

Research Article

Antiplasmid Potential of *Kalanchoe blossfeldiana* Against Multidrug Resistance *Pseudomonas aeruginosa*

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Abstract: This study concerned with the isolation of *Pseudomonas aeruginosa* from various clinical cases in human which include (burn, wound and urine) that admitted to Emergency hospital and internal lab of teaching hospital in Erbil city. Forty isolates of *P. aeruginosa* from out of 120 samples were identified by using cultured, morphological and biochemical tests in addition to vitek machine. According to the resistance of the isolates to these antibiotics they showed variation in their resistance; the highest resistance was for penicillin G with 85% while the lowest resistance was for ceftizoxime with 40% and for others ranged between 42.5-82.5%. On the other hand the isolates P9, P16 and P30 were resist to all antibiotics under study. RCR was used to detect Exotoxin A (ETA) structural gene sequence. The result revealed that 82.5% of the isolates were positive for ETA gene. Methanolic and aqueous crude extract of *Kalanchoe blossfeldiana* were used as medicinal plant for curing agent for the reduction of antibiotic resistance genes of *P. aeruginosa* isolate P9, this was done through determination of Minimum Inhibitory Concentration (MIC) of this medicinal plant that used as curing agent which was 1800 µg/mL for aqueous extract and 1400 µg/mL for methanolic extract. The effect of MIC of methanolic extract on antibiotic resistance genes for isolate P9 showed reduction of the resistance from 44-100% while the effect of MIC of aqueous extract on antibiotic resistance genes for isolate P9 revealed decreasing of the resistance from 36-86%, respectively.

Keywords: Antibiotic resistance, *Kalanchoe blossfeldiana*, plasmid curing, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen capable of infecting virtually all tissues. *P. aeruginosa* infections in hospitals mainly affect the patients in intensive care units and those having catheterization, burn and/or chronic illnesses (Brook *et al.*, 2007).

P. aeruginosa has a wide arsenal of virulence factors at its disposal. Among these virulence factors are a variety of secreted factors, such as proteases, phospholipases and the exotoxin A. *P. aeruginosa* strains also possess a type III secretion system that allows them to deliver toxins (effectors) directly into the cytoplasm of a host cell, exotoxin A causes tissue necrosis since it blocks protein synthesis (Rietisch *et al.*, 2005).

Exotoxin A (ExoA, *toxA*) is a 66 kDa protein acts as a major virulence factor of *P. aeruginosa*, analogous in action to that of diphtheria toxin. ExoA is a highly virulent protein, exhibiting and LD50 of 2.5 mg/kg in mice, it has been shown that $\Delta toxA$ mutants are less virulent than wild type strains and that vaccination against ExoA confers partial immunity to *P. aeruginosa* infection in animals (Engel, 2003). The highly toxic ETA is produced by the majority of *P. aeruginosa*

strains and can inhibit eukaryotic protein biosynthesis at the level of polypeptide chain elongation factor 2, similarly to diphtheria toxin (Wolfgang *et al.*, 2003).

In recent times, there have been increases in antibiotic resistant strains of clinically important *P. aeruginosa*, which have led to the emergence of new bacterial strains that are multi-resistant (Aibinu *et al.*, 2004). The non-availability and high cost of new generation antibiotics with limited effective span have resulted in increase in morbidity and mortality (Williams, 2000). Therefore, there is a need to look for substances from other sources with proven antimicrobial activity. Consequently, this has led to the search for more effective antimicrobial agents among materials of plant origin, with the aim of discovering potentially useful active ingredients that can serve as source and template for the synthesis of new antimicrobial drugs (Moreillion *et al.*, 2005).

Kalanchoe sp., (Family: Crassulaceae) is an erect, succulent, perennial shrub that grows about 1.5 m tall and reproduces through seeds and also vegetatively from leaf bubils. It has a tall hollow stems, freshly dark green leaves that are distinctively scalloped and trimmed in red and dark bell-like pendulous flowers. The plant is considered a sedative wound-healer, diuretic and cough suppressant. The plant is also

employed for the treatment of kidney stones, gastric ulcer and edema of legs (Okwu and Nnamdi, 2011). The plant, *Kalanchoe* is also widely used in ayurvedic system of medicine as astringent, analgesic, carminative and also useful in nausea and vomiting (Majaz *et al.*, 2011). It was found that this plant showed various pharmacological activities such as anthelmintic, immunosuppressive, wound healing, hepatoprotective, antinociceptive, anti-inflammatory and antidiabetic, nephroprotective, antioxidant activity, antimicrobial activity, analgesic, anticonvulsant, neuropharmacological and antipyretic. The main plant chemicals found in *Kalanchoe* alkaloids, triterpenes, glycosides, flavonoids, cardenolides, steroids, bufadienolides and lipids include: arachidic acid, astragalol, behenic acid, beta amyrin, benzenoids, beta-sitosterol, bryophollone, bryophollone, bryophyllin, bryophyllin A-C, bryophyllol, bryophynol, bryotoxin C, bufadienolides, caffeic acid, campesterol, cardenolides, cinnamic acid, clerosterol, clionasterol, codisterol, coumaric acid, epigallocatechin, ferulic acid, flavonoids, friedelin, glutinol, hentriacontane, isofucosterol, kaempferol, oxalic acid, oxaloacetate, palmitic acid, patuletin, pesterol, phosphoenolpyruvate, protocatechuic acid, pseudotaraxasterol, pyruvate, quercetin, steroids, stigmasterol, succinic acid, syringic acid, taraxerol and triacontane (Harlalka *et al.*, 2007). On the basis of this background, *in-vitro* antimicrobial activities of the extracts of *Kalanchoe* from two solvents were tested against clinically important *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial isolation and identification: Forty isolates of *P. aeruginosa* were identified among 120 sample were collected from clinical specimens including burn, wound and urine from Emergency hospitals and internal lab of teaching hospital in Erbil city during the period from march to the end of June 2013. All isolates were identified depending on morphological, cultural and biochemical tests in addition to vitek machine.

Antibiotic susceptibility test: Muller hinton agar was used as growth media to study the effect of different antimicrobial which include Amoxicillin, Amoxicillin/clavulanic acid, Ampicillin+cloxacillin, Cefotaxime, Cefoperazone, Ceftizoxime, Ceftriaxone, Cloxacillin, Penicillin G and Streptomycin on *P. aeruginosa* isolates, after sterilization and cooling at 45°C, final concentration of antibiotics were added to media and poured into sterile petri dishes. After solidification the plates were inoculated by streaking method with *P. aeruginosa* isolates then incubated at 37°C for 24 h. The results were recorded next day (Jerma *et al.*, 2005).

Isolation of plasmid DNA content from bacterial isolates under study:

Quantum prep plasmid miniprep kit (fermentes): The steps of plasmid DNA isolation are the following.

An overnight culture was transferred to a micro test tube centrifuged at 8000 round/min (rpm) for (30 sec). Supernatant was removed. Two hundred and fifty microlitre of re-suspension solution vortexed until the cell pellet is completely re-suspended. Two hundred and fifty microlitre lyses solution was added and mixed by inverting 10 times. Three hundred microlitre neutralization solution was added. Mixed by inverting 10 times then centrifuged for 5 min. A spin column inserted into a 2 mL wash tube. The supernatant transferred to a spin column, centrifuged at 8000 rpm for 1 min to pull fluid through the column. The spin column was removed, the filtrate discarded and the column replaced. Five hundred microlitre wash buffer added, centrifuge 2 min followed by a 2 min spin to remove all wash buffers. The spin column removed to a clean 1.5 mL tube, 100 µL sterilized H₂O was added. Centrifuged for 1 min.

Agarose electrophoresis technique (Sambrook *et al.*, 2006):

Preparation of 1% agarose gel: The gel (1%) was prepared by dissolving 1 g of agarose powder in 100 mL of 0.5 X TBE (Tris base ethidium bromide) buffer, boiled until all agarose was dissolved and left to cool at 50°C, 8 µL of ethidium bromide was added, the gel was poured in to the glass plate that contained appropriate comb, the gel was left to solidify and the comb was removed gently, the gel was soaked in a gel tank containing TBE buffer should cover the surface of the gel.

Sample loading: Ten microlitre of plasmid DNA samples were mixed with 5 µL of loading buffer and the mixture was slowly loaded in to the wells on the gel, also a molecular weight marker was loaded as control.

Running the electrophoresis: The electrophoresis apparatus was joined to power supply, turned on and the samples electrophoresed at 10 volt/cm for 1 h. The gel was visualized by UV-transilluminator and then photographed.

Genomic DNA purification protocol for gram negative bacteria: DNA was extracted from *P. aeruginosa* isolates and used for detection of *ETA* gene by PCR technique.

Three to five milliliter overnight culture in a 1.5 or 2 mL micro centrifuge tube harvested by centrifugation for 10 min at 10000 rpm, the supernatant discarded. The pellet resuspended in 180 µL of digestion solution, 20 µL of proteinase k solution was added and mixed thoroughly by vortexing or pipetting to obtain a uniform suspension. The sample incubated at 56°C for ~ 30 min in shaking water bath, until the cells are completely lysed. Twenty microlitre of RNase solution was added then mixed by vortexing and the mixture

incubated for 10 min at room temperature. Two-hundred microlitre of lyses solution was added to the sample, mixed thoroughly by vortexing for about 15 sec until homogeneous mixture was obtained. Four-hundred μL of 50% ethanol was added and mixed by pipetting or vortexing. The prepared lysate transferred to Gene JET™ genomic DNA purification column and inserted in a collection tube. The column centrifuged for 1 min at 6,000 rpm. The collection tube then discharged containing the flow-through solution. The Gene JET™ genomic DNA purification column placed into a new 2 mL collection tube. Five hundred microlitre of wash buffer I (with ethanol added) was added. Then centrifuged for 1 min at 8,000 rpm. The flow-through discarded and the purification column placed back into the collection tube. Five hundred microlitre of wash buffer II (with ethanol added) was added to the Gene JET™ genomic DNA purification column. Centrifuged for 3 min at maximum speed ($\geq 12,000$ rpm). Two-hundred μL of elution buffer was added to the center of the Gene JET™ genomic DNA purification column membrane to elute genomic DNA. Incubated for 2 min at room temperature and centrifuged for 1 min at 8000 rpm. The purified DNA immediately discarded in downstream applications or stored at -20°C .

PCR master kit: The reagent of master mix is an optimized ready to use 2 \times PCR mixtures of Taq DNA polymerase, PCR buffer, MgCl_2 and dNTPs. Master Mix contains all components for PCR, except DNA template and primer.

Protocol of PCR technique: DNA extract was used as a template in the PCR technique. PCR was performed in a 25 μL of reaction volume. Master Mix 12.5 μL , forward Primer 1 μL , reverse Primer 1 μL , template DNA 1 μL , sterile deionized water 9.5 μL .

PCR technique procedure: PCR was used to detect the *ETA* gene with amplicon size 363 bp in the genomes of the *P. aeruginosa* isolates. The *ETA* primers used were forward 5'-ACGCTCGACAATGCTCTCTC-3' and reverse: 5'-TGTCCTGGCGACTATCGAG-3'. The PCR cycles were: denaturation at 94°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min, repeated 35 times (Ahmed, 2013).

Detection of PCR product: The amplified product were visualized by ethidium bromide staining after gel electrophoresis of 7 μL of the final reaction mixture in 1.2% agarose. One hundred bp DNA ladder (Gene dire) was used as molecular markers (Sambrook and Russell, 2001).

Selection of medicinal plant: *Kalanchoe blossfeldiana* plant was obtained from local market in Erbil city, Iraq and the plant was classified in the Education Salahaddin University Herbarium (ESUH). The aerial part of the plant (stem and leaves) washed with tap water after

drying at 37°C for 24 h the plant were ground in grinding machine.

Preparation of watery crude extract: Fifty gram of the powdered plant material were put in conical flask then 250 mL of double D.W. was added to the flask and placed on magnetic stirrer, left to mix by magnetic bar at room temperature. After 72 h the solution was filtered by muslin cloth, then by filter paper, the above step were repeated 3-5 times to residue, until a clear colorless supernatant liquid was obtained indicating that no more extraction from the plant material was possible. The extracted liquid was subjected to Rota-evaporation to remove the water and the temperature adjusted at 55°C (Salah, 2007).

Preparation of methanolic crude extract: The extract was prepared using absolute methanol inspite of double distal water.

Determination of Minimum Inhibitory Concentration (MIC) of plant extract: The MIC of plant extract was determined by turbidity method (spectrophotometer method) at 600 nm and the following dilutions were prepared (200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and 2000 $\mu\text{g}/\text{mL}$, respectively). The MIC was used as a curing agent.

The MIC was determined for plant extract which inhibited bacterial growth, contrasted with control sample that consisted of 10 mL of nutrient broth and 0.1 mL of activated culture of bacterial suspension and then it was incubated at 37°C for 24 h (Crucishank *et al.*, 1975).

Plasmid curing: MIC of plant extract (methanol and water extract separately) and 0.1 mL of overnight bacterial suspension were added to 10 mL nutrient broth then incubated at 37°C for 24 h. Next day 0.1 mL of it was spreaded on nutrient agar plate and incubated for 24 h at 37°C , then 50 colonies transferred to antibiotic agar plate, after incubation for 24 h at 37°C the viable colonies were registered, then percent of cured colonies were calculated (Salah, 2007).

Chemical detection methods:

Alkaloids detection: The method followed was described by Hasan (2001). Ten milliliter of plant extract acidified with HCl was taken, then tested with picric acid: yellow participate refers to alkaloids.

Glycosides detection: Two parts of Fahlang's reagent was mixed with plant extract, left in boiling water bath for 10 min, red color means presence of glycosides (Hasan, 2001).

Flavonoids detection: Ten milliliter of 50% ethanol was added to 10 mL 50% KOH then this solution was mixed with equal volume of plant extract. Yellow color refers to positive result (Jaffer *et al.*, 1983).

Tannins detection: Ten milliliter from plant extract divided into two equal parts, then drops of 1% CH₃COOPb was added to the first part, the appearance of white pellet means positive result. To second part drops of 1% FeCl₃ was added, formation of green bluish color means positive result (Hasan, 2001).

Saponin detection: Five milliliter of plant extract was extremely shaken for half minute, then left in vertical case for 15 min, appearing of foam means presence of saponin (Hasan, 2001).

Resins detection: Ten milliliter of acidify D.W. with HCl was added to 10 mL of plant extract, if turbidity appears means positive reaction (Hasan, 2001).

Phenols detection: Three milliliter of plant extract was added to 2 mL of potassium hexacyanofrate and 2 mL of FeCl₃, the green bluish color means positive result (Harborne, 1984).

RESULTS AND DISCUSSION

Isolation and identification of *P. aeruginosa*: A total of 120 specimens were collected from patient attending Emergency hospital and internal lab of teaching hospital in Erbil city. Results showed only 40 isolates identified as *P. aeruginosa* from 120 sample representing 33.3% of the total (18 from burn, 15 from wound and 7 from urine). All the isolates were Gram negative rod shaped bacteria, non-spore former, colonies on 5% sheep blood agar were typically yellow-green and β-haemolytic. All isolates were positive for oxidative, catalase, simmon citrate and motility test, but negative for methyl red, Vogus-ProskauEr and Indol test (Raouf *et al.*, 2010).

Using vitek 2 systems showed that all isolates were belonging to one biotype according to 64 tests present in the vitek 2 system. This system selected the isolates to the 99% as *P. aeruginosa* (Harley, 2002).

These results were in agreement with those obtained from other studies which showed that *P. aeruginosa* has a highest percentage among burn infection followed by wound (Ahmed, 2013). Also in agreement with study of Al-Amir (1998) who reported that *P. aeruginosa* represent high percentage in burn. Our results also close to those reported by Sulaiman (2013) who showed that the highest percentage of

P. aeruginosa occur among burn infection. The burn wound is major site for infection because of loss of skin barrier and destroy of normal flora, presence of dead tissue due to impaired local blood flow and a general state of immunosuppression is caused by impaired functioning of neutrophil, cellular and humoral immune system. In these conditions, microorganism can easily multiply and colonize wounds (Bollero *et al.*, 2002).

Antibiotic resistance: All forty isolates of *P. aeruginosa* were exposed to ten different types of antibiotics. Results in Table 1 revealed the resistance pattern of *P. aeruginosa* isolates in which (85%) of the isolates were resistance for penicillin G, (82.5%) were resistance for Ampicillin-cloxacillin and Amoxicillin, while resistance for Amoxicillin-clavulanic acid, Cloxacillin, Cefoperazone, Streptomycin, Ceftriaxone, Cefotaxime and Cefizoxime were (77.5, 77.5, 70, 50, 52.5, 52.2, 42.5 and 40%, respectively). On other hand three isolates designated as P9, P16 and P30 were resistance to all antibiotics under study with 100%. Similar results obtained by Ahmed (2013) who reported a highest resistance to penicillin G with 100%. Sulaiman (2013) pointed out among 100 isolates of *P. aeruginosa* 81% were resistance to Amoxicillin, 95% to ampicillin, 62% to cefotaxime, 73% to ceftriaxone and 76% to streptomycin. Othman (2011) revealed that more than 50 isolates of *P. aeruginosa* among different clinical specimen 98% were resistance to Amikacin 70% to ampicillin, 70% to Augment and 60% to Doxycycline respectively. This resistance could result from a complex interaction between several mechanisms which tend to inactivate the antibiotics or prevent their intracellular accumulation to inhibitory levels (Hancock and Spreet, 2000). The major mechanism of resistance to β-lactam antibiotics including penicillin is related to β-lactam production. Both plasmid mediated and chromosomally mediated β-lactamase production can occur, which is responsible for resistance to penicillin (Carsenti-Etesse *et al.*, 2001). Vedel (2005) stated that *P. aeruginosa* produces an inducible cephalosporinase (class C enzyme), has low outer-membrane permeability and constitutive expression of Efflux pump transporters, which naturally resists aminopenicillins, first and second-generation of cephalosporins and some third-generation of

Table1: Resistance of *P. aeruginosa* to antibiotics

| Antibiotics | Abbreviation | No. of resistance isolates | Resistance (%) |
|----------------------------|--------------|----------------------------|----------------|
| Amoxicillin | AM | 33 | 82.5 |
| Amoxicillinclavulanic acid | ACL | 33 | 82.5 |
| Ampicillin+cloxacillin | AML | 33 | 82.5 |
| Cefotaxime | CTX | 17 | 42.5 |
| Cefoperazone | CPZ | 28 | 70.0 |
| Ceftizoxime | CAZ | 16 | 40.0 |
| Ceftriaxone | CRO | 21 | 52.5 |
| Cloxacillin | CLX | 31 | 77.5 |
| Penicillin G | PG | 34 | 85.0 |
| Streptomycin | S | 20 | 50.0 |

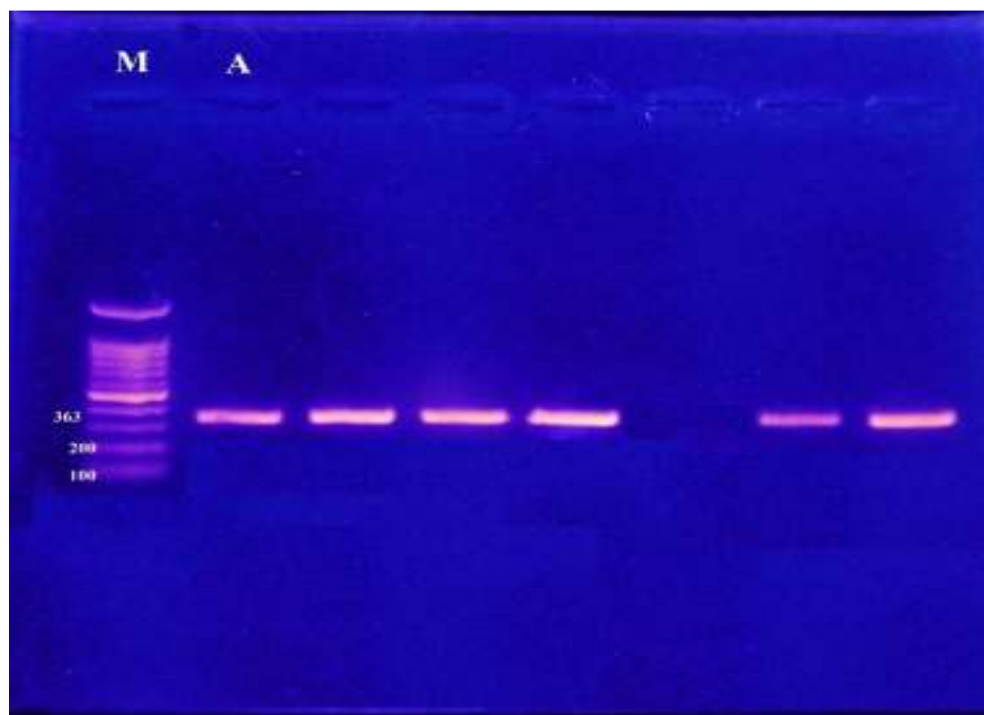


Fig. 1: PCR product of ETA gene for *P. aeruginosa* (M: Ladder; A: Positive control)

cephalosporins such as cefotaxime and show that certain wild-type strains of *P. aeruginosa* may acquire resistance to β -lactams during treatments, which firstly it was knowingly sensitive to these antibiotics. Also Henrichfreise *et al.* (2007) reported among 22 multi-resistant isolates of *P. aeruginosa*, intrinsic and acquired antibiotic resistance makes *P. aeruginosa* one of the most difficult organisms to treat, because of the high intrinsic antibiotic resistance of *P. aeruginosa* which is due to several mechanisms including a low outer-membrane permeability, the production of an AmpC β -lactamase and the presence of numerous genes coding for different multidrug resistance efflux pump.

Detection of exotoxin A gene in *P. aeruginosa*: In this study PCR assay were performed for the molecular detection of exotoxin A gene in 40 isolates of *P. aeruginosa* recovered from clinical sources. Results showed that 33 (82.5%) of the isolates harbored exotoxin a gene as in Fig. 1. Similar results obtained by Nikbin *et al.* (2012) that detected ETA gene with 90.6% among 268 clinical samples. In another study the ETA gene was detected in 25(55%) *P. aeruginosa* isolates from 48 isolates collected from different clinical sample (Ahmed, 2013). Al-Daraghi and Abdullah (2013) showed that 50 (100%) of the *P. aeruginosa* were positive by PCR for ETA gene. Similarly, Bahaael-Din *et al.* (2008) demonstrate that out 47 *P. aeruginosa* isolates ETA gene was found in 42 (89.4%). They found there was relation between appearance of bacteremia and positive blood culters and production of

ETA; this gives good idea about the virulence power of ETA that help the bacteria to invade deeper in the tissues and reaching blood stream.

Khan and Cerniglia (1994) also developed a PCR procedure to detect *P. aeruginosa* by amplifying the ETA gene. They reported that of 136 tested *P. aeruginosa* isolates, 125 (96%) contained the ETA gene. Studies that compare the virulence of ETA producing strains of *P. aeruginosa* to mutant strains that do not produce such toxin suggest that ETA is an important virulence factor (Fogle *et al.*, 1994). ETA mediates equally, local and systematic disease processes caused by *P. aeruginosa* and its cecrotizing allows for successful bacterial colonization and that purified ETA is extremely poisonous to animals including primates (Hamood *et al.*, 2004).

Curing of plasmid DNA in *P. aeruginosa* by *K. blossfeldiana* plant extract: The prevalence of resistant bacteria is significant and deserves more consideration. To overcome this constrain now we are taking shelter to our ancestor's medicinal practice. Reviewed studies stated that enormous work has done to screen the antibacterial activity of medicinal plants against human pathogen for this reason the antibacterial efficacy of *K. blossfeldiana* were tested and showed varied level of removing the antibiotic resistance gene in *P. aeruginosa*. Minimum Inhibitory Concentration (MIC) of aqueous and methanolic extract of *K. blossfeldiana* was estimated through the optical density reading at 600 nm by uv/light

Table 2: The MIC of aqueous and methanolic *K. blossfeldiana* extracts on P9 isolate

| Plant extract type | Concentration of <i>k. blossfeldiana</i> (µg/mL) | | | | | | | | | |
|--------------------|--|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| | 200 | 400 | 600 | 800 | 1000 | 1200 | 1400 | 1600 | 1800 | 2000 |
| Aqueous extracts | 1.053 | 1.038 | 0.940 | 0.915 | 0.890 | 0.870 | 0.726 | 0.377 | 0.298 | 0.000 |
| Methanolic extract | 1.001 | 0.913 | 0.863 | 0.751 | 0.345 | 0.188 | 0.180 | 0.000 | 0.000 | 0.000 |

Table 3: Phytochemical screening of kalanchoe extract by methanol and water

| Plant extract type | Compound detected | | | | | | |
|--------------------|-------------------|------------|------------|-------|---------|-------|--------|
| | Alkaloids | Glycosides | Flavonoids | Tanin | Saponin | Resin | Phenol |
| Aqueous extract | + | + | - | + | + | + | - |
| Methanolic extract | + | + | + | + | + | + | + |

-: Absent; +: Present

Table 4: Effect of MIC of aqueous and ethanolic extract on *P. aeruginosa* P9 resistance genes

| Antibiotics | Methanolic extract 1400 µg/mL | | Aqueous extract 1800 µg/mL | |
|-------------|-------------------------------|---------------------------|----------------------------|---------------------------|
| | Colonies growth (%) | Decreasing resistance (%) | Colonies growth (%) | Decreasing resistance (%) |
| *CAZ | 0 | 100 | 7 | 86 |
| CTX | 0 | 100 | 7 | 86 |
| PG | 4 | 92 | 8 | 72 |
| CPZ | 0 | 100 | 7 | 86 |
| ACL | 5 | 90 | 18 | 64 |
| CLX | 22 | 44 | 32 | 36 |
| ALX | 0 | 100 | 7 | 86 |
| AM | 3 | 94 | 10 | 80 |
| CRO | 0 | 100 | 7 | 86 |
| ST | 6 | 88 | 21 | 58 |

*: Abbreviations are given in Table 1

spectrophotometer, which is clarified in Table 2 and shows that the MIC of aqueous and methanolic extracts of kalanchoe were 1800 and 1400 µg/mL with spectrophotometric reading was 0.298 and 0.180, respectively. The MIC of both extracts was used as curing agent by transferring method for isolate P9.

A results in the Table 2 also indicates that the methanolic extracts were more active for inhibition of bacterial growth than the aqueous extract. Rashmi *et al.* (2012) found that the methanolic extract of *Kalanchoe pinnate* had a wide range of activity on pathogen than other extract like chloroform, petroleum, ether, acetone and ethylacetate.

Table 3 recognizes the phytochemical screening of kalanchoe that indicate the absence of both flavonoid and phenol in aqueous extract and presence of all compound detected in methanolic extract.

MIC of *K. blossfeldiana* methanolic extract was used against *P. aeruginosa* P9 (which is resistance to all antibiotics) as curing agent. The results revealed as in Table 4 that the methanolic extract affected the resistance genes of P9 isolate 100% for cefotaxime, ceftriaxone, cefoperazone, Amoxicillin-clavulnic acid and ceftizoxime, 94% for amoxicillin, 92% to penicillin G, 90% for ampicillin+Cloxacillin, 88% for streptomycin, 44% for Cloxacillin. On the other hand the aqueous extract lowered the resistance of P9 isolate in the following manner, 86% for ceftriaxone, cefoperazone, Amoxicillin-clavulnic acid, cefotaxime and cefetizome, 80% for amoxicillin, 72% for penicillin G, 64% for ampicillin+Cloxacillin, 58% for

streptomycin and 33% for Cloxacillin. From these results we can conclude that methanolic extract are more potent to remove the antibiotic resistance genes than watery extract.

To support the above results in which these antibiotic resistance genes were affected more by methanolic extract, the plasmid DNA of P9 was extracted and run on gel electrophoresis. Figure 2 shows only one plasmid remain after treating P9 with methanolic extract of *K. blossfeldiana* while three plasmids remain after aqueous treatment and this may explain why antibiotic resistance genes when treated with aqueous extract were less affected compared with methanolic extract. On the other hand the anti plasmid effect of methanol extract *P. aeruginosa* may be due to the ability of the methanol to extract some of the active properties of this plant like phenol and flavonoids which is absent in aqueous extract which is reported to be antimicrobial. The flavonoids have been found to be effective *in vitro* and acting as the antimicrobial substance against wide array of microorganism, Ozcelik *et al.* (2008) used six flavonoids against ESBL producing *Klebsiella pneumonia*, they were found that all showed *in vitro* antimicrobial activity against all the isolates of *K. Pneumonia* similar to the control antibacterial (afloxacin). Quazi *et al.* (2011) subjected the roots of *K. pinnata* to petroleum ether, chloroform, methanol and aqueous solvent respectively for extraction and *in vitro* evaluation of antimicrobial activity was done against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and

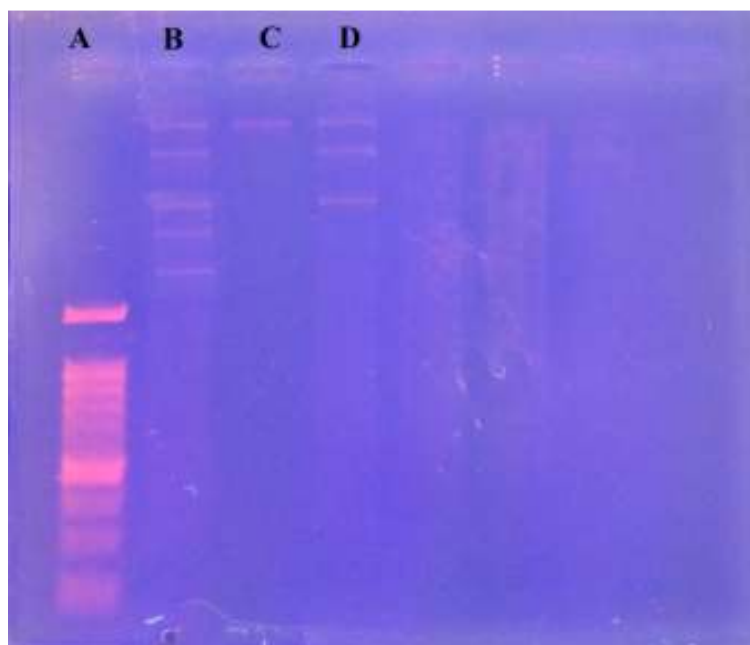


Fig. 2: Plasmid profile of P9 before and after curing

A: Ladder; B: Plasmid profile before treatment; C: Plasmid profile after treatment with methanolic extract; D: Plasmid profile after treatment with aqueous extract

Candida albicans. Methanolic extract of roots of *K. pinnata* was found to be most effective as antibacterial as compared to others while none of the extracts showed activity against *C. albicans*. Akinpelu (2000) in a study found that 60% methanolic leaf extract inhibits the growth of five out of eight bacteria used at a concentration of 25 mg/mL. *Bacillus subtilis*, *E. coli*, *Proteus vulgaris*, *Shigella dysenteriae*, *S. aureus* were found to be inhibited while *Klebsiella pneumoniae*, *P. aeruginosa* and *C. albicans* were found to resist the action of the extract. Chemical investigation of the bioactive constituents from the leaf of *K. pinnata* resulted in the isolation of two new novel flavonoids; 5'-methyl 4', 5, 7-trihydroxy flavone and 4I, 3, 5, 7-tetrahydroxy 5-methyl 5'-propenamine anthocyanidines. The antimicrobial observation of the aforementioned compounds could be responsible for the activity of *K. pinnata* and its use in herbal medicine in Nigeria (Okwu and Nnamdi, 2011). Odunayo *et al.* (2007) found that the methanolic squeezed-leaf juice of *Kalanchoe crenata* was the most active one with MIC of 8 mg/mL against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Bacillus subtilis*, 32 mg/mL against *Shigella flexneri*, 64 mg/mL against *Escherichia coli* and 128 mg/mL against the control strain *Staphylococcus aureus* compared with other solvents.

CONCLUSION

The implication of the broad spectrum action of some of these extracts is that they can be useful in

antiseptic and disinfectant formulation as well as in chemotherapy. The anti-pseudomonal activities of some of the effective extracts of these plants can be further explored.

REFERENCES

- Ahmed, A.J., 2013. Antibacterial profile and exotoxin: A gene detection of *P. aeruginosa* isolated from different sources. M.Sc. Thesis, College of Science, Salahaddin University, Erbil, Iraq.
- Aibinu, I., E. Adenipekun and T. Odugbemi, 2004. Emergence of quinolone resistance amongst *Escherichia coli* strains isolated from clinical infections in some Lagos State hospitals in Nigeria. *Niger. J. Health Biomed. Sci.*, 3(2): 73-78.
- Akinpelu, D.A., 2000. Antimicrobial activity of *Bryophyllum pinnatum* leaves. *Fitoterapia*, 71(2): 193-194.
- Al-Amir, L.A.K., 1998. Molecular study of virulence factors in *P. aeruginosa*. Ph.D. Thesis, College of Science, University of Baghdad, Iraq.
- Al-Daraghi, W.A. and Z.H. Abdullah, 2013. Detection of exotoxin A gene in *Pseudomonas aeruginosa* from clinical and environmental samples. *Sci. Al-Nahrain Univ.*, J., 16(2): 167-172.
- Bahaael-Din, A., A.E. Magda, B. Rawia and A. El-Sabagh, 2008. *Pseudomonas aeruginosa* exotoxin A: Its role in burn wound infection, and wound healing. *Egypt J. Plast. Reconstr. Surg.*, 32(1): 59-65.

- Bollero, D., M. Cortellini, M. Stella, R. Carinino, G. Magliacani and M. Iorio, 2002. Pan-antibiotic resistance and nosocomial infection in burn patients: Therapeutic choices and medico-legal problems in Italy. *Burns Fire Disaster.*, 13(7): 185-202.
- Brook, G.F., J.S. Butel, S.A. Mores, K.C. Carroll and E. Jawetz, 2007. *Jawetz, Melnick and Adelberg's Medical Microbiology*. 24th Edn., Lange Medical Books/McGraw-Hill, Medical Pub. Division, Toronto, New York, pp: 224-249.
- Carsenti-Etesse, H., J.D. Cavallo, P.M. Roger, Z. Zarifi, P. Plesiat, E. Garrebe and P. Dellamonica, 2001. Effect of β -lactam antibiotics on the *in vitro* development of resistance in *Pseudomonas aeruginosa*. *Clin. Microbiol. Infec.*, 3(7): 144-151.
- Cruickshank, R.W., D.L. Duguid, B.P. Marmion and R.H.A. Swain, 1975. *Medical Microbiology*. 12th Edn., Churchill Livingstone, London.
- Engel, J.N., 2003. Molecular Pathogenesis of Acute *Pseudomonas aeruginosa* Infections. In: Hauser, A.R. and J. Rello (Eds.), *Severe Infections Caused by Pseudomonas aeruginosa*. Kluwer Academic Publishers, Dordrecht, pp: 201-229.
- Fogle, M., R. Griswold, J.A. Oliver and A.N. Hamood, 1994. Anti-ETA IgG neutralizes the effects of *Pseudomonas aeruginosa* exotoxin A. *J. Surg. Res.*, 106: 86-98.
- Hamood, A.N., J.A. Colmer-Hammod and N.L. Carty, 2004. Regulation of *Pseudomonas aeruginosa* exotoxin A synthesis. In: Ramos, J.L. (Ed.), *Pseudomonas: Virulence and Gene Regulation*. Vol. 2, Kluwer Academic/Plenum Publishers, New York, pp: 389-423.
- Hancock, R.E. and D.P. Sreet, 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: Mechanisms and impact on treatment. *Drug Resist. Update.*, 3: 247-255.
- Harborne, J.B., 1984. *Phytochemical Methods*. 2nd Edn., Chapman and Hall, NY.
- Harlalka, G.V., C.R. Patil and M.R. Patil, 2007. Protective effect of *Kalanchoe pinnata* pers. (Crassulaceae) on gentamicin-induced nephrotoxicity in rats. *Indian J. Pharmacol.*, 39(4): 201-205.
- Harley, P., 2002. *Laboratory Exercises in Microbiology*. 5th Edn., McGraw-Hill, pp: 333-338.
- Hasan, M.K.A., 2001. The use of some plant extracts for inhibition of genotoxic effect for some anticancer drugs in mouse. Ph.D. Thesis, College of Science, University of Babylon, Iraq.
- Henrichfreise, B., I. Wiegand, W. Pfister and B. Wiedemann, 2007. Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. *Antimicrob. Agents Ch.*, 51(11): 4062-4070.
- Jaffer, H.J., M.J. Mahaed, A.M. Jawad, A. Naji and A. Al-Naib, 1983. Phytochemical and biological screening of some Iraqi plant. *Fitoterapialix*, 18: 299 (In Arabic).
- Jerman, B., M. Butala and D. Zgur-Bertok, 2005. Sublethal concentrations of ciprofloxacin induce bacteriocin synthesis in *Escherichia coli*. *Antimicrob. Agents Ch.*, 49(7): 3087-90.
- Khan, A.A. and C.E. Cerniglia, 1994. Detection of *Pseudomonas aeruginosa* from clinical and environmental samples by amplification of the exotoxin A gene using PCR. *Appl. Environ. Microb.*, 60: 3739-3745.
- Majaz, Q.A., S. Nazim, Q. Asir, Q. Shoeb and G.M. Bilal, 2011. Screening of *In-vitro* anthelmintic activity of *Kalanchoe pinnata* roots. *Int. J. Res. Ayurveda Pharm.*, 2(1): 221-223.
- Moreillon, P., Y.A. Que and M.P. Glauser, 2005. *Staphylococcus aureus* (Including Staphylococcal Toxic shock). In: Mandell, G.L., J.E. Bennett and R. Dolin (Eds.), 6th Edn., *Principles and Practice of Infectious Diseases*. Churchill Livingstone Philadelphia, 2: 2333-2339.
- Nikbin, V.S., M.M. Aslani, Z. Sharafi, M. Hashemipour, F. Shahcheraghi and G.H. Ebrahimipour, 2012. Molecular identification and detection of virulence genes among *Pseudomonas aeruginosa* isolated from different infectious origins. *Iranian J. Microbiol.*, 4(3): 118-123.
- Odunayo, R.A., E. Ibukun, A. Tayo, T. Adelowotan and T. Odugbemi, 2007. *In vitro* antimicrobial activity of crude extracts from plants *Bryophyllum pinnatum* and *Kalanchoe crenata*. *Afr. J. Tradit. Complem.*, 4(3): 338-344.
- Okwu, D.E. and F.U. Nnamdi, 2011. Two novel flavonoids from *Bryophyllum pinnatum* and their antimicrobial activity. *J. Chem. Pharm. Res.*, 3(2): 1-10.
- Othman, A., 2011. Anti plasmid (curing) effect of alcoholic extract of *rosmarinusofficinalis* on resistant isolate of *Pseudomonas aeruginosa*. M.Sc. Thesis, College of Medecine, Hawler Medical University, Erbil, Iraq.
- Ozcelik, B., D.D. Orhan, S. Ozgen and F. Ergun, 2008. Antimicrobial activity of flavonoids against extended-spectrum β -lactamase (ESBL)-producing *Klebsiella pneumonia*. *Trop. J. Pharm. Res.*, 7(4): 1151-1157.
- Quazi, M.A., S. Nazim, S. Afsar, S. Siraj and M.S. Patel, 2011. Evaluation of antimicrobial activity of roots of *Kalanchoe pinnata*. *Int. J. Pharmacol. Bio. Sci.*, 5(1): 93-96.
- Raouf, W.M., L. Rahman and A. Ibraheem, 2010. *In vitro* study of the swarming phenomena and antimicrobial activity of pyocyanin produced by *Pseudomonas aeruginosa* isolated from different human infections. *Eur. J. Sci. Res.*, 47(3): 405-421.

- Rashmi, A., A. Singh, K. Priyanka, S. Sangeeta, B. Divya and M.K. Deepak, 2012. Antimicrobial and antioxidant activities of *Kalanchoe pinnata* against pathogens. *J. Pharm. Res.*, 5(10): 5062-5063.
- Rietisch, A., V.I. Gely, L.S. Dove and J.J. Mokalane, 2005. Exs E a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *J. Harvard Med. School.*, 102(22): 8006-8011.
- Salah, H.F., 2007. Effect of some medicinal plant extracts on antibiotic resistance by plasmid of *E. coli* isolated from different sources. M.Sc. Thesis, College of Science Education, Salahaddin University, Erbil, Iraq.
- Sambrook, J. and E. Russell, 2001. *Molecular Cloning: A Laboratory Manual*. 3rd Edn., Cold Spring Harbor Laboratory Press, USA.
- Sambrook, J., M. Fritsch and T. Maniatis, 2006. *Molecular Cloning: A Laboratory Manual*. Cold Spring Laboratory, New York, pp: 233-455.
- Sulaiman, S.H.S., 2013. Antibiotic resistance studies and curing analysis by ascorbic acid in *P. aeruginosa*. Ph.D. Thesis, College of Medicine, Hawler Medical University, Erbil, Iraq.
- Vedel, G., 2005. Simple method to determine β -lactam resistance phenotype in *Pseudomonas aeruginosa* using the disc agar diffusion test. *J. Antimicrob. Chemoth.*, 56: 657-664.
- Williams, R., 2000. Antimicrobial resistance-a global threat. *Essential Drug Monitor*, 1: 28-29.
- Wolfgang, M.C., B.R. Kulasekara, X. Liang, D. Boyd, K. Wu and Q. Yang, 2003. Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*. *P. Natl. Acad. Sci. USA*, 100: 8484-8489.