Research Article

Synergistic Effect of Combined Antibiotic and Methanol Extract of *Eucalyptus camaldulensis* leaf Against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract

A total of seventy-five medical specimens were collected from different patients admitted to Zagazig university hospital, Egypt. The selected bacterial isolates were distributed as 45 Gram negative bacterial isolates and 30 Gram positive bacterial isolates. The most effective antibiotic was gentamycin followed by amikacin and nitrofurantoin. The multi-drug resistant (MDR) bacterial isolates were selected and identified to four groups; *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Escherichia coli* and *Staphylococcus aureus.* The selected isolates were screened for their capability of biofilm, hemolysins, lecithinase and protease production. *S. aureus* AM23 and *P. aeruginosa* AM41 were selected as the highest biofilm and degrading enzymes producers. Furthermore, twenty-four methanolic and aqueous crude extracts derived from different medicinal plants in Egypt were screened for their antibacterial activity against both selected MDR strains. Methanolic extract of *Eucalyptus camaldulensis* leaf showed the greatest effect on the tested bacteria. Also, there was a synergistic effect of combined *E. camaldulensis* leaf extract and antibiotics (Gentamycin and Ceftriaxone) against tested organisms. Transmission electron microscopy showed changes in cell shapes, size, contents and cell envelop of antimicrobials treated bacterial cells compared to the control. Our findings proved that the leaf extracts of *E. camaldulensis* had great potential as antimicrobial agents in the treatment of infectious organisms.

Keywords: Plant extract; *E. camaldulensis;* Synergistic effect; Virulence factor; TEM investigation.

Introduction

The virulence factor concept has unquestionably led to the identification of important microbial attributes of virulence that have greatly furthered to understanding of microbial pathogenesis. Furthermore, the approach of defining virulence factors by the use of the molecular postulates has provided an experimentally rigorous approach to the study of virulence in certain microbes (Falkow, 2004). Nevertheless, the virulence factor concept has significant limitations for a global understanding of microbial virulence. Increasing hospital and community-acquired infections due to bacterial multidrug-resistant (MDR)

pathogens for which current antibiotic therapies are not effective represent a growing problem. Antimicrobial resistance is one of the major threats to human health (Franci *et al.,* 2015).

One of the defense mechanisms of *S. aureus* is the capacity to form biofilm. A biofilm is an aggregate of microorganisms in which cells adhere to each other on a surface, these adherent cells are frequently embedded within a self- produced matrix of extracellular polymeric substance (Medora *et al.,* 2010). Microbial biofilm has been associated with a variety of persistent infections which respond poorly to conventional antibiotic therapy. This also helps in the spread of antibiotic resistance traits in nosocomial pathogens by increasing mutation rates and by the exchange of genes which are responsible for resistance (Pramodhini *et al.,* 2012). Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. They exhibit resistance to antibiotics by various methods like restricted penetration of antibiotic in to biofilm, decreased growth rate and expression of resistance genes (Hassan *et al.,* 2011). As a consequence of biofilm development, it is said that the ability of organisms to transfer genes horizontally will be enhanced within these micro communities thus facilitating the spread of antibiotic resistance (Sasirekha *et al.,* 2012).

Biofilm formation is believed play an important role in infection immunity and protection toward antimicrobial agents. For example, the gram-positive *Staphylococcus epidermidis* and the gram-negative *Pseudomonas aeruginosa* are the most prevalent pathogens involved in clinical chronic infections (Vuong *et al*. 2004). Their growth and proliferation within a biofilm provides protection from antibiotics and provides them a host defense mechanism by slowing down or preventing penetration of different agents through the biofilm (Costerton *et al.,* 2003).

Numerous enzymes have been implicated in microbial virulence. Although the number of enzymes in this categories vast we will discuss several examples to illustrate their mechanism of action. Enzymes that are considered virulence factors are generally active against host components and contribute to virulence by damaging host tissues. Tissue damage makes the host permissive for microbial infection. Enzyme virulence factors that damage tissue include proteases, licithinases and hemolysines. These enzymes damage cells and provide nutrients by digesting substrates into smaller components that can be assimilated by microbes (Cox *et al.,* 2000).

Eucalyptus camaldulensis is one of such medicinal plants belonging to the family Myrtaceae includes 140 genera species distributed in tropical and subtropical reign of the world (Ali *et al.,* 2011). *Eucalyptus* species constitute a major reservoir for a wide range of secondary metabolites

many of which have been found to harbor a diverse range of biological activities (Huang *et al.,* 2015 and Sebei *et al.,* 2015). A number of constituents isolated from *Eucalyptus* trees have been shown to have antibacterial, antifungal, antioxidant and repellent activities (Amini *et al.,* 2012).

The rich chemical diversity in plants has been reported to be a promising source of antibacterial compounds (Ishnava *et al.,* 2012 and Okoth *et al.,* 2013), raising hopes of obtaining novel antibiotics that can aid the fight against drug resistant infections. Synergism due to combination of plant extracts with antibiotics help to minimize the minimum inhibitory concentrations (MICs), consequently reduce the side effects, 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 (Jouda, 2013).

Eucalypts are valued for their wood and some are also valuable sources of polyphenols, Terpenoids and the base composition (70 to 80 mg/ml) of the leaves are the Eucalyptol or Cineole (Ayepola and Adeniyi, 2008), proteins, tannins, gum, and dyes although their most valuable product is the eucalyptus oil that is readily distilled from their leaves (Sartorelli *et al.,* 2007).Antibacterial activity of the leaf extracts of *E. camaldulensis* can be attributed to the action of the phytochemical compounds it contains. Babayi *et al.*, (2004) reported the presence of saponins, saponin glycosides, steroid, cardiac glycosides, tannins, volatile oils, phenols and balsam (gum) in *E. camaldulensis*. This study aims to evaluate the antibacterial efficacy of some medicinal plants in Egypt against multi-drug resistant pathogenic bacteria and improving the efficacy of available antibiotics by the combination of plant extracts with these antibiotics.

Materials and Methods

Samples Collection
The medical specimens of pus, sputum, vaginal and urine collected from patients admitted to Zagazig University Hospital, Egypt. Samples were obtained in period from March to May 2014.The specimens were collected and transported according to Miller (1999).

Isolation and Purification of Bacteria
The swabs were streaked on the surface of nutrient agar, MacConkey agar and Mannitol salt agar plates until pure single colonies were obtained according to Murray *et al.* (2007).

Antibiotic Susceptibility Test
 Susceptibility of the bacterial isolates to ten antibiotics (Conc.μg/disc) (Amikacin, Amoxycillin, Cefotaxime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Gentamycin, Nitrofurantoin, Norfloxacin and Tetracycline) 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 Bacteria

The purified cultures of the selected MDR were characterized morphologically and biochemically according to standard clinical laboratory methods reported and recommended by Bergey's manual of Systematic Bacteriology (Holt *et al.,* 1994 and Vos *et al.,*2009). Identification of selected MDR isolates were confirmed molecularly by investigation of 16S rRNA sequencing according to Liu *et al.,* (2002) and Zhang *et al.,* (2000).

Screening for Virulence Factors Produced by MDR Bacteria

The MDR bacteria were screened for their capability to produce hemolysin, protease and lecithinase enzymes on blood agar, casein agar and egg yolk agar media, respectively, at 35°C for 24 h using agar well diffusion assay according to Klaenhammer (1988). Also, biofilm formation using Congo red method was tested (Mathur *et al.,* 2006).

Preparation of Plant Extracts

Twenty grams of every dried powdered plant material was soaked in 100 ml of distilled boiled water or 80% methanol in a sterile conical flask for 48 h 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 45ºC) to make the final volume half of the original volume (stock solutions) (Al-Daihan *et al.,*2013).

Antibacterial Activity of the Tested Plant Extracts

The antibacterial activity of plant extracts was determined against two multi-drug resistant strains; *S. aureus* AM23 and *P. aeruginosa* AM41, using disc diffusion method. Sterile filter paper discs (Whatman No.3, 6 mm diameter and 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 (Arokiyaraj *et al.,*2013).

Combination of Gentamycin and Ceftriaxone with E. Camaldulensis Leaf Extract against MDR Bacteria

The most effective methanolic plant extract of *E. camaldulensis* was tested in combination with Gentamycin (CN) and Ceftriaxone (CRO) as the highest and lowest active antibiotics, respectively against *P. aeruginosa* AM41*, S. aureus* AM23 according to Jouda (2013). Each antibiotic disk was loaded with 10µl of the extract. Synergistic effect occurs when the effect of two drugs together is greater than the effect of either alone. Indifference occurs when the effect of two drugs together is

the same of either alone. Antagonism occurs when the effect of two drugs together is less than the effect of both alone (Rakholiya and Chanda, 2012).

Minimum Inhibitory Concentrations of Ceftriaxone, Gentamycin and E. Camaldulensis Leaf Extract against MDR Bacteria

The minimum inhibitory concentration (MIC) of the methanol leaf extract of *E. camaldulensis,* ceftriaxone and gentamycin were carried out using the agar dilution method described by Lajubutu *et al.,* (1995). Different concentrations were prepared to give a final 50 to 0.048 mg /ml, 2500 to19.5 µg/ml, 2000 to1.95 µg/ml of the plant extracts, ceftriaxone (CRO), gentamycin (CN), respectively. The 2ml of each dilution was mixed with 2ml of Mueller-Hinton agar, poured into Petri dishes and allowed to set. The agar medium was streaked with an overnight broth culture of the test organisms and incubated overnight. The lowest concentration inhibiting growth was regarded as the minimum inhibitory concentration of the extract and antibiotics.

Transmission Electron Microscope (TEM)

Collected non treated cells of *S. aureus* AM23 and *P.aurgenasa* AM41 and treated cells (1/2 MIC of *E. camaldulensis* methanol extract and 1/2 MIC of CRO) were prepared for investigated by TEM according to Karnovsky (1965). Ultrathin sections were observed at 80 kV using a JEOL 2100 TEM at 80 KV, Faculty of Agriculture, Mansoura University, Dakhalia governorate, Egypt.

Results and Discussion

Understanding which bacterial characteristics contribute most to disease is a major area of research in microbiology and infection biology (Lebeaux *et al.,* 2014). Bacterial characteristics that reduce host health and/or survival are considered 'virulence factors' (Kazmierczak *et al.,* 2015). In the present study, seventy-five bacterial isolates which isolated from wound, urine, vaginal and respiratory infections of different patients were distributed as 30 Gram-positive bacterial isolates and 45 Gram negative bacterial isolates (Table 1). This indicated that Gram-negative bacteria were more distributed pathogens than the gram positive. These results are in line with EL-Zawahry *et al.,* (2013) who isolated 50 Gram negative bacterial isolates and 30 Gram positive bacterial isolates from different clinical sources. The increasing spread of antibiotic resistance in Gram-negative bacteria, particularly in *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae,* represents a major global medical challenge (Bergen *et al.,* 2012). The situation is exacerbated by the lack of progress with respect to the clinical development of new antibiotics for Gram-negative bacteria over the last few decades (Carlet *et al.,* 2012).

Table 1: Distribution of 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.	%
Genital tract infections (Vaginal)	4	5	9	12	13	17
Wound infections (Pus)	8	11	12	16	20	27
Respiratory tract infections (Sputum)	6	8	11	15	17	23
Urinary tract infections (Urine)	12	16	13	17	25	33
Total	30	40	45	60	75	100

Table 2: Comparative susceptibility of bacterial isolates against different antibiotics.

Antibiotic	Symbol	Conc. (µg/disc)	Resistant (R)		Intermediate (I)		Susceptible (S)	
			No.	%	No.	%	No.	%
Amikacin	AK	30	27	36	8	11	40	53
Amoxycillin	AX	25	43	57	9	12	23	31
Cefotaxime	CTX	30	51	68	5	7	19	25
Ceftriaxone	CRO	30	61	81	6	8	8	11
Chloramphenicol	C	30	40	53	1	2	34	45
Ciprofloxacin	CIP	5	41	54	5	7	29	39
Gentamicin	CN	10	16	21	2	3	57	76
Nitrofurantoin	F	30	38	51	3	4	34	45
Norfloxacin	NOR	10	39	52	3	4	33	44
Tetracycline	TE	30	46	61	11	15	18	24

Antibiotic sensitivity testing (AST) aims to determine the susceptibility of an isolate to a range of potential therapeutic agents (Sahloffand Martin, 2002). The antibiotic susceptibility of seventy-five collected bacterial isolates to 10 antibiotics representing different groups showed that the tested isolates were highly susceptible to gentamycin with susceptibility percentage so that it represented the most effective antibiotic followed by amikacin, nitrofurantoin, chloroamphenicol, and norfloxacin with 53%, 45%, 45% and 44% susceptibility, respectively (Table 2). On the other hand, the data showed that 81% of bacterial isolates were resistant to ceftriaxone while 68%, 61% and 57% of bacterial isolates were resistant to Cefotaxime, tetracycline and amoxicillin, respectively**.** These results are in agreement with El-Zawahry *et al.,* (2013) who reported that the most effective antibiotic for clinical bacterial isolates was amikacin followed by nitrofurantoin, norfloxacin, streptomycin and ciprofloxacin with 80%, 76.25%, 71.25%, 70% and 60% susceptibility. Also, Wagih *et al.,* (2016) showed that the lowest resistance was recorded for amikacin. The resistances of all tested bacterial isolates for cephalosporin's groups was for cephalexin, while the resistances of the tested bacterial isolates to the aminoglycoside drugs, which included gentamicin and tobramycin were 69 and 54% respectively. In explanation, antibiotics have played important role to treat various bacterial infections since their time of introduction, but

wide use of antibiotics has led to development of resistant strains, which is becoming a global problem. There is requirement to find some alternatives to tackle the problem of emergence of drug resistant microorganisms (Rakholiya and Chanda, 2012).

In the present study, 28 multi-drug resistant bacterial isolates which resistant to more than 80% of the tested antibiotics were selected and preliminary identified according to the keys of Bergey's manual of Systematic Bacteriology (Holt *et al.,* 1994 and Vos *et al.,* 2009)**.** Selected isolates were divided into four groups: *P. aeruginosa, K. pneumoniae, E. coli* and *S. aureus***.** *S. aureus* found to be the most frequent pathogen within MDR bacterial isolates representing 39 % of MDR isolates followed by *P. aeruginosa, K. pneumoniae, E. coli* with percentage, 25 %, 18%, and 18%, respectively. These results are in agreement with Desouky *et al.,* (2014) who demonstrated that total of 200 clinical samples, 50 (25%) *Staphylococcus* strains were isolated and primarily defined as *Staphylococcus* spp., which reflects the importance of this type of microorganism that transmitted readily in hospital community and different medical tools. Moreover, Yasidi *et al.* (2015) demonstrated that the frequency of bacterial pathogens isolated, *S. aureus* accounted for 53% of the total pathogens isolated, followed by coliforms 22%, *P. aeruginosa,* 20%*, E. coli* 2% and *Klebsiella* spp.*, Proteus*

spp. and *Streptococcus* spp. accounted for 1% each respectively.

The virulence factors of pathogenic bacteria are divided into several groups on the basis of the mechanism. Virulence and function of the important ones are secretary proteins such as toxins and enzymes (Wu *et al.,* 2008). These virulence factors were considered as bacterial tools for cause pathogenicity and could be detected easily in laboratories (Lee *et al.,* 2012). Bacterial biofilm has long been considered as a virulence factor contributing to infection associated with various medical devices and causing nosocomial infection (Arciola *et al.,* 2001). In the current study, twenty-eight multi-drug resistant bacterial isolates were screened for their capability of biofilm, hemolysins, lecithinase and protease production. The results in Fig. 1 revealed that *S. aureus* and *P. aeruginosa* were highly producer for the tested virulence factors than *E. coli* and *K. pneumoniae*. Our results consistent to Bakir and Ali, (2016) who demonstrated that *S. epidermidis*, *S. aureus* and *P. aeruginosa* showed 100%, 94.3% and 72.7%, respectively for their biofilm production. In addition, Maroui *et al.,* (2016) reported that among 68 clinical isolates, 92.6% were β hemolysin producers, 100% for lipase, lecithinase production, and 98.53% for caseinase.

New molecular techniques, novel culture techniques have tried to sweep the basic methods of microbiology, but isolation culture methods remain used in diagnosis of infection (Dickson *et al.,* 2014). Identification of the selected multi-drug resistant bacteria was confirmed using 16S *rRNA* sequencing. They identified as *S. aureus* and *P. aeruginosa,* respectively and their partial nucleotide sequences of amplified genes were submitted in DDBJ/EMBL/GenBank at web server (http://www.ddbj.nig.ac.jp/sub/ref8-e.html) under accession number (s) LC218434 and LC218433, respectively. BLAST program (www.ncbi.nlm.gov/blast) for phylogenetic analysis was used to assess the DNA similarities of obtained 16S rDNA gene sequence. Multiple

sequence alignment and molecular phylogeny were performed using Bio Edit software. The phylogenetic tree was displayed using TREEVIEW program (Fig. 2). In addition to highly conserved primer binding sites, 16S *rRNA* sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification (Pereira *et al.,* 2010).

Herbal plants are frequently used in popular medicine as remedies for many infectious diseases (Plowman, 2000). In recent years, antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world (Al-Daihan *et al.,* 2013). In the current study, twenty-four methanolic and aqueous crude extracts derived from different parts of twelve medicinal plant species traditionally used in Egyptian folk medicine belonging to different genera and botanical families. Extracts of these plants were screened for their antibacterial activity against selected MDR bacterial strains using the disc diffusion method. The results in Table (3) showed that the methanol extract of *E. camaldulensis* leaf exhibited high antibacterial activity against tested MDR bacteria; meanwhile, *White turnip* and *Rucola arugula* seeds and leaves had no activities. Also, the results indicated that methanolic extracts of most samples showed the best antibacterial activities against the tested strains as compared with aqueous extract. In an explanation, Saleem *et al.,* (2010) reported that the tested methanolic plant extracts contain anthocyanins, tannins, polyphenols, terpenoids, saponins, xanthoxyllines, totarol, quassinoids, lactones, flavones, and phonons have antimicrobial activity than water extracts which contain only anthocyanins, starches, tannins, saponins, terpenoids, polypeptides, and lectins. Furthermore, Potdara *et al.,* (2015) reported that the *Eucalyptus* showed antimicrobial activity against *E. coli, S. aureus, P. vulgaris, S. typhi, P. aeruginosa, B. subtilis, K. pneumonia, S. paratyphi,* with zones of inhibition 19mm, 20mm, 18mm, 18mm, 22mm, 20mm, 19mm, 19mm, respectively (Babayi *et al.,* 2004).
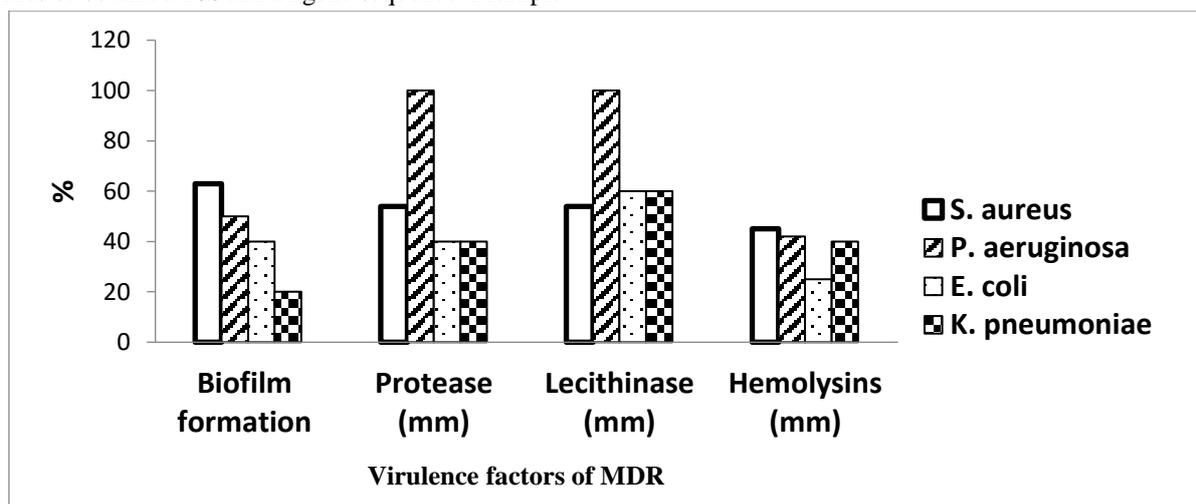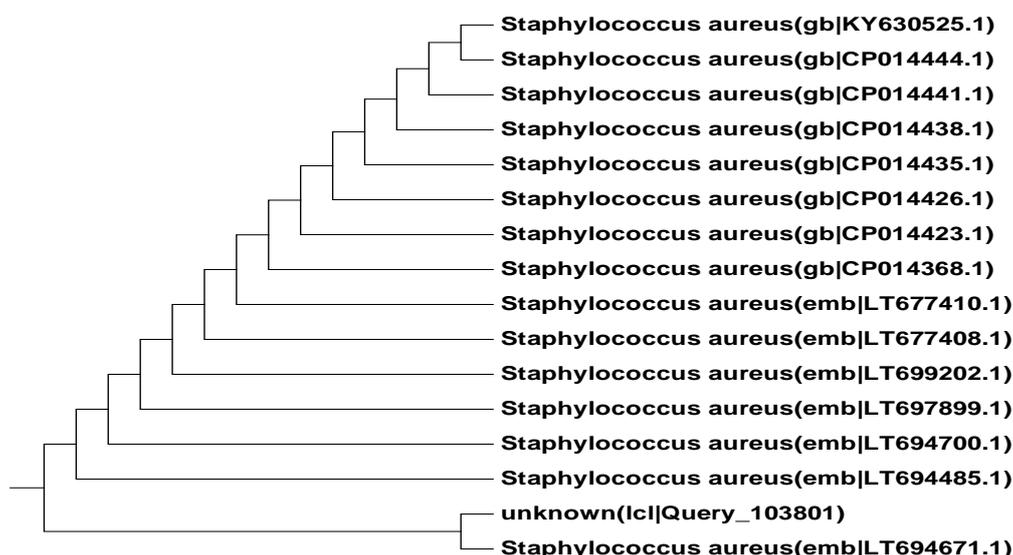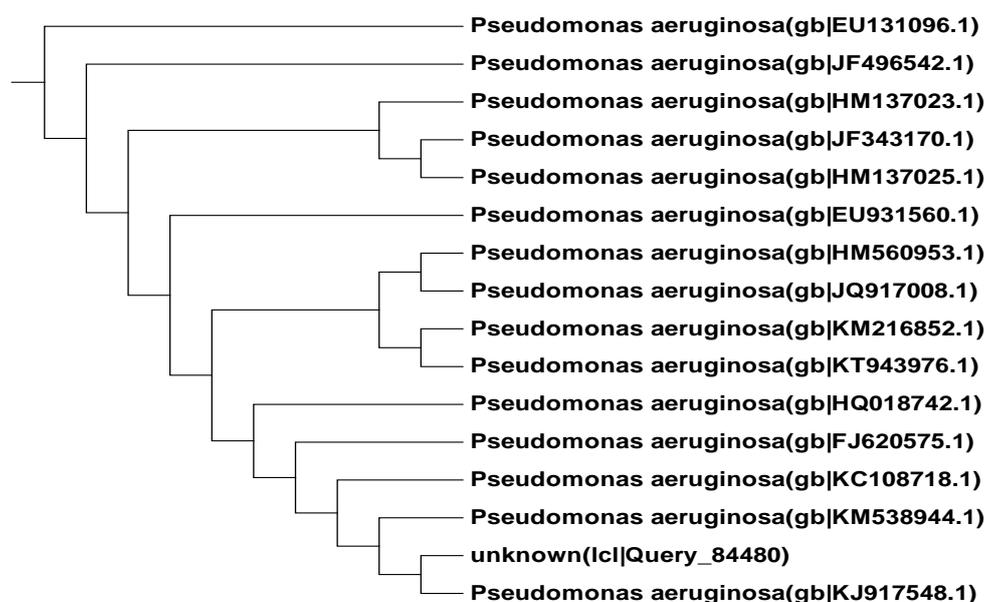

Fig 1: Distribution of virulence factors of different selected MDR pathogenic bacterial groups.

Staphylococcus aureus(gb|KY630525.1)
Staphylococcus aureus(gb|CP014444.1)
Staphylococcus aureus(gb|CP014441.1)
Staphylococcus aureus(gb|CP014438.1)
Staphylococcus aureus(gb|CP014435.1)
Staphylococcus aureus(gb|CP014426.1)
Staphylococcus aureus(gb|CP014423.1)
Staphylococcus aureus(gb|CP014368.1)
Staphylococcus aureus(emb|LT677410.1)
Staphylococcus aureus(emb|LT677408.1)
Staphylococcus aureus(emb|LT699202.1)
Staphylococcus aureus(emb|LT697899.1)
Staphylococcus aureus(emb|LT694700.1)
Staphylococcus aureus(emb|LT694485.1)
unknown(lcl|Query_103801)
Staphylococcus aureus(emb|LT694671.1)

(A)

Pseudomonas aeruginosa(gb|EU131096.1)
Pseudomonas aeruginosa(gb|JF496542.1)
Pseudomonas aeruginosa(gb|HM137023.1)
Pseudomonas aeruginosa(gb|JF343170.1)
Pseudomonas aeruginosa(gb|HM137025.1)
Pseudomonas aeruginosa(gb|EU931560.1)
Pseudomonas aeruginosa(gb|HM560953.1)
Pseudomonas aeruginosa(gb|JQ917008.1)
Pseudomonas aeruginosa(gb|KM216852.1)
Pseudomonas aeruginosa(gb|KT943976.1)
Pseudomonas aeruginosa(gb|HQ018742.1)
Pseudomonas aeruginosa(gb|FJ620575.1)
Pseudomonas aeruginosa(gb|KC108718.1)
Pseudomonas aeruginosa(gb|KM538944.1)
unknown(lcl|Query_84480)
Pseudomonas aeruginosa(gb|KJ917548.1)

(B)

Fig 2: The phylogenetic tree of selected multi-drug resistant strains (A) *S. aureus* AM23 and (B) *P. aeruginosa* AM41.

Combination therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity and to obtain synergistic antimicrobial activity; it could be an alternative to monotherapy for patients with invasive infections that are difficult to treat, such as those due to multi-resistant species and for those who fail to respond to standard treatment. Plants antimicrobials have been found to be synergistic enhancers in that though they may not have any antimicrobial properties alone, but when they are taken concurrently with standard drugs they enhance the effect of that drug (Kamatou *et al.,* 2006). In the present study, the antibacterial activity of combination between selected antibiotics and *E. camaldulensis* extract showed high efficacy against the tested MDR clinical strains than each of them alone (synergistic effect), (Fig.3). The *E. camaldulensis* extract showed the best synergism with ceftriaxone (22mm) and (20mm), against *S. aureus* AM23 and *P. aeruginosa* AM41, respectively. 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 (Garvey *et al.,* 2011). Moreover, Kaur *et al.,* (2016) reported that natural substances can produce synergistic effects, 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 (Adwan *et al.,*2008).

Table 3: Antibacterial activity of methanolic plant extracts and aqueous plant extracts on the selected strains.

Family	plant extract (Scientific name	plant extract (English name)	Part used	Methanolic extract inhibition zone (mm)		Aqueous extract inhibition zone (mm)	
				S. aureus AM23	*P. aeruginosa* AM41	*S. aureus* AM23	*P. aeruginosa* AM41
Apiaceae (Umbelliferea)	*Anethum graveolens*	Dill	Seeds	6	4	5	0
	Apium graveolens	Celery	Seeds	12	9	10	6
Apocynaceae	*Nerium oleander*	Oleander	Leaves	10	7	9	7
Asteraceae Compositae	*Conyza canadensis*	Horseweed	Leaves	13	9	10	8
Brassicaceae (Cruciferae)	*Anastatica hierochuntica*	Rose of Jericho	Leaves	14	11	11	9
	Brassica rapa var rapa	White turnip	Seeds	0	0	0	0
	Eruca sativa	Rucola arugula	Seeds	0	0	0	0
	Eruca sativa	Rucola arugula	Leaves	0	0	0	0
Convolvulaceae	*Convolvulus arvensis*	Field bindweed	Aerial parts	8	6	4	0
Euphorbiaceae	*Ricinus communis*	Castor oil plant	Seeds	13	9	12	10
Fabaceae (Leguminosa)	*Cassia fistula*	golden shower tree	Pods	12	10	9	6
Myrtaceae	*Eucalyptus camaldulensis*	Eucalyptus camaldulensis	Leaves	18	16	12	14

Fig 3: Influence of combination between selected antibiotics and methanolic extract of *E. camaldulensis* leaf against MDR bacteria (CRO= Ceftriaxone (30 μg/disc); CN= Gentamicin (10 μg/disc); EU= E. *camaldulensis extract* only. Each antibiotic disk loaded with 10μl of plant extract).

The MICs of two antibiotics (gentamycin and ceftriaxone) and the most effective extract of *E. camaldulensis* were determined against the tested MDR strains. The results in Table (4) showed that the highest MIC was observed in Ceftriaxone against *P. aeruginosa* AM41 (1225 µg/ml), meanwhile, the lowest MICs were observed in plant extract of *E. camaldulensis* for both tested strains. Also, the results demonstrated that *S. aureus* AM23 was more sensitive against positive tested antibiotics and *E. camaldulensis* extract than *P. aeruginosa* AM41. This observation also, 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. These results agree with George *et al.,* (2002) who observed differences to be due to the fact that while synthetic antibiotics are in a pure form, crude plant extracts contains some impure substances that may be inert and do not have any antibacterial activities. In this connection, Jouki and Khazaei, (2010) reported that the MIC of *E. camaldulensis* extract was 1.25 mg/ml for *S. aureus* and 5mg /ml for *B. subtilis*, while *E. coli* was resistant to all the concentrations of plant extract. Furthermore, Potdara *et al.,* (2015) demonstrated that the MIC values of the *E. camaldulensi*s extract were 0.5mg/ml for *S. aureus,* 10mg/ml for *P. aeruginosa* and 75mg/ml for *E. coli.*

Table 4: Minimum Inhibitory Concentrations (MICs) of selected antibiotics and methanolic extract of *E. camaldulensis* leaf against selected multi-drug resistance strains.

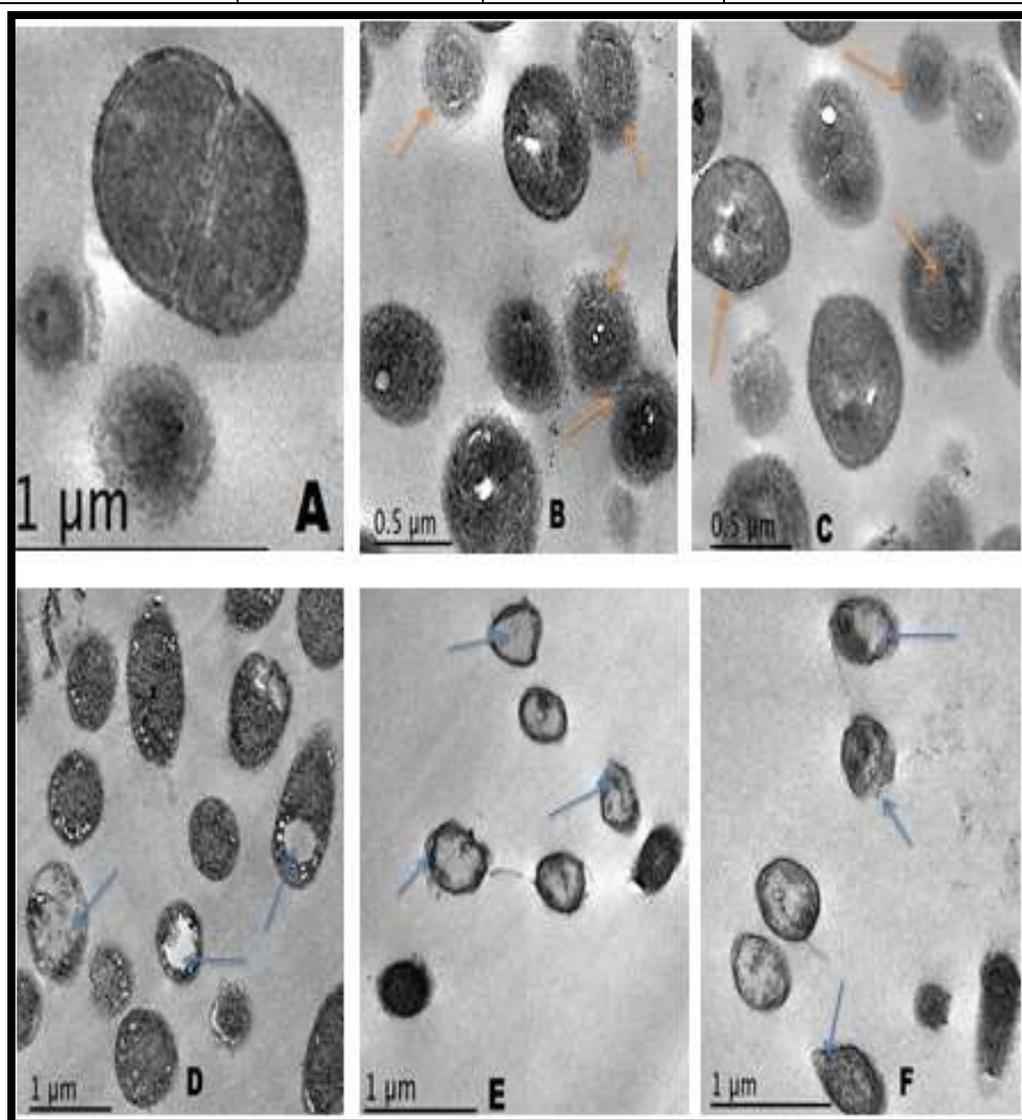| Bacterial strain	Ceftriaxone (µg/ml)	Gentamicin (µg/ml)	*E. camaldulensis* (mg/ml)
S. aureus AM23	625	125	0.78
P. aeruginosa AM41	1225	1000	3.12

Fig 4: TEM photograph showing mode of action of Ceftriaxone and *E. camaldulensis* leaf extract against *S. aureus* AM23; (A) control cell without treatment, (B) and (C) treated cells with ceftriaxone; (D), (E) and (F) treated cells with *E. camaldulensis* leaf extract.

Transmission electron microscopy of untreated *S. aureus* AM23 and *P. aeruginosa* AM41and treated cells (1/2 MIC of CRO and methanolic extract of *E. camaldulensis* leaf) was tested. It was observed that treatments of *S. aureus* AM23 cells with CRO induced cell wall lyses and formed protoplast, (Fig. 4B & C). Meanwhile, the cells treated with plant extract was induced cells with large vacuoles, numerous bright holes around cytoplasmic membrane and vacuoles, presence of completely lysed cell content enclosed with thick envelop and formation of reduced of *S. aureus* AM23cells comparing to control, (Fig. 4D & F). Also, treatment of *P. aeruginosa* AM41with 1/2 MIC of CRO showed that formation of large central and peripheral vacuoles in beside to cell wall lyses, (Fig. 5B & C). On the other hand, the cells treated with tested plant extract observed that formation of filament cells with partially shrunked cytoplasm, increase the presence of peripheral bright holes around the cytoplasmic membrane, change in cell ends and formation of pointed pool and apparent of lysed cell content (Fig. 5D & F), comparing to control cell. Completely changing the shape of bacterial cell and partially distribution and rough of cell wall. Our findings proved that methanolic extract of *E. camaldulensis* leaf have potential antimicrobial activity against tested strains than tested antibiotic. These results are in line with Stern *et*

al., (1996) reveled the antimicrobial activity of the extracts could be explained by the presence of tannins. The mechanism of action of tannins is based on their ability to bind proteins thereby inhibiting cell protein synthesis. Moreover, the mechanism of action for the antimicrobial activity of natural plants is not fully understood, however, membrane disruption by terpenoids and phenolics; metal chelation by phenols and flavonoids; and effect on genetic material by alkaloids are thought to inhibit growth of microorganisms (Cowan, 1999). In explanation, 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).Williamson (2001) observed there was no significant difference in the antimicrobial activity of the extracts on Gram-negative and Gram positive bacteria despite the differences in their cell wall components. It is thought that phenolic compounds such as flavonoids may increase the biological activity of other compounds by synergistic or other mechanisms.
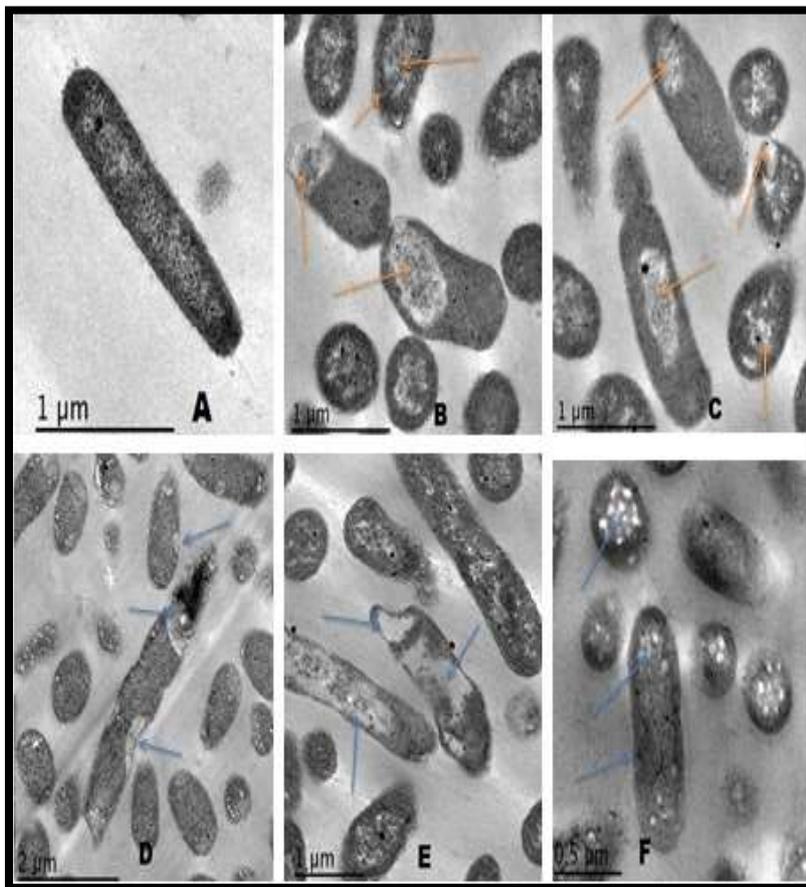

Fig 5: TEM photograph showing mode of action of *E. camaldulensis* leaf extract against *P. aeruginosa* AM41*;*(A) control cell without treatment, (B) and (C) treated cells with ceftriaxone; (D), (E) and (F)treated cells with *E. camaldulensis* leaf extract.

Conclusion

This study proved the importance of plant extracts to inhibit the growth of microorganisms and it indicate that plant extract may be new source of alternatives to conventional antibiotics. The results have shown that the leaf extracts of *E. camaldulensis* had great potential as antimicrobial agents in the treatment of infectious organisms. Further detailed investigation of the active components of the plant for the exact mechanism of action will contribute greatly to the development new pharmaceuticals. Combination of plant extract with antibiotics leads to new choice for treatment of infectious diseases and plant extracts may be act as active modified agent for antibiotics.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Reference

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