92					
15- EC	75					
16-CF	83	-ve			+ve	
17- EC	100	+ve			-ve	
18- EC	75	-ve				

KA=*Klebsiella aerogenes*, EC=*E. coli*, CF= *Citrobacter freundii*

Table 4: Antibacterial activity of petroleum ether extracts on the tested bacterial isolates

Plant extract	Resistant isolates%	sensitive isolates %	Mean of inhibition zone mm
Foeniculum vulgare	35	65	13
Rosmarinus officinalis	39	61	12
Psidium guajava	40	60	8.5
Vinca difformis	61	39	8
Ocimum basilicum	77	23	7.7

Table 5: Antibacterial activity of methanol plants extracts on the tested bacterial isolates

Plant extract	Resistant isolates%	Sensitive isolates %	Mean of inhibition zone mm
Rosmarinus officinalis	16	84	10.6
Foeniculum vulgare	20	80	10.1
Psidium guajava	27	73	9.1
Vinca difformis	70	30	8.5
Ocimum basilicum	71	29	8.6

Table 4 represented the antibacterial activity of the methanol extract of *Ocimum basilicum*, *Vinca difformis*, *Psidium guajava*, *Foeniculum vulgare* and *Rosmarinus officinalis* against the seventy tested bacterial isolates. Petroleum ether extract of *Foeniculum vulgare* and *Rosmarinus officinalis* showed mean inhibition zone of 13 and 12 mm respectively for resistant bacterial isolates. Methanol extract of *Rosmarinus officinalis* and *Foeniculum vulgare* showed mean inhibition zone of 10.6 and 10.1 mm for bacterial isolates (Table 5). Methanol extracts of *Ocimum basilicum* and *Vinca difformis* had a weak antibacterial activity on bacterial isolates 29 and 30% respectively. On the other hand, methanol extracts of *Rosmarinus officinalis*, *Foeniculum vulgare* and *Psidium guajava* showed high growth inhibition of all bacterial isolates. Also the extract of *Rosmarinus officinalis* revealed the highest inhibitory effect on bacterial isolates growth accounted by 84% of all isolates followed by *Foeniculum vulgare* (80%) and *Psidium guajava* (73%) as shown in Table 5.

Twelve antibiotic disks were screened for antibiotic resistance of 18 MDR bacterial isolates using the disk diffusion method (Table 6 a and b). *E. coli* bacterial isolates number 13 and 17 showed 100% resistance against all tested antibiotics. Antibiotics combined with *Rosmarinus officinalis* had synergistic inhibitory effect on *E. coli* bacterial isolates number 13 and 17.

Table 6a: Effect of combination between *Rosmarinus officinalis* methanol extract and antibiotics on MDR bacteria

Bacterial strain	Association rosemary antibiotic																					
K. aerogenes	AMP R- AMP	- -	OX R- OX	- -	CTX R- CTX	+ -	GN RGN	- -	TOB RTOB	- -	E ER	- -	TE RTE	- -	VA VAR	- ++	CIP R CIP	- ++	C RC	- -	AK RAK	+ ++	DA RDA	- ++
K. aerogenes	AMP R- AMP	- -	OX R- OX	- -	CTX R- CTX	- -	GN RGN	- -	TOB RTOB	- -	E ER	- -	TE RTE	- -	VA VAR	- ++	CIP R CIP	- ++	C RC	+ +	AK RAK	+ ++	DA RDA	- -
E. col	AMP R- AMP	- -	OX R- OX	- -	CTX R- CTX	- -	GN RGN	++ -	TOB RTOB	- -	E ER	- -	TE RTE	- -	VA VAR	- ++	CIP R CIP	- -	C RC	- -	AK RAK	+ ++	DA RDA	- -
E. coli	AMP R- AMP	- -	OX R- OX	+ +	CTX R- CTX	- -	GN RGN	- -	TOB RTOB	- -	E ER	- -	TE RTE	- -	VA VAR	- +	CIP R CIP	+ +	C RC	+ -	AK RAK	+ +	DA RDA	- +
E. coli	AMP R AMP	- -	OX R- OX	- -	CTX R- CTX		GN RGN	+ -	TOB RTOB	- -	E ER	- -	TE RTE	- +	VA VAR	- -	CIP R CIP	+ ++	C RC	+ +	AK RAK	- +	DA RDA	- -
Citrobacter freundii	AMP R- AMP	- -	OX R- OX	- -	CTX R- CTX	- -	GN RGN	- -	TOB RTOB	- -	E ER	- -	TE RTE	- -	VA VAR	- -	CIP R CIP	+ ++	C RC	+ +	AK RAK	+ +	DA RDA	- -
E. coli	AMP R- AMP	- -	OX R- OX	- -	CTX R- CTX	- -	GN RGN	- -	TOB RTOB	- -	E ER	- -	TE RTE	- -	VA VAR	+ +	CIP R CIP	- -	C RC	- -	AK RAK	- +	DA RDA	- -
E. coli	AMP R- AMP	- -	OX R- OX	- -	CTX R- CTX	- +	GN RGN	+ -	TOB RTOB	+ +	E ER	- -	TE RTE	- -	VA VAR	- -	CIP R CIP	+ ++	C RC	- +	AK RAK	- ++	DA RDA	- -
K. aerogenes	AMP R- AMP	- -	OX R- OX	- -	CTX R- CTX	- -	GN RGN	- -	TOB RTOB	+ +	E ER	- -	TE RTE	+ -	VA VAR	- +	CIP R CIP	- ++	C RC	+ ++	AK RAK	+ ++	DA RDA	- -
E. coli	AMP R- AMP	- -	OX R- OX	- -	CTX R- CTX	- -	GN RGN	- -	TOB RTOB	+ -	E ER	- -	TE RTE	- +	VA VAR	- -	CIP R CIP	- ++	C RC	- -	AK RAK	+ +	DA RDA	- -
E. coli	AMP R AMP	- -	OX R- OX	- -	CTX R- CTX	- -	GN RGN	- ++	TOB RTOB	- ++	E ER	- -	TE RTE	- -	VA VAR	- +	CIP R CIP	- ++	C RC	+ -	AK RAK	+ +++	DA RDA	+ -
E. coli	AMP R AMP	- -	OX R- OX	- -	CTX R- CTX	- -	GN RGN	+ +	TOB RTOB	- -	E ER	- -	TE RTE	- -	VA VAR	- -	CIP R CIP	- ++	C RC	- -	AK RAK	- ++	DA RDA	- -
E. coli	AMP R - AMP	- -	OX R- OX	- -	CTX R- CTX	- -	GN RGN	- -	TOB RTOB	- -	E ER	- -	TE RTE	- -	VA VAR	- -	CIP R CIP	- ++	C RC	- -	AK RAK	- ++	DA RDA	- -
E. coli	AMP R- AMP	- -	OX R- OX	- -	CTX R- CTX	- -	GN RGN	- -	TOB RTOB	- -	E ER	- -	TE RTE	- -	VA VAR	- +	CIP R CIP	- ++	C RC	- -	AK RAK	+ +	DA RDA	- -
E. coli	AMP R - AMP	- -	OX R- OX	- -	CTX R- CTX	- +	GN RGN	- +	TOB RTOB	+ +	E ER	- -	TE RTE	- -	VA VAR	- -	CIP R CIP	+ ++	C RC	- +	AK RAK	+ +	DA RDA	- -

Ampicillin (AMP), Oxacillin (Ox), Cephalexin (CL), Gentamicin (G), Tobramycin (Tob), Chloramphenicol (C), Erythromycin (E), Ciprofloxacin (Cip), Vancomycin (VA), Tetracycline (TE), Clindamycin (DA), Amikacin (AK) and R(rosemary)

Table 6b: Antibacterial response of tested antibiotics combined with *Rosmarinus officinalis* methanol extract against the multidrug resistant bacteria

Bacterial isolate	Bacterial isolate No.	Antibiotic resistance%	*Combination resistance%
Klebsiella aerogenes	1	83	67
Klebsiella aerogenes	2	83	67
E. coli	3	92	75
E. coli	4	92	67
E. coli	5	75	67
Citrobacter freundii	6	75	58
E. coli	7	92	83
E. coli	8	75	58
Klebsiella aerogenes	9	67	58
E. coli	10	83	75
E. coli	11	75	58
E. coli	12	92	75
E. coli	13	100	83
E. coli	14	92	75
E. coli	15	75	58
Citrobacter freundii	16	83	75
E. coli	17	100	85
E. coli	18	75	67

* Antibiotics combined with *Rosmarinus officinalis* methanol extract

DISCUSSION

Infectious diseases represent an important cause of morbidity and mortality in developing countries. Therefore, pharmaceutical companies have been motivated to develop new antimicrobial drugs in recent years, especially due to the constant emergence of microorganisms resistant to conventional antimicrobial agents. Apparently, bacterial species present the genetic ability to acquire and transmit resistance against currently available antibacterial agents and became multi resistant to other medications available on the market. In the present study, a total of seventy bacterial isolates were isolated from 70 patients in Tanta University Hospital, Egypt, Gram-negative bacilli were the most commonly detected pathogens. These results are in agreement with Madigan and Martinko [18] and Kohler *et al*. [19]. The antimicrobial susceptibility of the seventy bacterial isolates against 12 antibiotics ranged from 16-100%. Most of bacterial isolates were sensitive to amikacin antibiotic [20]. The results obtained from the present study revealed that the bacterial isolates were sensitive to a lot of tested plant extracts. This indicates that these extracts might have different modes of action than that of tested antibiotics. This observation agrees with the hypothesis of Eloff [21] who expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. The results showed that methanol was the best solvent of extraction followed by petroleum ether [22, 23]**.** The activity of the extract of *Rosmarinus officinalis* by methanol was the most efficient on the seventy bacterial isolates and multidrug resistant *E. coli*, followed by the extract of *Foeniculum vulgare*, these results are in agreement with Erdogrul [24] and Abubakar [25].

Natural products using as a proven template for the development of new scaffolds of drugs, phytochemical researches involving the discovery of herbal-drug interactions of new biologically active compounds for medicinal uses are encouraging nowadays. The success of natural products in drug discovery has been credited to their high chemical density, the effect of evolutionary pressure to create biologically active molecules and the structural similarity of protein targets across many species The synergy between the methanol extract of *Rosmarinus officinalis* and the antibiotics demonstrated that there are exportable phytochemicals in the plant that acted synergistically with each of the antibiotics to produce significant antibacterial effects at their supposed target sites [26]. Antibiotics combined with *Rosmarinus officinalis* methanol extract revealed synergistic inhibitory effect on MDR bacterial isolates. These results are in agreement with those obtained by Bishnu *et al* [6] and Elbashiti *et al.* [27].

CONCLUSION

It could be finally concluded that the multi-drug resistant Gram negative bacteria were sensitive to most of the tested plant extracts due to their strong antimicrobial activities.

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