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Full Length Research Paper

Inhibition of beta-lactamase enzyme of *Pseudomonas aeruginosa* by clavulanic acid of *Rumex vesicarius* L.

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This study plays an important role in solution of beta-lactam resistance problem. This aim has been achieved by clavulanic acid production as beta-lactamase inhibitor. Beta-lactam-resistant *Pseudomonas aeruginosa* possesses beta-lactams resistance machinery by secretion of extracellular beta-lactamase enzyme. The present study was begun with collection of eighty nosocomial bacterial isolates from Riyadh hospitals through one year. Nosocomial bacterial isolates were treated by amoxicillin (250 µg ml^{-1}) to determine resistant isolates. Among them fifty amoxicillin-resistant isolates could be found with different minimum inhibitory concentrations (MICs). However, one isolate considers the most potent which has MIC over than 1000 µg ml^{-1} and also was identified by using polymerase chain reaction (PCR) technique as *P. aeruginosa*. Isolated gene of *P. aeruginosa* has 870 bp. The second phase of this study was begun with collection of six plants which are growing in Saudi Arabia, then were identified by professionals of Science College herbarium, King Saud University. These plants were extracted by methanol and then dried. These plants are *Rhazya stricta*, *Calotropois procera*, *Maerua crassifolia*, *Haloxylon salicornicum*, *Rumex vesicarius* L. and *Lycium shawii*. The plant extracts were tested to detect and determine their antagonistic effect for amoxicillin-resistant *P. aeruginosa* and of course for its beta-lactamase enzyme. Only one extract of *R. vesicarius* L. has antagonistic effect for beta-lactamase enzyme. Beta-lactamase inhibitor was purified and characterized as clavulanic acid by using nuclear magnetic resonance (NMR) spectroscopy analysis, infra-red (IR) spectroscopy analysis and elemental analysis. Eventually, amoxicillin-resistant *P. aeruginosa* was inhibited by amoxicillin-clavulanic acid combination at 125 µg ml^{-1} and 128 mg ml^{-1}, respectively.

Key words: Amoxicillin, nosocomial infections, rapid polymerase chain reaction, structure elucidation and xerophyte plants.

INTRODUCTION

Nosocomial infections are an important source of morbidity and mortality in many hospitals affecting millions of patients each year (Empel et al., 2007). *Pseudomonas aeruginosa* is one of the most important nosocomial pathogens, being responsible for various types of infections with more and more limited therapeutic options (Sardelic et al., 2003). Infection due to *P. aeruginosa* continues to be a major cause of mortality among critically ill and immunocompromised patients despite the development of newer and more powerful antibiotics.

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Anti-microbial resistance is a main problem in worldwide. The common mechanism of antibiotics resistance involves production of enzymes which modify the antibiotics (Medeiros, 1997). The most common mechanism of beta-lactam resistance among both Gram-positive and Gram-negative bacteria involves the production of beta-lactamases, enzymes which cleave β-lactam ring (Doran et al., 1990). Since discovery of antibiotics and their uses as chemotherapeutic agents, there are a belief in the medical fraternity that this will lead to eradication of infectious diseases. However diseases and disease agents are controlled by antibiotics then returning in new forms resistant to antibiotic therapies (Levy and Marshall, 2004). *P. aeruginosa*, is a prevalent opportunistic human pathogen and the most common Gram-negative bacterium is found in

nosocomial infections (Bodey et al., 1983). The resistant organisms multiply and spread to other geographic locations as well as to other microbes by transferring of resistance genes (Levy and Marshall, 2004). Selection of resistant strains occurs so rapid for some bacteria that clinical usefulness of the antibiotics is lost within a 5 year period (Bush, 2004). Pneumonia is an illness, usually causes infection in which the lungs become inflamed and congested, reduces oxygen exchange and also cough and breathlessness (Anthony et al., 2008). It affects individuals of all ages but occurs most frequently in children and the elderly. Among children, pneumonia is the most common cause of death worldwide (Bryce et al., 2005; Wardlaw et al., 2006). One from the methods to reduce the resistance of antibiotics is using enzyme inhibitors from plant origin (Kim et al., 1995). The potential antihypertensive activity of Brazilian plants is evaluated *in vitro* by its ability to inhibit the angiotensin converting enzyme (ACE). Forty-four plants belonging to 30 families are investigated. Plants are selected based on their popular use as antihypertensive and/or diuretics. The following plants are presented significant ACE inhibition rates: *Calophyllum brasiliense, Combretum fruticosum, Leea rubra, Phoenix roebelinii* and *Terminalia catappa* (Fernão et al., 2007).

Certain study is undertaken to evaluate the effect of extraction from *Hemidesmus indicus* roots on five different solvents activity against the pathogenic and non-pathogenic organisms. *H. indicus* (L.) is one of the plants used in Ayurveda for several remedies it belongs to the family Asclepiadaceae (Sujatha and Anusha, 2011). Ethanolic and ether extracts of *Commiphora myrrha* are evaluated for their antimicrobial activity against two Gram-negative organisms (*Escherichia coli* and *Pseudomonas aeruginosa*), two Gram-positive organisms (*Bacillus subtilis* and *Staphylococcus albus*) and fungi are represented by *Candida albicans* which isolated from gazelles held at King Khalid Wildlife Research Centre (Omer et al., 2011). Antibacterial properties of different extracts of *Chlorophytum borivilianum* are studied. Ethanol, ethyl acetate, acetic acid and water are used to prepare the extract. The antibacterial activity of different extracts is carried out against four bacteria, S*taphylococcus aureus, E. coli*, *P. aeruginosa* and *B. subtilis*, by agar cup diffusion method (Sundaram et al., 2011). The ethanolic extracts of *Cassia alata* (CA), Walnut-*Juglan nigra* (JN), *Ocimum basilicum* (OB) and *Aloe vera* (AV) are studied for their *in vitro* antimicrobial activity against tested pathogenic microorganisms using agar diffusion method. Preliminary phytochemical screening showed the presence of tannin, fats and oil, saponins and glycosides in the ethanolic extracts of all tested plants. *J. nigra* has a highest activity against all tested organisms *E. coli*, *S. aureus* and *C. albicans*. While the least activity against tested organism is shown by OB, ethanolic extracts of AV is the most effective against *S. aureus*, while JN is the most effective against *E. coli* and *C. albicans* (Nebedum et al., 2009). Phytochemical analysis of the extracts showed presence of tannins, flavonoids, alkaloids, terpenoids and saponins with some of these constituents showing variations across the seasons. Broad spectrum antibacterial activity is observed for all the extracts (Osadebe et al., 2008).

Clavulanic acid is beta-lactamase inhibitor which can combine with beta-lactam antibiotics to eradicate anti-microbial resistance. Clavulanic acid biosynthetically generates from the amino acid arginine and the sugar glyceraldehyde-3-phosphate (Arulanantham et al., 2006; Tahlan et al., 2004). Clavulanic acid is negligible intrinsic antimicrobial activity. However, it has beta-lactam ring that characterizes beta-lactam antibiotics (Sutherland, 1990). However, the similarity in chemical structure allows to the molecule acts as a competitive inhibitor of beta-lactamases which secretes by certain bacteria to confer resistance of beta-lactam antibiotics. Augmentin, a brand name, containing a combination of amoxicillin and potassium clavulanate, is one of the best selling antibiotics (Lee et al., 2002; Rosa et al., 2005).

MATERIALS AND METHODS

Clinical isolates

We examined twenty clinical isolates of *P. aeruginosa* from the clinical hospital for infectious diseases in urinary tract. The bacterial isolates were collected between male and female from different patients.

Determination of MIC

The minimum inhibitory concentration (MIC) was determined for each bacterial isolate against amoxicillin according to Jennifer (2001).

Identification of *P. aeruginosa*

Identification of amoxicillin-resistant *P. aeruginosa* was carried out by using PCR technique according to Luiz et al. (1999).

Preparation of samples and DNA amplification

Urinary tract isolate was first incubated in nutrient broth medium (Oxoid) at 37°C for 24 h (Luiz et al., 1999). Bacterial cells were precipitated by centrifugation at 3000 rpm for 15 min, and then collected. Bacterial cells were washed by a solution of 100 mM Tris, 90 mM boric acid and 1 mM EDTA, pH 8.0 (TBE). Bacterial cells were soaked in 1 ml of the same solution and shaked vigorously, and then frozen instantaneously in liquid nitrogen, boiled for 30 min and centrifuged at 13,800 rpm. for 5 min. The supernatant fraction was separated and 5 µl used directly for the PCR reactions.

Primer selection

The *algD* GDP mannose dehydrogenase gene of *P. aeruginosa*

contains 2032 bp (GenBank access no. 400337, identification no. g45267). The selected primers were [VIC1 (5′ TTCCCTCGCAGAGAAAACATC 3′) and VIC2 (5′ CTGGTTGATCAGGTCGATCT 3′)] designed to amplify a 520 bp segment of the *algD* GDP mannose dehydrogenase gene of *P. aeruginosa*.

PCR protocol

PCR technique was performed in a 25 µl reaction mixture containing 150 ng of DNA template or 5 µl of the processed clinical sample, 0.4 µmol/L of each primer, 200 µmol/L each of the four nucleotides triphosphates, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.0) and 1 U of Tag DNA polymerase. Sample was subjected to the following thermocycling process (Perkin Elmer 2400): 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. A final extension step at 72°C was continued for another 7 min. A tube containing the reaction mixture and sterile water was included in all reactions as a negative control.

Plant extraction

The selected plants were collected from soils and identified in herbarium of Botany Department, Faculty of Science, King Saud University in Saudi Arabia. Air dried and finely powdered plant materials were extracted in a Soxhlet with 2 L. of methanol for 4 h. Solvent was evaporated under reduced pressure and the different extracts conserved in tightly sealed glass vials. Completely dried plant extract was dissolved in ethyl ether (v/v). The concentrations of the plant extracts were chosen to have no effect on the growth of the microorganisms based on preliminary experiments (Aburjai et al., 2001).

Determination of the resistant inhibitory effect of plant extracts *(in vitro)*

Each plant extract solution was added to the nutrient agar to make a concentration of 200 gm ml^{-1} in the final mixture (Aburjai et al., 2001). The concentration of amoxicillin used in the experiment was half MIC so it has no effect on microorganisms. At this concentration ethyl ether containing plant extract, amoxicillin solutions mixed with nutrient media and *P. aeruginosa* as a control. The results were showed that the amoxicillin did not inhibit the growth of *P. aeruginosa*. Bacterial suspension was added to give inoculum size of 10^4 cells in the final mixture of the plant extract and the amoxicillin. The medium was mixed thoroughly, poured in the plates and incubated at 37°C for 24 h. Control plates containing the plant sample alone in the nutrient agar with *P. aeruginosa* were also incubated. The blank plates were prepared by mixing ethyl ether (solvent) with nutrient agar and *P. aeruginosa*. Preliminary experiments were showed that ethyl ether did not exhibit antimicrobial activity. The experiments were carried out in duplicate, three times and correlated for the blank plates.

Precipitation of clavulanic acid

Potassium 2-ethyl hexanoate salt was used for clavulanic acid precipitation and prepared from 2-ethyl hexanoic acid (Hirata et al., 2009).

Purification of β-lactamase inhibitory agent

Purification of β-lactamase inhibitory agent was carried out by using diethylaminoethyl-cellulose (DEAE-cellulose) and sephadex G-200 according to Andrews (1969).

Characterization of β-lactamase inhibitory agent

Elemental analysis

Elemental analysis was carried out to all carbon, hydrogen, nitrogen and oxygen. This experiment was done in Microanalyses center of Science College, Cairo University.

Spectroscopic analysis

High performance liquid chromatography (HPLC) analysis: Clavulanic acid was quantified by HPLC, using a reversed-phase column (C-18). The mobile phase contained KH_2PO_4 (50 mM, 70%, 0.348 ml min^{-1}) and methanol (30%, 0.157 ml min^{-1}) and the pH was adjusted to 3.2 (Foulstone and Reading, 1982).

IR spectroscopic analysis

IR spectroscopy was performed by Shimadzu FT-IR 8101 PC infrared spectrophotometer. The sample (that is, clavulanic acid) was ground and mixed thoroughly with potassium bromide at 1:5 (sample:KBr) ratio, respectively. The KBr disc was prepared by compressing the powders at 20 psi for three minutes on KBr-press. The spectrum was scanned over wave number range of 4500 to 500 cm^{-1} (BLANCO, 2004).

NMR spectroscopic analysis

All NMR experiments were conducted on Varian mercury VX-300 NMR spectrophotometer operating at 300 MHz for proton and 75 MHz for ^{13}C (Spyros, 2000). NMR experiments were done in Microanalyses center of Science College, Cairo University.

Susceptibility test

Preparation of amoxicillin stock solutions

Suitable range of amoxicillin concentrations was chosen against *P. aeruginosa* (31.25 to 1000 µg ml^{-1}). Stock solutions were prepared by using the formula W = C × V × 1000 / P; where W is the weight of amoxicillin (mg), C is the concentration of amoxicillin (mgL^{-1}) multiply in 1000, V is the volume of distilled water as a solvent (ml) and P is the potency of amoxicillin which equal 600 µg mg^{-1} (Michael et al., 1993).

Preparation of β-lactamase inhibitory agent stock solutions

Suitable range of β-lactamase inhibitory agent concentrations was chosen (0.015 to 128 mgL^{-1}). Stock solutions were prepared by using the same formula that mentioned before.

Preparation of agar dilution plates

Nutrient agar medium was prepared and cooled at 50°C and then added amoxicillin concentrations (39.06 to 1000 µgml^{-1}) to each concentration of β-lactamase inhibitory agent (0.015 to 128 mgL^{-1}). Poured plates (each one contains 20 ml) and left at solidification, inoculated by *P. aeruginosa*, and incubated at 30°C for 24 h.

Table 1. Collection and differentiation of nosocomial bacterial isolates.

Hospital	Geog. area	Str. no.	Male no.	Res. cases	%	Sen. cases	%	Female no.	Res. cases	%	Sen. cases	%
Soliman prince	West	10	6	4	66.7	2	33.3	4	1	25	3	75
Al-Eiyman general	South	15	9	7	77.8	2	22.2	6	1	16.6	5	83.4
King Khalid	North	20	18	15	83.4	3	16.6	2	0	0	2	100
Society insurances	East	15	12	10	83.4	2	16.6	3	3	100	0	0
King Saud	Middle	20	12	7	58.4	5	41.6	8	2	25	6	75
Total	5	80	57	43	-	14	-	23	7	-	16	-

Geo = Geographic, Str = strain, Res = resistance; Sen = sensitive.

Table 2. Ethnobotanic data of studied plants.

Plant name	Weight of plant (g)	Weight of extract (g)	Activity (inhibition zone mm)
Rhazya stricta	550	110	0
Calotropois procera	535	42	0
Maerua crassifolia	670	46	0
Haloxylon salicornicum	950	123	0
Rumex vesicarius L.	1050	74	40
Lycium shawii	450	30	0

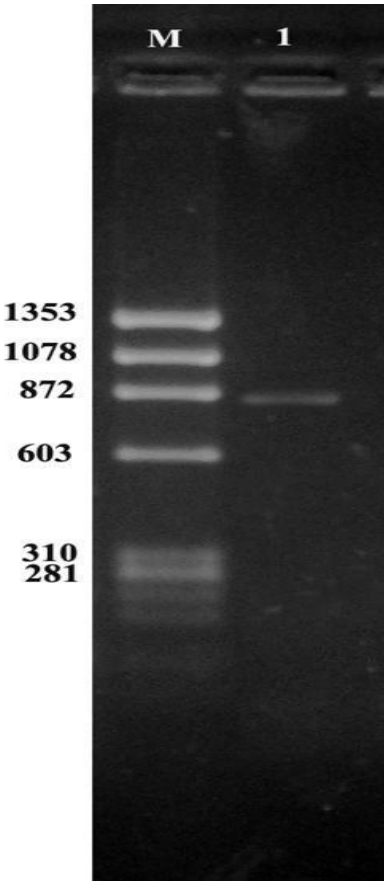

Figure 1. Agarose gel electrophoresis of PCR products from *P. aeruginosa.*

RESULTS

There were eighty nosocomial bacterial isolates collected from different hospitals in Riyadh city. These hospitals were selected according to their geographical sites, and with each hospital total number of bacterial isolates was determined. Also, with each hospital nosocomial isolates the number of infected cases was determined for both male and female as well as percent of resistant and sensitive isolates was determined by treatment of isolates with amoxicillin 250 µg ml^{-1} as shown in Table 1. The total number of amoxicillin-resistants was fifty ones including twenty *Pseudomonas aeruginosa* isolates which were characterized preliminary by using Analysis profile Index kits (20 NE API). The minimum inhibitory concentration (MIC) was determined for each resistant isolate. Certain isolate was considered most potent amoxicillin-resistant due to its highest MIC over than 1000 µg ml^{-1}. This bacterial isolate was identified before by API as *Pseudomonas aeruginosa*, furthermore it was identified genetically by PCR as confirmation. The isolated *algD* GDP mannose dehydrogenase gene has 870 bp as illustrated in Figure 1.

There were six plants grown in Saudi Arabia collected and identified by professionals of Science College herbarium, King Saud University. These plants (leaves) were extracted by methanol, dried and tested for inhibition the growth of amoxicillin-resistant *Pseudomonas aeruginosa* but only one extract of *Rumex vesicarius* L has an inhibitory effect as shown in Table 2.

Thereby, inhibiting agent of *R. vesicarius* L. extract was purified by diethylaminoethyl cellulose and sephadex

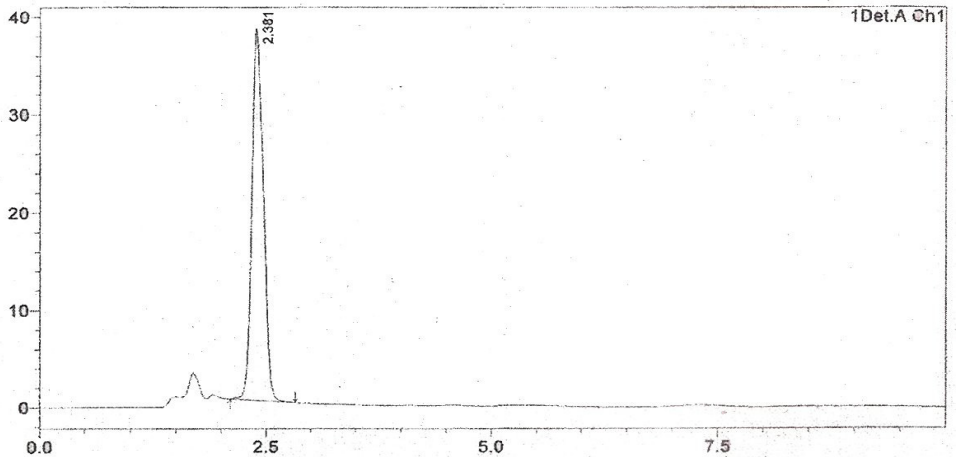

Figure 2. HPLC chromatogram of clavulanic acid.

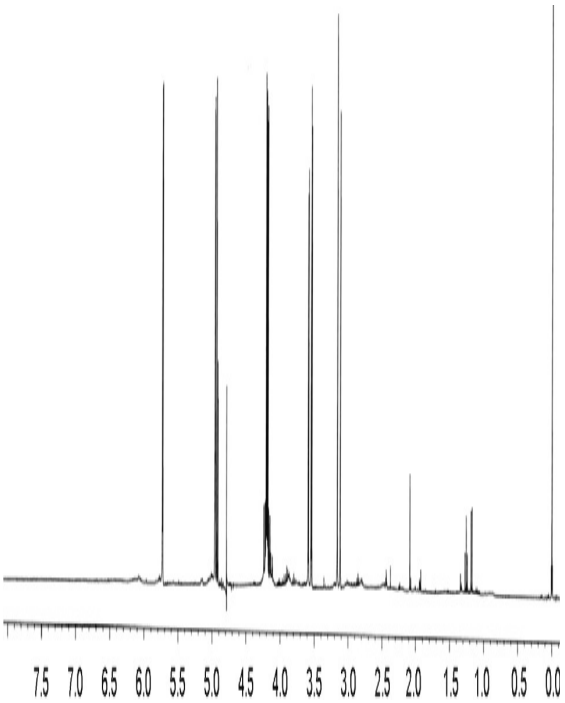

Figure 3. Spectrum ^{1}H NMR of purified β-lactamase inhibitory agent.

G-200 column chromatography. With each step of purification process (diethylaminoethyl cellulose and sephadex G-200 column chromatography) fifty fractions were collected (5 ml/fraction). With each fraction beta-lactamase inhibitory activity was tested. By using diethylaminoethyl cellulose column chromatography activity was constricted in two frequent fractions while with sephadex G-200 column chromatography it was constricted in only one fraction. The homogeneity of active fraction was checked by the analytical HPLC by injection 20 µl into the analytical (C_{18}) column (25 × 0.46 cm) packed with 5 µ hypersile octadecyl silan (ODS), at a flow rate of 1 ml min^{-1} Figure 2. The pure fraction was subjected to the spectroscopic analysis: 300 MHz ^{1}H NMR as shown in Figure 3, 75 MHz ^{13}C NMR and IR Figure 4. Figure 4 show an increase in the spectra at 1080 and 800 nm of the 5:5 binary system, which were the –NH and C=O spectra. This data support the prediction that there is a formation of hydrogen bonding between –NH/-C=O site of clavulanic acid. Elemental analysis was carried out for all carbon, hydrogen, nitrogen and oxygen where the percentages are 40.5, 3.8, 5.9 and 33.7% respectively. So, beta lactamase inhibitory agent was characterized as clavulanic acid.

Susceptibility test was done according to Checkerboard method with amoxicillin-resistant *P. aeruginosa* against different concentrations of amoxicillin (31.25 to 1000 μgml^{-1}), as well as different concentrations of clavulanic acid (128 to 0.015 mgL^{-1}). Combination of clavulanic acid (128 mgL^{-1}) and amoxicillin (125 μgml^{-1}) could inhibit the growth of *P. aeruginosa* at lowest concentrations as shown in Table 3.

DISCUSSION

In the present study clavulanic acid as potential beta-lactamase inhibitor was produced from xerophyte which grown naturally in Riyadh city. The choice was fallen on beta-lactam antibiotics due to their widespread and intervention in the treatment of many indications. Amoxicillin was chosen due to its easy absorption and safety, so led to its resistance by various Gram-negative bacteria especially *P. aeruginosa* which has a machinery of multi-resistance antibiotics. Others reported that *P. aeruginosa* isolates were most resistants to ciprofloxacin. Even though the isolates which were taken from healthy individuals exhibited similar resistance trends as those isolated from hospital patients (Bouza et al., 1999; Cavallo et al., 2000; Strateva et al., 2007). Bouza et al.

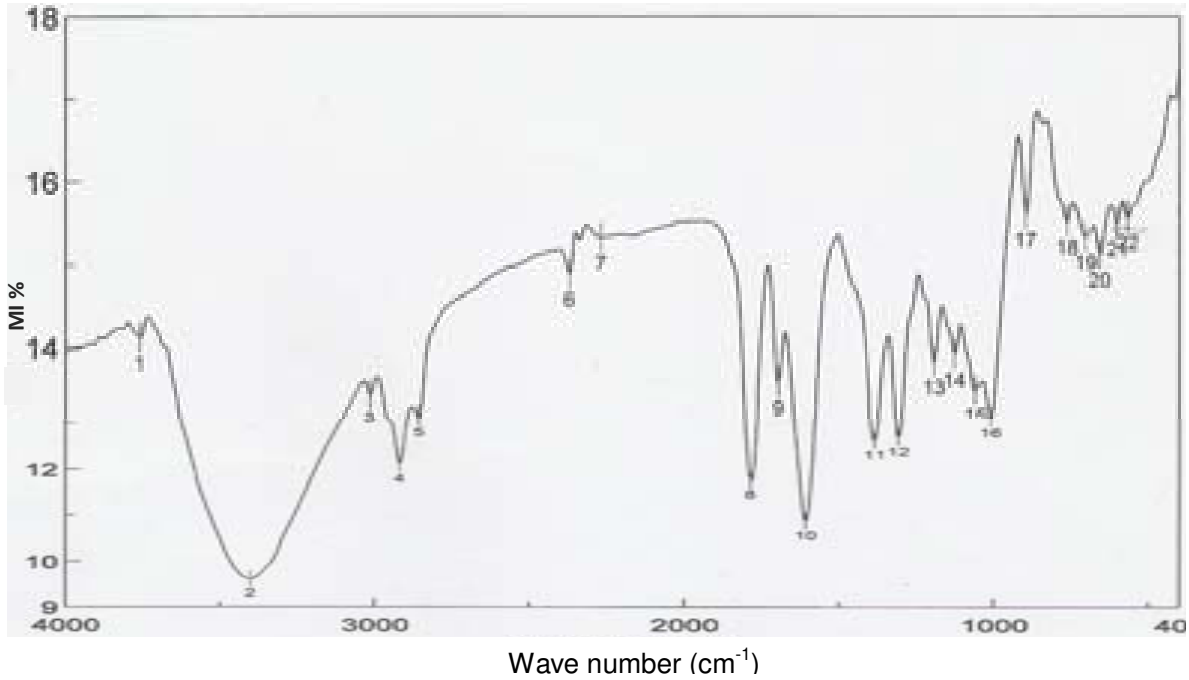

Figure 4. IR spectrum of clavulanic acid.

Table 3. Susceptibility of *P. aeruginosa* to amoxicillin and clavulanic acid.

Concentration of amoxicillin (µgml^{-1})	Concentration of clavulanic acid (mgl^{-1})													
	128.0	64.0	32.0	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.032	0.015
1000	-	-	-	-	-	+	+	+	+	+	+	+	+	+
500	-	-	-	+	+	+	+	+	+	+	+	+	+	+
250	-	-	+	+	+	+	+	+	+	+	+	+	+	+
125	-	+	+	+	+	+	+	+	+	+	+	+	+	+
62.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
31.25	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = Bacterial growth, - = no bacterial growth.

(1999) reported that further resistant to ciprofloxacin was more in out patients than in the nosocomial isolates. The incidence of resistance appeared by bad usage of antibiotics. Although *P. aeruginosa* strains exhibit an intrinsic sensitivity to β-lactams, imipenems, aminoglycosides and flouroquinolones, resistance for these antibiotics was emerged and widespread by some *P. aeruginosa* strains (Pagani et al., 2005).

There were six plants grown in Saudi Arabia collected, identified and extracted in dry form. The plant extracts were tested to inhibit the growth of amoxicillin-resistant *P. aeruginosa*. The extract of *R. vesicarius* L. only has beta-lactamase inhibiting effect. Similar results were recorded by various workers (Tagwi et al., 2001). The inhibitory agent was purified and characterized as clavulanic acid. Clavulanic acid was precipitated by potassium 2-ethyl hexanoate salt and exposed to purification. The purification was done by diethylaminoethyl cellulose using phosphate buffer (pH = 7.5) for elution and sephadex G-200 equilibrated with phosphate buffer (pH = 7.5). Similar results were obtained by (Huang et al., 1996). The purified agent was characterized as clavulanic acid by using NMR spectroscopy, IR spectroscopy and elemental analysis. Clavulanic acid at 128 mgL^{-1} combined with amoxicillin at 125 µgml^{-1} to inhibit *P. aeruginosa*.

Conclusion

Amoxicillin-resistant *P. aeruginosa* was isolated from clinical field. Also, was identified by rapid PCR method

and inhibited by amoxicillin-clavulanic acid combination. Clavulanic acid was produced by xerophyte called *R. vesicarius* L. which is growing in a desert of Riyadh, Saudi Arabia. Clavulanic acid was characterized by spectroscopic analyses including nuclear magnetic analysis (NMR), infra-red analysis (IR) and elemental analysis. Eventually, it has been proved that amoxicillin acts synergistically with clavulanic acid to inhibit nosocomial resistant pathogenic *P. aeruginosa* at 125 and 128 $mgml^{-1}$ respectively.

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