Synergistic Effect of Combination Treatment by Certain Plant Extracts and Some Antibiotics on the Resistance of Pathogenic Bacteria to Some Common Antibiotics

EL-Zawahry, Y. A.; Reda, F. M. and Azazy, W. M

Department of Botany, Faculty of Science, Zagazig University,44519, Zagazig, Egypt.
afm67@yahoo.com, Fifi.reda133@yahoo.com

Abstract: A total of eighty bacterial isolates were isolated from pus, sputum, blood, stool and urine of different patients admitted to Sidnawy Hospital, Zagazig University, Egypt. These bacterial isolates were distributed as 50 Gram negative bacterial isolates (62.5%) and 30 Gram positive bacterial isolates (37.5%). The antibiotic susceptibility showed that the most effective antibiotic was amikacin followed by nitrofurantoin, norfloxacin, streptomycin and ciprofloxacin with 80%, 76.25%, 71.25%, 70% and 60% susceptibility respectively. On the other hand, 87.5% of bacterial isolates were resistant to aztreonam while 77.5% and 67.5% were resistant to clindamycin and oxacillin respectively. The four tested isolates; *Escherichia coli 3, Staphylococcus aureus 20, Pseudomonas aeruginosa 58* and *Klebsiella pneumoniae 65* were selected as multi-drug resistant (MDR) isolates against the tested antibiotics. Identification of the four selected isolates was confirmed molecularly by investigation of 16S *rRNA* gene sequences. The minimum inhibitory concentrations (MICs) of the most three effective antibiotics; amikacin, nitrofurantoin and norfloxacin were determined against the four multi-drug resistant(MDR) isolates. Furthermore, a total of 488 methanolic and aqueous crude extracts derived from different parts of 235 medicinal plant species traditionally used in Egyptian folk medicine belonging to 209 genera and 88 botanical families, were screened for their antibacterial activity against the highly resistant bacterial isolates. Out of 235 tested plants, 30 plant species belonging to 21 botanical families showed highly significant antibacterial activity by inhibiting all tested MDR isolates, and were more effective against Gram-positive than Gram-negative isolates. The microorganisms' susceptibility to different extracts did not correlate with the susceptibility or resistance to particular antibiotics. In most cases the organic extracts (80% methanol, 80% ethanol, 80% butanol, acetone, petroleum ether or chloroform) showed the same or greater activity than the aqueous extracts. Also, the methanolic extracts showed the strongest and broadest spectrum. The combination between the most potent plant extracts (*Rhus coriaria*, *Acacia nilotica* or *Tamarindus indica*) and antibiotics (amikacin, norfloxacin, vancomycin, tetracycline or amoxycillin) showed synergistic effect against the tested bacteria than each of them alone.
[EL-Zawahry, Y. A.; Reda, F. M. and Azazy, W. M. **Synergistic Effects of Combination Treatment between Certain Plant Extracts and Some Antibiotics on the Resistance of Pathogenic Bacteria against Some Common Antibiotics.** *Life Sci J* 2013;10(4):3477-3489]. (ISSN:1097-8135). http://www.lifesciencesite.com. 463

Key words: Antibiotic resistance, Antibacterial activity, Plant extracts, Synergistic effects.

1.Introduction

Throughout the history of mankind, infectious diseases have remained a major cause of death and disability accounting for about 22% of the global disease burden **(Murray and Lopez, 1997).** The discovery of penicillin in the 1940s and several other antibiotics in subsequent years led to great improvements in the management of infectious diseases particularly in developed countries. However, despite this success, the increased use of antibiotics led to the inevitable development of resistance, with the effect that diseases were hitherto thought to have been controlled by antibiotics later re-emerged as resistant infections **(Norrby *et al.*, 2005).** The occurrence and spread of antibiotic-resistant bacteria are pressing public health problems worldwide. Many bacteria have become and continue to be resistant nearly against all antimicrobial agents **(Simon *et al.*, 2009).**

Infectious diseases are the second leading cause of death worldwide. Treatment of infections continues to be a problem in modern time because of several side effects of some drugs and growing resistance to antimicrobial agents. One part of the problem is that bacteria causing infections are remarkably resilient and have developed several ways to resist antibiotics and other antimicrobial drugs. Another part of the problem is due to increasing use and misuse of existing antibiotics in human and veterinary medicine and in agriculture **(Bronzwear *et al.*, 2002)**. Several other factors like poverty, unsafe health practices, over-crowding, lack of education, misuse and over the counter availability of antibiotics also contribute to make the wonder weapons useless. A number of multidrug resistant (MDR) extracellular and intracellular pathogens are increasingly observed in normal community and/ or hospital settings **(Farooqui, 2008).** Hence, search for newer, safer and more potent antimicrobials is a pressing need. Herbal medicines have received much attention as a source of new antibacterial drugs since they are considered as time-

tested and comparatively safe both for human use and for environment **(Bazzaz *et al.*, 2005)**.

Plant-derived drugs remain an important resource, especially in developing countries, to combat serious diseases. Approximately 60-80% of the world's population still relies on traditional medicines for the treatment of common illnesses **(Dev, 2010 and Tekeli *et al.*, 2011).** Plants produce a wide variety of secondary metabolites many of which have been reported to be of therapeutic value **(Tshibangu *et al.*, 2001),** and a promising source of antibacterial compounds **(Rath *et al.*, 2012 and Al-Daihan *et al.*, 2013)**, raising hopes of obtaining novel antibiotics that can aid the fight against drug resistant infections. These compounds are believed to play a role in the plant's defense against infection by working in synergy with intrinsic antimicrobials **(Tegos *et al.*, 2002)**. It has therefore been suggested recently, that such compounds can potentially be used to improve the efficacy of antibiotics against MDR bacterial pathogens.

The action mechanisms of plant extracts and their natural components are related to: degradation of the cell wall; damage to cytoplasmic membrane and membrane proteins; leakage of intracellular contents; coagulation of cytoplasm; interference with active transport or metabolic enzymes; dissipate cellular energy in ATP form and depletion of proton motif force (PMF), electron flow which can cause cell death **(Tiwari *et al.*, 2009 and Saleem *et al.*, 2010).**

Antibacterial compounds such as thymol, eugenol, and carvacrol have been shown to cause disruption of the cellular membrane, inhibition of ATPase activity, and release of intracellular ATP and other constituents of microorganisms **(Raybaudi-Massilia *et al.*, 2009 and Negi, 2012)**. Thymol binds to the membrane proteins hydrophobically and changes the permeability characteristics of membrane **(Negi, 2012)**.

Combination of plant extracts with antibiotics help to minimize the minimum inhibitory concentrations (MICs), synergistic activity and this reduces the side effects, the economic cost and reduce sensory impact. Furthermore, these combinations may also control some bacteria that are known to show consistently high resistance to antimicrobials, i.e.: improving the efficacy of antibiotics against resistant bacterial pathogens, (modifying agents) **(Aiyegoro *et al.*, 2010 and Jouda, 2013)**. Those findings provided a basis to believe that plants can be potential sources of natural MDR inhibitors that can potentially improve the performance of antibiotics against resistant strains.

The plants from different geographical locations act synergistically with common antibiotics and exhibited greater antimicrobial activity against MDR pathogens **(Elbashiti *et al*., 2011; Rakholiya & Chanda, 2012 and Mabeku *et al.*, 2013).**

Production of efflux pump inhibitors by the plant would be one way to ensure delivery of the antimicrobial compound (**Stermitz *et al.*, 2000a,b)**. They observed the MDR inhibitors facilitated the penetration of berberine (produced from *Berberis* plants) into a model Gram positive bacterium, *S. aureus*. Moreover, the ability of plant extracts to potentiate antibiotics has not been well explained. It is speculated that inhibition of drug efflux, increasing permeability, and inhibition of β-lactamase and alternative mechanisms of action could be responsible for the synergistic interactions between plant extracts and antibiotics **(Smith *et al.* 2007 and Garvey *et al.*, 2011).**

This study aims to evaluate the antibacterial effect of the famous medicinal plants used in Egypt against multi-drug resistant pathogenic bacteria and improving the efficacy of available antibiotics, particularly the older and cheaper ones against the resistant bacteria pathogens by the combination of plant extracts with these antibiotics.

2.Materials and Methods

Collection of samples: The medical specimens of pus, sputum, blood, stool and urine were collected from inpatients admitted to Sidnawy Hospital, Zagazig, Egypt in the period from April to August 2012. The specimens were collected and transported according to **Miller (1999).**

Isolation and purification of bacteria: The swabs were streaked on Nutrient agar surface and different diagnostic and selective media namely; cystine lactose electrolyte deficient agar medium (C.L.E.D), MacConkey agar, Mannitol salt agar and Blood agar until pure single colonies were obtained according to **Murray *et al*. (2007).**

Antibiotic susceptibility test: Susceptibility of the bacterial isolates to seventeen antibiotics (Conc. µg/disc) (Amikacin, Amoxycillin, Amoxycillin/clavulanic acid, Azithromycin, Aztreonam, Cefotaxime, Chloramphenicol, Ciprofloxacin, Clindamycin, Nitrofurantoin, Norfloxacin, Oxacillin, Rifampicin, Streptomycin, Sulphamethoxazole/trimethoprim, Tetracycline and Vancomycin) was carried out by Kirby-Bauer disk diffusion technique according to **Bauer *et al*. (1966)**. The antibiotic disks were purchased from Oxoid Company.

Identification of the selected multi-drug resistant (MDR) bacterial isolates:

The purified cultures of the selected MDR were identified after investigating morphological and biochemical tests according to standard clinical laboratory methods reported and recommended by

Bergey's manual of Determinative Bacteriology **(Holt *et al.*, 1994; Garrity *et al.*, 2005 and Vos *et al.*, 2009)** and others **(Murray *et al.*, 2007 and Mahon *et al.*, 2011)**. The identification of MDR isolates were molecularly confirmed by investigation of 16S *rRNA* gene sequencing according to **Zhang *et al.* (2000) and Liu *et al.* (2002).**

Determination of the minimum inhibitory concentrations (MICs) and Minimum bactericidal concentrations (MBCs):

The MICs and MBCs of the most effective antibiotics; amikacin, nitrofurantoin and norfloxacin (which are members of aminoglycosides, nitrofurans and fluoroquinolones groups, respectively) against the multi-drug resistant isolates; *E. coli 3, S. aureus* 20, *P. aeruginosa 58* and *K. pneumoniae 65* were carried out using the standard broth dilution technique according to **Marie (2005)**.

Preparation of plant extracts: Twenty grams of every dried powdered plant material was soaked in 100 ml of distilled boiled water or the selected organic solvent (80% methanol, 80% ethanol, 80% butanol, acetone, petroleum ether or chloroform) in a sterile conical flask for 48 hours with continuous shaking. Then after filtration through 8 layers of muslin cloth and centrifuged at 5000g for 10 min the supernatant was collected and concentrated (in oven at 45 ºC) to make the final volume half of the original volume (stock solutions) **(Parekh and Chanda, 2006 and Al-Daihan *et al.*, 2013).**

Determination of antibacterial activity of the medicinal plant extracts against multi-drug isolates: The antibacterial activities of plant extracts were determined against 9 multi-drug resistant isolates (included within the seven bacterial groups which have variable resistance rate) namely; *E. coli 3, E. coli 44*, *K. pneumoniae 65, P. aeruginosa 58, Proteus vulgaris 11, Citrobacter freundii 16,* Methicillin-resistant *S. aureus 55 (*MRSA), Vancomycin-resistant *S. aureus 20 (*VRSA), and *Enterococcus faecalis 33* and only one sensitive isolate; Methicillin-sensitive *S. aureus 48* (MSSA), using disc diffusion method. Sterile filter paper discs (Whatman No.3, 6 mm diameter & three layers) were saturated by plant extracts and allowed to dry for one hour then placed on the surface of inoculated agar plates. After incubation the entire diameters of the inhibition zones were measured including the diameter of the disk (6mm) **(Kumara *et al.*, 2009 and Korcan *et al.*, 2013).**

Influence of combination between antibiotics and plant extracts against selected multi drug resistant strains: The most active methanolic plant extracts (*Rhus coriaria*, *Acacia nilotica* and *Tamarindus indica*) were tested in combination with five antibiotics, two of the highest active antibiotics (amikacin, norfloxacin) and three of the lowest active antibiotics (vancomycin, tetracycline & amoxycillin) against four selected MDR strains*; E. coli 3,* Vancomycin-resistant *S. aureus* 20, *K. pneumoniae 65* and *P. aeruginosa* 58 by using disk diffusion according to **Rakholiya and Chanda (2012) and Jouda (2013).** Each antibiotic disk was loaded with 10µl of the extract**.**

3.Results and Discussion

Distribution of collected isolates:

The occurrence and spread of antibiotic-resistant bacteria are pressing public health problems worldwide. Many bacteria have become and continue to be resistant nearly against all antimicrobial agents. The resistance rates are higher in developing countries **(Simon *et al.*, 2009)**. Nosocomial infections affect nearly 10% of hospitalized patients and represent a major problem in health care facilities, resulting in prolonged hospital stays, substantial morbidity and mortality, and excessive costs **(Burke, 2003)**.

In the present study, the results demonstrated that Gram-negative bacteria were the most common pathogens in the examined clinical specimens (Table 1). The eighty bacterial isolates were distributed as 50 Gram negative bacterial isolates and 30 Gram positive bacterial isolates, representing (62.5%) and (37.5%), respectively. These results are in agreement with **Abou-Zied (2011)** who demonstrated that Gram negative bacteria represented 52.95 % of total identified clinical bacteria while Gram positive bacteria represented about 47.05 % of total identified clinical ones.

Table (1): Distribution of collected bacterial isolates according to their Gram's stain reaction and source of isolation:

Source of isolation	Gram positive isolates		Gram negative isolates		Total	
	No.	%	No.	%	No.	%
Urinary tract infections (urine)	10	12.5	20	25	30	37.5
Wound infections (pus)	11	13.75	9	11.25	20	25
Respiratory infections (Sputum)	4	5	12	15	16	20
Blood infections (blood)	2	2.5	5	6.25	7	8.75
Stool	3	3.75	4	5	7	8.75
Total	30	37.5	50	62.5	80	100

Antibiotic susceptibility of bacterial isolates:

The antibiotic susceptibility of the collected isolates to 17 antibiotics was studied in the present investigation. The results in Table (2) showed that the most effective antibiotic was amikacin followed by nitrofurantoin, norfloxacin, streptomycin and ciprofloxacin with 80%, 76.25%, 71.25%, 70% and 60% susceptibility respectively. On the other hand, 87.5% of bacterial isolates were resistant to aztreonam while 77.5%, 67.5% and 67.5% of bacterial isolates were resistant to clindamycin, oxacillin and amoxycillin/clavulanic acid, respectively. **Das *et al.* (2006)** showed that the susceptibility rate of clinical isolates was the highest for amikacin (87.2%), followed by ciprofloxacin (74.8%), ceftazidime (71.5%), gentamicin (70.4%) and nitrofurantoin (53%).While, **Yuksel *et al.* (2006)** showed that nitrofurantoin was the most active agent (2.2% resistant isolates), followed by amikacin (4.9 %) and ciprofloxacin (12%).

Table (2): Susceptibility of bacterial isolates to different antibiotics:

Antibiotic	Symbol	Conc. µg/disc	Resistant % R	Intermediate % I	Susceptible% S
Amikacin	AK	30	15	5	80
Nitrofurantoin	F	300	20	3.75	76.25
Norfloxacin	NOR	10	25	3.75	71.25
Streptomycin	S	10	22.5	7.5	70
Ciprofloxacin	CIP	5	40	0	60
Chloramphenicol	C	30	25	18.75	56.25
Sulphamethoxazole/trimethoprim	SXT	25	48.75	1.25	50
Vancomycin	VA	30	51.25	2.5	46.25
Tetracycline	TE	30	51.25	3.75	45
Azithromycin	AZM	15	38.75	18.75	42.5
Rifampicin	RF	30	58.75	0	33
Oxacillin	OX	1	67.5	0	32.5
Amoxycillin/clavulanic acid	AMC	30	67.5	7.5	25
Clindamycin	DA	2	77.5	0	22.5
Cefotaxime	CTX	30	46.25	32.5	21.25
Aztreonam	ATM	30	87.5	5	7.5
Amoxycillin	AX	25	50	50	0

No. of Resistant isolates
% R = ———— x 100
Total count of isolates

No. of Sensitive isolates
% S = ———— x 100
Total count of isolates

No. of Intermediate isolates
% I = ———— x100
Total count of isolates

The multi-drug resistant bacterial isolates (34 isolates) which were resistant to more than 50% of tested antibiotics were selected and preliminary identified. According to the keys of Bergey's manual of Determinative Bacteriology and others the tested isolates were divided into seven groups as *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus and Enterococcus faecalis*. The results in Fig. (1) revealed that, *E. coli* was found to be the most frequent pathogen within multi-drug resistant (MDR) bacterial isolates representing 23.5%, followed by *K. pneumoniae*, *P. aeruginosa* and *S. aureus* with equal percentage, (17.6%) each. On the other hand, *E. faecalis* was the least frequent pathogen within MDR isolates (5.9%), followed by *P. vulgaris* (8.8%) and *Citrobacter freundii* (8.8%).

Out of 34 multi-drug resistant isolates, four isolates (*E. coli 3, S. aureus* 20, *P. aeruginosa 58* and *K. pneumoniae 65*) were selected as the multi-drug resistant (MDR) isolates against the tested antibiotics. These results are in agreement with **Das *et al.* (2006)**. They reported that the most common isolated pathogens were *E. coli* (59.4%), *Klebsiella spp.* (15.7%), *E. faecalis* (8.1%). *Escherichia coli* infection is one of the major public health problems in many developing countries and has contributed exceedingly to morbidity, mortality and increased health costs **(Ogata *et al.*, 2002)**. *Pseudomonas aeruginosa* was identified as one of the most common pathogen causing hospital acquired infections **(Geffers *et al.*, 2004)**. Also, the common resistance to methicillin in *S. aureus* caused alarming reports with regard to the spread of *S. aureus* in hospitals and the community **(Götz, 2010).**

The tested isolates showed high resistance to β-lactam antibiotics (amoxicillin, amoxycillin/clavulanic acid, oxacillin, cefotaxime and aztreonam). The results agree with those got by **Zaid (2001)** who reported that the resistance of 94 strains of *P. aeruginosa* and *E. coli* studied to 12 β-lactam antibiotics and found that all tested isolates were resistant to at least 7 β-lactam antibiotics. The resistance of isolates to β-lactam antibiotics may be due to drug inactivation by β-lactamases like AmpC cephalosporinase (beta-lactamase enzyme that open the β-lactam ring) as an intrinsic resistance, target site

modification (i.e. change in penicillin binding proteins (PBPs) as mutational resistance and acquired resistance represented in drug inactivation **(Abigail and Dixie, 1994).**

Moreover, the resistance of isolates to aminoglycoside antibiotics and azithromycin macrolides antibiotics may be due to inaccessibility of the target as an intrinsic resistance, reduced permeability or uptake as mutational resistance and acquired resistance represented in drug inactivation. The resistance of isolates to tetracycline antibiotic may be due to efflux system as acquired resistance. Also, the resistance of isolates to fluoroquinolones antibiotics may be due to reduced permeability or uptake as mutational resistance **(Fange *et al*., 2009).**

The identification of four selected isolates; *E. coli 3, S. aureus 20, P. aeruginosa 58* and *K. pneumoniae 65* was molecularly confirmed by investigation of 16S *rRNA* gene sequences (Fig. 2 and 3a,b,c,d). Sequence data were submitted to GenBank at NCBI web site (www.ncbi.nlm.nih.gov) with the following accession numbers; KF771030, KF771028, KF771032 and KF771031, respectively.

The MICs and MBCs of the most effective antibiotics against the four selected MDR isolates:

Three most effective antibiotics (amikacin, nitrofurantoin and norfloxacin) were tested against the four MDR isolates: *E. coli 3, S. aureus 20, P. aeruginosa 58* and *K. pneumoniae 65*. The MICs and MBCs were determined using the standard broth dilution technique.

The results in Table (3) showed that, the highest MIC and MBC were observed in nitrofurantoin antibiotic against *E. coli 3* (131.68 and 250 µg/ml, respectively), followed by *P. aeruginosa 58*. Meanwhile, the lowest MIC and MBC were observed in norfloxacin and amikacin antibiotics for *P. aeruginosa 58,* followed by *S. aureus 20*. **Discotto *et al*. (2001)** reported that the resistance of *S. aureus* to quinolones arises primarily from mutation in quinolone resistance determining region of DNA gyrase.

Antibacterial activity of medicinal plant extracts on the MDR strains:

Medicinal plants continue to play a central role in the healthcare systems of large proportions of the world's population, particularly in developing countries, where herbal medicine has a long and uninterrupted history of use **(Koduru *et al.*, 2007)**. In the present investigation, 488 methanolic and aqueous crude extracts derived from different parts of 235 medicinal plant species traditionally used in Egyptian folk medicine belonging to 209 genera and 88 botanical families. Extracts of these plants were screened for their antibacterial activity against ten isolates from different bacterial groups (9 multi-drug resistant and one sensitive) using the disc diffusion method.

The results of screening were encouraging as out of 235 tested plants, 30 plant species belonging to 21 botanical families showed high significant antibacterial activity against all tested MDR bacteria (both Gram-positive and Gram-negative bacteria) with inhibition zones from 8 to 50mm (Table 4). Out of all plant extracts, the methanolic extract of *Rhus coriaria* (sumac) was the most active. The recorded inhibition zone diameters ranged between 25-50 mm, and *Acacia nilotica* (Egyptian thorn) caused inhibition zones ranged between 18 to 45 mm. Followed by *Tamarindus indica* (tamarind), *Garcinia cambogia* (gambooge), *Citrus limon* (lemon), *Quercus infectoria* (Aleppo oak gall), *Syzygium aromaticum* (clove), *Hibiscus sabdariffa* (roselle), *Alpinia galanga* (galangal) and *Phyllanthus emblica* (Indian gooseberry, aamla) respectively (Table 4 and photo 1). These results are in agreement with **Riaz *et al.* (2011); Rath *et al.* (2012) and Al-Daihan *et al.* (2013)** with varying degrees of potency. The difference in potency may be due to the stage of collection of the plant sample, soil nature, other environmental factors, storage conditions, the part of plant used, method of extraction, method of screening, solvent used, concentration of extract and different sensitivity of the test strains.

In the present study, the multi-drug resistant isolates were found to be sensitive to a lot of tested plant extracts. This clearly indicates that these extracts might have different modes of action than that of tested antibiotics. This observation agrees with the hypothesis of **Eloff (1998)** who expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. Also, the present results showed that, the plant extracts were more effective against Gram-positive than Gram-negative bacteria (except for *E. faecalis 33* which had high resistance against tested plant extracts).This observation was reported by many authors **(Gibbons, 2004 and Suffredini *et al.*, 2006)**. These differences may be attributed to the fact that the cell wall in Gram-positive bacteria consists of a single layer, whereas in Gram-negative the cell wall is a multi-layered structure, bounded by an outer cell membrane and is quite complex **(Burt, 2004)**.

Moreover, the results in the same table revealed that, the microorganisms' susceptibility to different extracts did not correlate with the susceptibility or resistance to particular antibiotics. For example; *Rhus coriaria, Paeonia emodi* and *Terminalia chebula* caused 35mm, 15mm, 8mm inhibition zones respectively against MSSA, meanwhile caused 50mm, 30mm, 15mm inhibition zones respectively against MRSA although MSSA was more sensitive to different antibiotics. Such results are in agreement with **Ahmad & Beg (2001)**.They reported that the microorganism susceptibility to

different extracts did not correlate with the susceptibility or resistance to a particular antibiotic.

Whereas, the aqueous extract of *Tamarindus indica* (tamarind) was the most active of all tested aqueous extracts against tested MDR isolates. The recorded inhibition zones diameter ranged between 15 to 33 mm and *Rhus coriaria* (sumac) caused inhibition zones ranged between 15 to 40mm. Followed by *Hibiscus sabdariffa* (roselle), *Garcinia cambogia* (gambooge), *Terminalia catappa* (tropical almond), *Citrus reticulata* (mandarin), *Acacia nilotica* (Egyptian thorn), *Allium sativum* (garlic), *Sinapis arvensis* (wild mustard), *Ceratonia siliqua* (carob tree) and *Alpinia galanga* (galangal) respectively (Table 5).

In this study, the aqueous and organic extracts from the same plants showed different activities. There were no common rules for this, but in most cases the organic extracts showed the same or greater activity than the aqueous extracts. The results in Tables (4 & 5) indicate that methanolic extracts of most samples showed the best antibacterial activities against the tested isolates as compared with aqueous extract. In an explanation, the methanolic plant extracts contain: anthocyanins, tannins, polyphenols, terpenoids, saponins, xanthoxyllines, totarol, quassinoids, lactones, flavones, and phonons, while water extracts could contain only anthocyanins, starches, tannins, saponins, terpenoids, polypeptides, and lectins **(Saleem *et al.*, 2010)**.

Among six organic solvents (methanol, ethanol, butanol, acetone, petroleum ether and chloroform) used in extraction of the most active plants, the methanolic plant extracts in general gave the maximum inhibition zones against tested strains. Extracts of chloroform registered the least antibacterial activities, as compared to the other five solvent-extracts (Data not shown). The results showed that methanol was the best solvent of extraction followed by ethanol, acetone, butanol, petroleum ether and chloroform, respectively. These results confirmed the results obtained in previous studies which have reported that methanol was the better solvent for more consistent extraction of antimicrobial substances from medical plants compared to other organic solvents and water **(Emad *et al.*, 2009 and Al-Daihan *et al.*, 2013)**. On the other hand, **Mahasneh and El-Oqlah (1999)** showed that butanol extracts have superior antimicrobial activity whereas; **Buwa and Staden, (2006)** reported that the aqueous extracts were more active against bacteria if compared with ethanol and ethyl acetate extracts. **Talib and Mahasneh (2010)** concluded that the activity was mainly concentrated in the butanol and aqueous extracts.

The action mechanisms of plant extracts and their natural components are related to: degradation of the cell wall; damage to cytoplasmic membrane and membrane proteins; leakage of intracellular contents; coagulation of cytoplasm; interference with active transport or metabolic enzymes; dissipate cellular energy in ATP form and depletion of proton motive force (PMF) and electron flow, which can cause cell death **(Negi, 2012).**

Influence of combination between selected antibiotics and the most effective methanolic plant extracts:

While the routine practice had been screened the plant extracts for direct antimicrobial compounds, the second option of searching for resistance modifying compounds that can improve the efficacy of antibiotics when used in combination with plant extracts, appeared more attractive as it allows recycling of old and relatively cheaper antibiotics that had been rendered ineffective due to the growing resistance to them **(Gibbons, 2004)**. In the current study, the antibacterial activity of combination between selected antibiotics and sumac extract (Table 6a and photo 2a) showed high efficacy against the four selected MDR strains than each of them alone (synergistic effect) except in case of combination of sumac extract and amikacin which produced 22mm inhibition zone equal to that of the extract only against *P. aeruginosa 58* (no synergistic effect) and less than that of the norfloxacin (antagonistic effect). Sumac extract had the best synergism with norfloxacin and amoxicillin against VRSA *S. aureus* 20, also norfloxacin and tetracycline against *K. pneumoniae 65,* whereas vancomycin and amoxicillin against *P. aeruginosa 58*.

Table (3): The MICs and MBCs of the most effective antibiotics against the MDR isolates:

Bacterial isolate	**Amikacin (AK)**		**Nitrofurantoin (F)**		**Norfloxacin (NOR)**	
	MIC(µg/ml)	MBC(µg/ml	MIC(µg/ml)	MBC(µg/ml	MIC(µg/ml)	MBC(µg/ml)
E. coli 3	125	125	131.687	250	17.341	26.012
S. aureus 20	87.791	87.791	26.012	31.25	17.341	31.25
P. aeruginosa 58	15.625	17.341	125	250	3.906	5.138
K. pneumoniae 65	58.527	62.5	62.5	125	31.25	31.25

Table (4): Antibacterial activity of methanolic plant extracts on the nine MDR isolates and one sensitive isolate:

Plant Family	Plant species	Part used	Inhibition zone (mm) of tested isolates									
			E.c1	E.c2	K.p	P.a	P.v	C.f	*MSSA	MRSA	VRSA	E.f
Anacardiaceae	*Rhus coriaria*	Fruits	35	30	30	25	25	30	35	50	40	30
Fabaceae	*Acacia nilotica*	Pods	25	20	25	22	25	25	30	40	45	18
Fabaceae	*Tamarindus indica*	Pods	30	25	30	28	20	15	32	20	32	25
Clusiaceae	*Garcinia cambogia*	Fruits	27	25	28	20	19	12	27	32	24	30
Rutaceae	*Citrus limon*	Fruits	25	15	20	22	25	20	25	35	30	15
Fagaceae	*Quercus infectoria*	Gall	20	20	20	20	20	25	30	30	40	10
Myrtaceae	*Syzygium aromaticum*	Floral buds	25	15	15	15	15	25	25	25	25	25
Malvaceae	*Hibiscus sabdariffa*	red calyces	12	12	15	20	20	20	20	30	30	15
Zingiberaceae	*Alpinia galangal*	Rhizomes	15	15	15	15	20	20	30	25	20	18
Phyllanthaceae	*Phyllanthus emblica*	Fruits	25	15	15	20	13	15	20	15	15	25
Nitrariaceae	*Peganum harmala*	Seeds	20	15	10	20	15	20	22	30	22	13
Lythraceae	*Lawsonia inermis*	Leaves	15	10	10	20	20	25	35	30	30	10
Asteraceae	*Calendula officinalis*	Flowers	15	15	12	12	15	20	15	15	30	15
Portulacaceae	*Portulaca oleracea*	Seeds	15	15	15	15	15	15	20	20	15	15
Rubiaceae	*Rubia tinctorum*	Roots	15	10	20	10	15	20	25	20	25	15
Lamiaceae	*Rosmarinus officinalis*	Aerial parts	18	20	10	12	15	25	25	22	15	10
Menyanthaceae	*Menyanthes trifoliate*	Aerial parts	15	15	15	15	15	15	20	25	15	10
Zingiberaceae	*Zingiber officinale*	Rhizomes	15	12	15	10	15	15	25	20	30	10
Lythraceae	*Punica granatum*	Peels	15	15	10	15	12	15	15	15	15	15
Lauraceae	*Laurus nobilis*	Leaves	15	8	15	15	15	15	20	20	20	8
Combretaceae	*Terminalia catappa*	Fruits	15	10	10	15	20	15	20	25	20	10
Fabaceae	*Senna alexandrina*	Pods	15	15	10	15	10	10	25	35	25	10
Lamiaceae	*Origanum vulgare*	Aerial parts	15	15	12	10	12	15	20	20	20	10
Schisandraceae	*Illicium verum*	Seeds	10	15	15	10	15	10	25	30	12	12
Asteraceae	*Saussurea costus*	Roots	15	10	15	8	15	15	20	20	15	10
Apiaceae	*Carum Copticum*	Seeds	10	15	15	10	10	15	15	20	15	15
Zingiberaceae	*Elettaria cardamomum*	Pods	15	10	15	10	8	15	20	25	20	8
Lamiaceae	*Thymus vulgaris*	Aerial parts	12	10	10	10	15	10	20	30	16	11
Paeoniaceae	*Paeonia emodi*	Roots	10	10	8	20	25	10	15	30	15	8
Xanthorrhoeaceae	*Aloe vera*	Leaves	10	8	8	15	15	10	20	30	25	10

E.c1= *Escherichia coli 44,* E.c2= *Escherichia coli 3*, K.p= *Klebsiella pneumoniae 65*
P.a= *Pseudomonas aeruginosa 58,* P.v= *Proteus vulgaris 11,* C.f= *Citrobacter freundii 16,*
*MSSA= Methicillin-sensitive *Staphylococcus aureus48,*
MRSA= Methicillin-resistant *Staphylococcus aureus 55* VRSA= Vancomycin-resistant *Staphylococcus aureus 20* E.f = *Enterococcus faecalis 33*

The antibacterial activity of combination between selected antibiotics and *Acacia nilotica* extract against the four selected MDR strains was represented in Table (6b) and photo (2b). This combination was exhibited a synergistic effect against the tested MDR strains. *Acacia nilotica* extract showed the best synergism with norfloxacin and amoxicillin against *P. aeruginosa 58*, and tetracycline and norfloxacin against VRSA *S. aureus* 20, followed by norfloxacin and amikacin against *K. pneumoniae 65*, while the antibacterial activity of combination between selected antibiotics and *Tamarindus indica* extract showed wide variation in their antibacterial activity (Table 6c and photo 2c). The combinations showed synergistic effect against the multi-drug resistant strains. While in case of combination of tamarind extract and amikacin gave effect equal to that of the antibiotic alone against *P. aeruginosa 58* (no synergistic effect) and in case of combination of tamarind extract and tetralecycline against *K. pneumoniae 65*, *P. aeruginosa 58* and VRSA 20 produced 10mm, 9mm and 9mm inhibition zones respectively less than that of the tamarind extract only (antagonistic effect). Also combination of tamarind extract with amoxicillin or vancomycin

showed antagonistic effect against *P. aeruginosa 58*. Tamarind extract showed the highest synergism with norfloxacin against *P. aeruginosa 58,* followed by amikacin, amoxicillin and vancomycin against VRSA *S. aureus* 20.

Furthermore, the results in the present investigation, showed that there is an increased activity in case of combination of methanolic plant extracts and the tested antibiotics. The observed synergism was not specific to a particular class of antibiotics and the synergistic interactions were observed largely against Gram positive organisms. The strongest synergism was observed between the extract of *Acacia nilotica* and selected antibiotics. Also, synergistic interactions in case of plant extracts and the highest active antibiotics were lower than that of plant extracts and the lowest active antibiotics. Our results agree with the findings of **Aiyegoro *et al.* (2010); Garvey *et al.* (2011); Rakholiya and Chanda (2012) and Jouda (2013).**They confirmed that indeed plants can be sources of compounds that can potentiate the activity of antibiotics against resistant bacterial pathogens. These compounds have variably been termed resistance modifying, modulating or reversal agents. This ability of plant extracts to potentiate antibiotics has not been well explained. It is speculated that inhibition of drug efflux, increasing permeability, inhibition of β-lactamase and alternative mechanisms of action could be responsible for the synergistic interactions between plant extracts and antibiotics **(Smith *et al.*, 2007 and Garvey *et al.*, 2011).**

Table (5): Antibacterial activity of aqueous plant extracts on the nine MDR isolates and one sensitive isolate:

Plant Family	Plant species	Part used	Inhibition zone (mm) of tested isolates									
			E.c1	E.c2	K.p	P.a	P.v	C.f	*MSSA	MRSA	VRSA	E.f
Fabaceae	*Tamarindus indica*	Pods	20	22	30	30	23	15	30	25	33	25
Anacardiaceae	*Rhus coriaria*	Fruits	30	15	20	20	20	20	25	40	35	20
Malvaceae	*Hibiscus sabdariffa*	red calyces	22	25	25	25	20	15	30	30	25	25
Clusiaceae	*Garcinia cambogia*	Fruits	10	15	15	15	20	8	25	20	27	25
Combretaceae	*Terminalia catappa*	Fruits	10	8	15	15	20	8	20	20	20	20
Rutaceae	*Citrus reticulate*	Peels	22	16	8	9	12	9	30	23	25	10
Fabaceae	*Acacia nilotica*	Pods	0	8	0	15	22	25	20	30	40	0
Amaryllidaceae	*Allium sativum*	Bulbs	10	0	20	10	15	0	25	10	22	0
Brassicaceae	*Sinapis arvensis*	Seeds	15	0	10	10	8	15	10	20	12	0
Fabaceae	*Ceratonia siliqua*	Pods	0	12	20	15	15	0	12	10	0	0
Phyllanthaceae	*Phyllanthus emblica*	Fruits	20	0	10	0	0	0	15	10	20	15
Zingiberaceae	*Alpinia galangal*	Rhizomes	0	10	0	0	0	10	20	20	15	15
Fagaceae	*Quercus infectoria*	Gall	0	0	10	0	0	0	25	20	30	0
Apiaceae	*Cuminum cyminum*	Seeds	0	0	12	0	0	0	20	20	22	0
Brassicaceae	*Raphanus sativus*	Seeds	15	0	0	8	8	8	10	15	20	0
Nitrariaceae	*Peganum harmala*	Seeds	10	0	0	12	10	0	10	20	18	0

Table (6): Influence of combination between the selected antibiotics and methanolic plants extract of *(a) Rhus coriaria* (sumac), (b) *Acacia nilotica* and (c) *Tamarindus indica* (tamarind) on the four selected MDR strains:

(a) ***Rhus coriaria* (sumac)**

Selected MDR strains	Inhibition zone (mm)										
	Ex	VA	VA+ex	AX	AX+ex	TE	TE+ex	AK	AK+ex	NOR	NOR+ex
E. coli 3	**19**	**0**	**22**	**0**	**23**	**0**	**20**	**0**	**24**	**0**	**24**
K. pneumoniae 65	**20**	**0**	**25**	**0**	**25**	**0**	**29**	**0**	**27**	**0**	**30**
P. aeruginosa 58	**22**	**0**	**30**	**0**	**28**	**0**	**26**	**20**	**22**	**25**	**20**
VRSA 20	**19**	**0**	**28**	**0**	**26**	**0**	**29**	**0**	**2**	**0**	**33**

(b) ***Acacia nilotica***

Selected MDR strains	Inhibition zone (mm)										
	Ex	VA	VA+ex	AX	AX+ex	TE	TE+ex	AK	AK+ex	NOR	NOR+ex
E. coli 3	17	0	21	0	20	0	19	0	20	0	21
K. pneumoniae 65	19	0	23	0	21	0	22	0	23	0	25
P. aeruginosa 58	21	0	27	0	28	0	26	20	28	25	29
VRSA 20	22	0	25	0	26	0	28	0	25	0	27

(c) ***Tamarindus indica*** **(tamarind)**

Selected MDR strains	Inhibition zone (mm)										
	ex	VA	VA+ex	AX	AX+ex	TE	TE+ex	AK	AK+ex	NOR	NOR+ex
E. coli 3	14	0	17	0	18	0	21	0	19	0	20
K. pneumoniae 65	17	0	21	0	18	0	10	0	18	0	18
P. aeruginosa 58	17	0	12	0	16	0	9	20	20	25	33
VRSA 20	16	0	21	0	22	0	9	0	22	0	20

VRSA(20) = Vancomycin-resistant *Staphylococcus aureus 20*

AX= amoxicillin (25 µg/disc); AK= amikacin (30 µg/disc); TE= tetracycline (30 µg/disc); VA= vancomycin (30 µg/disc); NOR= norfloxacin (10 µg/disc)

and ex= methanolic extract only (10µl). Each antibiotic disk loaded with 10µl of extract.

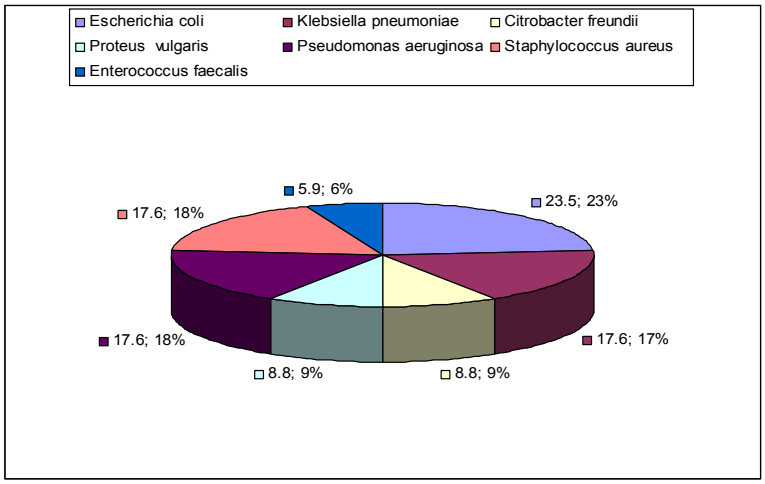


Fig. (1): The percentage of distribution of bacterial species within multi-drug resistant bacterial isolates.

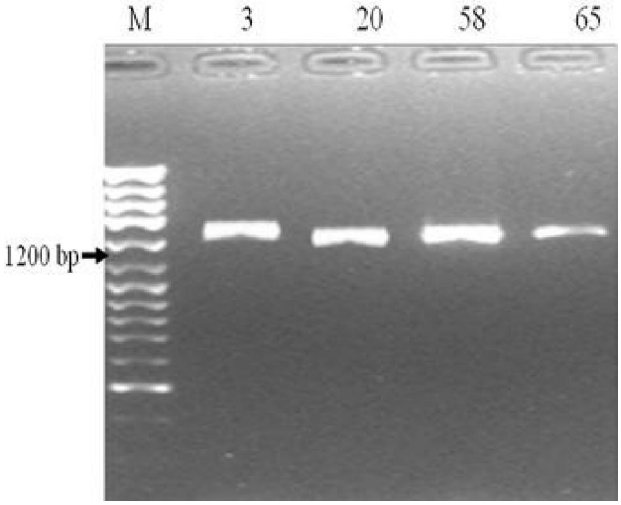


Fig. (2): The 16S rRNA gene amplified by polymerase chain reaction (PCR) of the selected MDR strains; *E. coli 3, S. aureus 20, P. aeruginosa 58* and *K. pneumoniae 65*

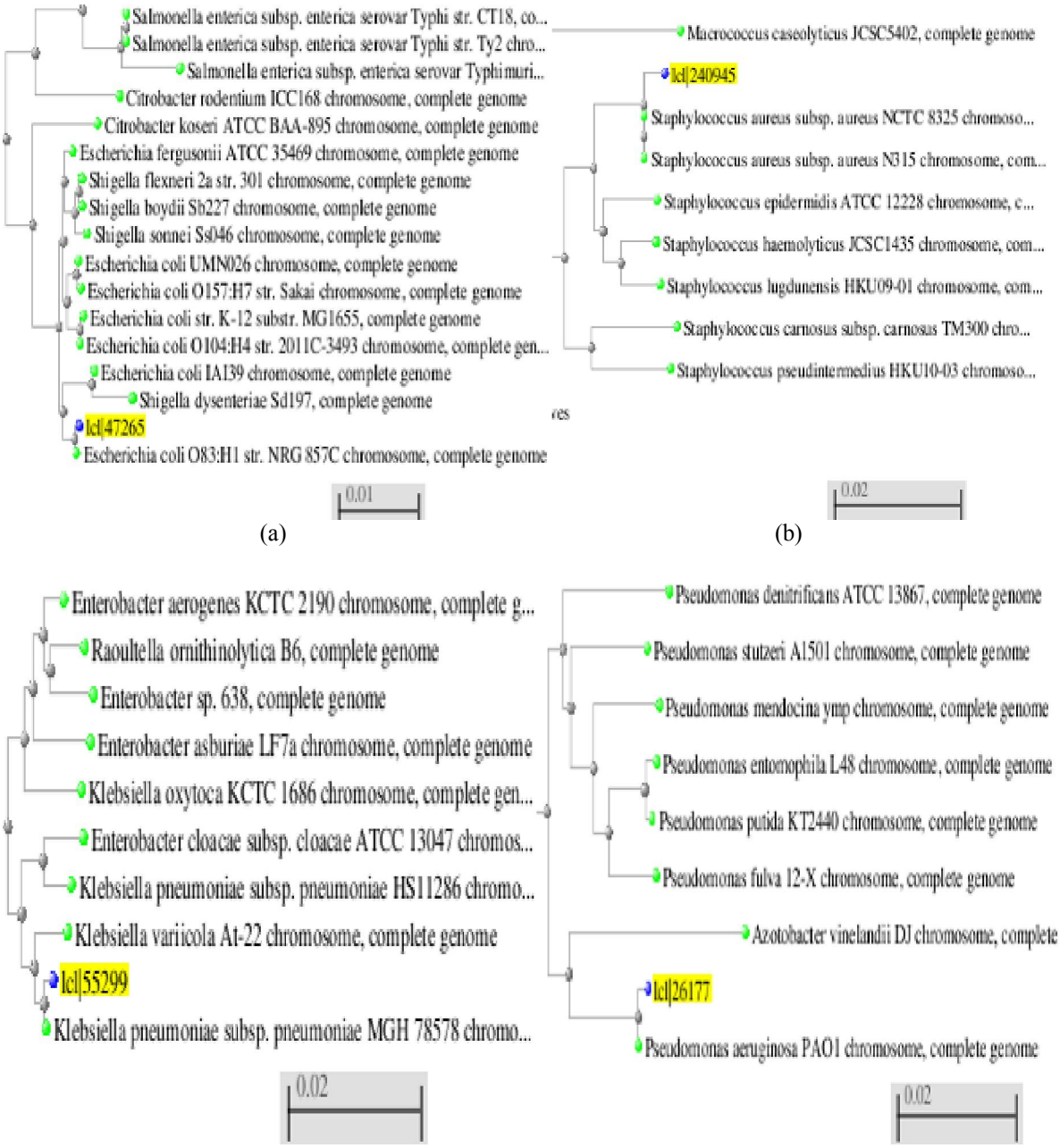
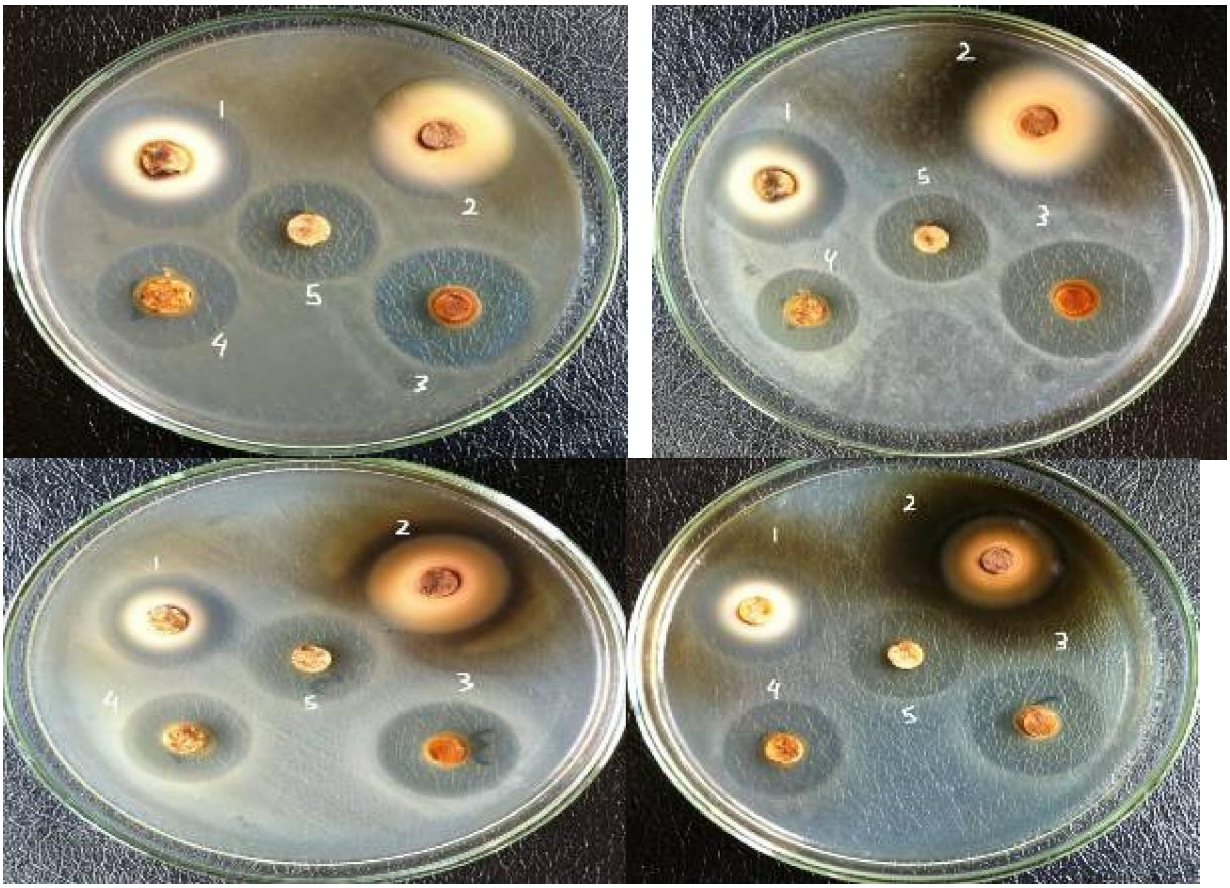


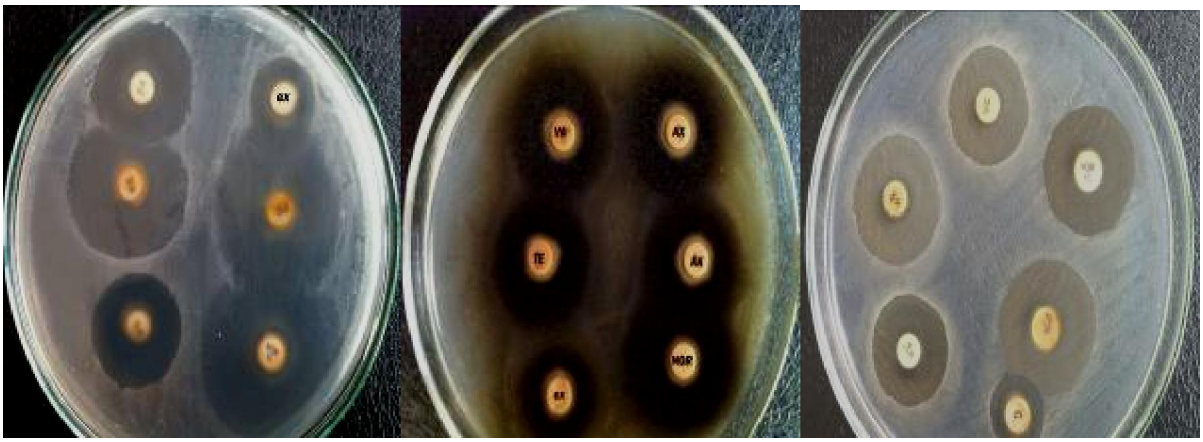
Fig. (3): The phylogenetic tree of selected multi-drug resistant strains (a) *E. coli* 3, (b) *S. aureus* 20, (c) *K. pneumoniae 65* and (d) *P. aeruginosa* 58.

(a) (b)



(c) **(d)**

Photo (1): Antibacterial activity of methanolic plant extracts against the four selected MDR strains; (a) *Staphylococcus aureus 20,* (b) *Klebsiella pneumoniae 65*, (c) *Escherichia coli 3*, (d) *Pseudomonas aeruginosa 58*, 1: *Rhus coriaria*, 2: *Acacia nilotica*, 3: *Tamarindus indica,* 4: *Garcinia cambogia*, and 5: *Citrus limon*



(a) (b) (c)

Photo (2): Synergistic effect between plant extracts and antibiotics against the selected MDR strains.
(a) Combination between antibiotics and methanolic extract of *Rhus coriaria* against VRSA *S. aureus 20*, (b) Combination between antibiotics and methanolic extract of *Acacia nilotica* against *Pseudomonas aeruginosa 58*, (c) Combination between antibiotics and methanolic extract of *Tamarindus indica* against *Escherichia coli 3*.
AX= amoxycillin; AK= amikacin; TE= tetracycline; VA= vancomycin; NOR= norfloxacin and ex= methanolic extract only (10µl). Each antibiotic disk loaded with 10µl of extract.

Conclusion

It is interesting to note that even crude extracts of some plants showed good activity against multi-drug resistant bacterial strains whilst modern antibiotic therapy has limited effect. Hence, these plants can be a potential source for evolving newer antimicrobial compounds for treating serious infections caused by MDR bacteria.

The synergistic effect of the association of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the older and cheaper antibiotic when it is no longer effective by itself as an effective treatment. In addition, the plant extracts can be a potential source of broad spectrum resistance modifying compounds that can potentially improve the performance of antibiotics in the treatment of multi-drug resistant infections.

Corresponding author
Fifi M. Reda
Department of Botany, Faculty of Science, Zagazig University, 44519, Zagazig, Egypt
E-mail: afm67@yahoo.com Fifi.reda133@yahoo.com

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