Antiplasmid Potential of Kalanchoe Blossfeldiana Against Multidrug Resistance Pseudomonas Aeruginosa

Zirak Faqe Ahmed Abdulrahman
Department of Biology, College of Education, University of Salahaddin

Abstract:

This study is concerned with the isolation and identification 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. Antibiotic resistance test was used ,the isolates 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 resisted 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 as 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 was reduction of the resistance from 44-100% while the effect of MIC of aqueous extract on antibiotic resistance genes for isolate P9 was decreasing of the resistance from 36-86 % respectively and these results were supported through gel electrophoresis process.

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

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 Δ*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,2011). It was found that this plant showed

various pharmacological activities such as anthelmentic, 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, cardienolides, steroids, bufadienolides and lipids include: arachidic acid, astragalin, behenic acid, beta amyrin, benzenoids, beta-sitosterol, bryophollenone, 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, peposterol, 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 Amoxycillin , Amoxycillinclavulanic acid, Ampicillin+cloxacillin, Cefotaxime, Cefoperazone, Ceftizoxime, Ceftriaxone, Cloxacillin, Penicillin G,and Streptomycin on *P.aeruginosa* isolates, after sterilization and cooling at 45^{o}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^{o}C for 24 hours. The results were recorded next day (Jerman *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 per minute (rpm) for (30 second). Supernatant was removed. Two hundred and fifty µl of re-suspension solution vortexed until the cell pellet is completely re-suspended. Two hundred and fifty µl lyses solution was added and mixed by inverting 10 times.Three hundred µl neutralization solution was added. Mixed by inverting 10 times then centrifuged for 5mint. A spin column inserted into a 2 ml wash tube. The supernatant to transferred to a spin column, centrifuged at 8ooo rpm for 1 mint to pull fluid though the column. The spin column was removed, the filtrate discarded, and the column replaced. 500 µl wash buffer added, centrifuge 2 minutes followed by a 2 minutes spin to remove all wash buffers. The spin column removed to a clean 1.5 ml tube, 100 µl sterilized H_2O was added. Centrifuged for one minute.

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

Preparation of 1% agarose gel: The gel (1%) was prepared by dissolving 1 gm 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 ºC, 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 µl 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 hour.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 ml 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 ^{o} C for 30 min in shaking water bath, until the cells are completely lysed.Twenty µl of RNase solution was added then mixed by vortexing and the mixture incubated for 10 min at room temperature. Two-hundred µl 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 1min 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 µl 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 µl of wash buffer II (with ethanol added) was added to the Gene JET™ genomic DNA purification column. Centrifuged for 3 min at maximum speed (≥ 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 1min at 8000 rpm. The purified DNA immediately discarded in downstream applications or stored at -20 ^{o} C.

PCR master kit: The reagent of master mix is an optimized ready to use 2 × 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 25µl 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 products were visualized by ethidium bromide staining after gel electrophoresis of 7 µl of the final reaction mixture in 1.2% agarose. 100 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 hours 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 hours 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 *Kalanchoe blossfeldiana* 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) µg/ml .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 suspention, and then it was incubated at 37ºC for 24 hours (Cruickshank *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 37ºC for 24hours .Next day 0.1 ml of it was spreaded on nutrient agar plate and incubated for 24 hours at 37 ºC , then 50 colonies transferred to antibiotic agar plate , after incubation for 24 hours at 37ºC 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 ml 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 minutes, red color means presence of glycosides (Hasan, 2001).

Flavonoids detection: Ten ml of 50% ethanol was added to 10ml 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 ml from plant extract divided into two equal parts, then drops of 1%CH_3COOPb 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 ml of plant extract was extremely shaked for half minute, then left in vertical case for 15 minutes, appearing of foam means presence of saponin (Hasan, 2001).

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

Phenols detection: Three ml of plant extract was added to 2ml of potassium hexacyanofrrate and 2ml 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 (Raoof, 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* occure 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 genaral state of immunosuppresion is caused by impaired functioning of neutrophil , celluler 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 Amoxycillin,while resistance for Amoxycillin-clavulanic acid, Cloxacillin, Cefoperozone, Streptomycin, Ceftriaxone, Cefotaxime, and Ceftizoxime 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 pencillin G with 100% .Sulaiman (2013) pointed out among 100 isolates of *P. aeruginosa* 81% were resistance to Amoxycillin, 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 inactive the antibiotics or prevent their intracellular accumulation to inhibitory levels (Hancock and Speert, 2000). The major mechanism of

Table1: resistance of *P.aeruginosa* to antibiotics

Antibiotics	abbreviation	No. Of resistance isolates	% of resistance
Amoxycillin	AM	33	82.5
Amoxycillinclavulanic acid	ACL	33	82.5
Ampicillin+cloxacillin	AML	33	82.5
Cefotaxime	CTX	17	42.5
Cefoperazone	CPZ	28	70
Ceftizoxime	CAZ	16	40
Ceftriaxone	CRO	21	52.5
Cloxacillin	CLX	31	77.5
Penicillin G	PG	34	85
Streptomycin	S	20	50

resistance to β-lactam antibiotics including pencillin is related to β-lactam production. Both plasmid mediated and chromosomally mediated β-lactamase production can occur, which is responsible for resistance to pencillin (Carsent-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 aminopencillins, first and second-generation of cephalosporins and some third-generation of 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-lactamse, 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 figure (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.

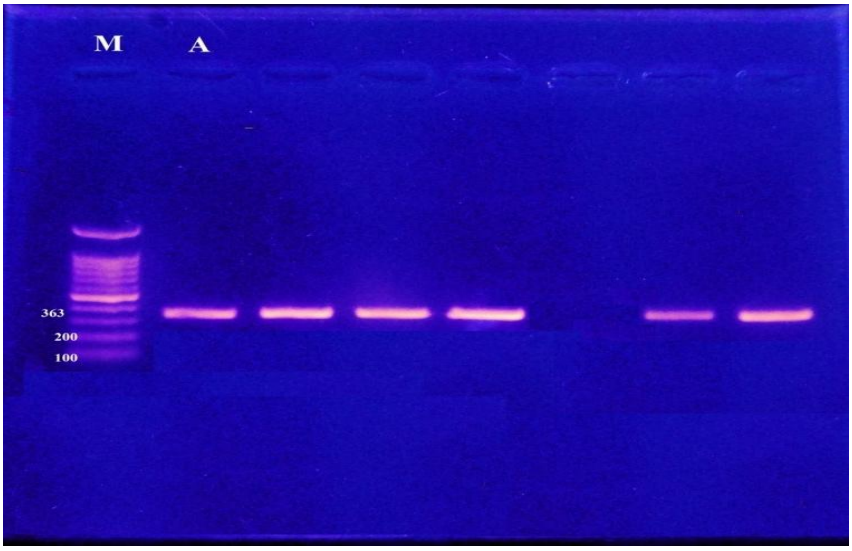


Figure (1): PCR product of ETA gene for *P.aeruginosa*

M:ladder, A:positive control

Khan and Cerniglla (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*., 2002). 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 spectrophotometer, which is clarified in table (2) and shows that the MIC of aqueous and methanolic extracts of kalanchoe were 1800 µg/ml 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.

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

Plant extract type	**Concentration of *k. blossfeldiana* (µg/ml)**									
	200	400	600	800	1000	1200	1400	1600	1800	**2000**
Aqueous extracts	1.053	1.038	0.940	0.915	0890	0.870	0.726	0.377	0.298	**0.000**
Methanolic extract	1.001	0.913	0.863	0.751	0.3450	0.188	0. 180	0.000	0.000	**0.000**

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 ehylacetate.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.

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

The results revealed as in table (4) that the methanolic extract affected the resistance genes of P9 isolate 100% for cefotaxime, ceftriaxone,cefoperazone,Amoxycillin-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, Amoxycillin-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 is more potent to remove the antibiotic resistance genes than watery extract.

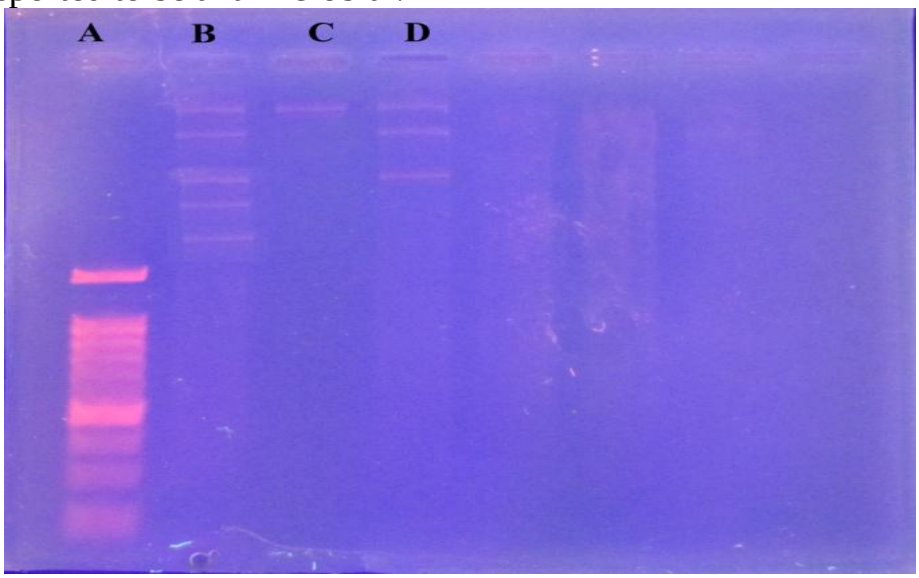
Table 4: Effect of MIC of aqueous and ethanolic extract on *P. aeruginosa* P9 resistance genes.

Antibiotics	methanolic extract 1400µg/ml		Aqueous extract 1800µg/ml	
	% of colonies growth	% decreasing resistance	% of 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).

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 component of this plant like phenol and flavonoids which is absent in aqueous extract which is reported to be antimicrobial.



Figure(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

The flavonoids have been found to be effective invitro 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 invitro 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 *Candida albicans*. Methanolic extract of roots of *K. pinnata*was found to be most effective as antibacterial as compare to others while none of extract showed the 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 25mg/ml. *Bacillus subtilis, E. coli, Proteus vulgaris, Shigelladysenteriae, 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 trihydroxyl 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.

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الخلاصة

القوة ضد البلازميدية لنبات الكلنكوة ضد بكتريا *Pseudomonas aeruginosa* متعدد المقاومة للمضادات الحيوية

تهدف هذ ه الدراسة الى عزل وتشخيص بكتريا ***Pseudomonas aeruginosa*** من حالات سريرية مختلفة والتى تشمل (الجروح , الحروق و الادرار) للمرضى الوافدين الى مستشفى الطوارئ والمختبر الداخلي للمستشفى التعليمي في مدينة اربيل .تم تشخيص اربعين عزلة من البكتريا المذكورة من بين ١٢٠ نموذج باستعمال الصفات المظهرية , المزرعية والبايوكيميائية بالاضافة الى تقنية vitek .تم استعمال اختبار الحساسية للمضادات الحيوية واظهرت النتائج تباين العزلات فى مقاومتها للمضادات الحيوية وكانت اعلى مقاومة لبنسلين G فى حين كانت اقل مقاومة لسيفتيزوزيم وكانت ٤٠% في حين بلغت النسبة لبقية المضادات ما بين ٤٢,٥-٨٢,٥%. على جانب اخر كانت عزلات P9,P16,and P30 مقاومة لجميع المضادات .تم استعمال تقنية التفاعل البلمرى المتسلسل للكشف عن جين ETA واظهرت النتائج بان ٨٢,٥% من العزلات كانت حاوية على تلك الجين. تم استعمال المستخلص المائي والكحولي لنبات *K. blossfeldiana* كعامل تحييد لتقليل الجينات المقاومة للمضادات الحيوية في عزلة p9 , وتم ذلك من خلال تحديد التركيز المثبط الادنى لهذا النبات والتى استعملت كعامل تحييد وكانت ١٨٠٠ µg/mlللمستخلص المائي و ١٤٠٠ µg/ml للمستخلص الكحولي. كان تاثير التركيز المثنط الادنى للمستخلص الكحولي على الجينات المقاومة للمضادات الحيوية تقليل المقاومة بنسبة ٤٤-١٠٠% بينما كان تاثير التركيز المثنط الادنى للمستخلص المائي على الجينات المقاومة للمضادات الحيوية انخفاض المقاومة بنسبة ٣٦-٧٨% على التوالي. وقد اكد ذلك استعمال تقانة الترحيل الكهربائي.