POTENTIAL OF AQUEOUS EXTRACTS OF *Allium sativum, Mentha spicata, Myrtus communis* AND *Thymus vulgaris* AS ANTIMICROBIALS AND CURING OF ANTIBIOTIC RESISTANT GENES IN *Klebsiella pneumoniae.*

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Abstract

Eighty six clinical isolates of Klebsiella pneumoniae were isolated from various pathogenic cases from Erbil hospitals. The isolates were varied in their resistance to antibiotics, and ranged between 13-18 antibiotics out of 22. Genetic transformation between purified plasmid from K. pneumoniae and E. coli JM83 demonstrated that all tested antibiotic resistant genes were plasmid DNA born except Doxycilin resistance gene was chromosomal born. The watery extracts of Allium sativum, Mentha spicata Myrtus communis and Thymus vulgaris were proved to be most powerful against K. pneumoniae K11 and K32 isolates. A comparative evaluation of plasmids elimination from K. pneumoniae isolates by sub-MIC of plant extracts showed that these extracts could cure plasmids effectively at their respective sub-MIC concentration. Plasmid cured was observed by sub-MIC of Allium sativum and Thymus vulgaris at 350 µg ml^{-1} cured 2, 4 and 3 plasmids, Mentha spicata and Myrtus communis extract at 250 µg ml^{-1}cured 2, 3 and 2, 4 plasmids from K. pneumoniae K11 and K32 isolates. Curing of plasmid DNA from K. pneumoniae isolates confirmed by determining the loss of resistance markers in cured cultures.

Keywords: *Klebsiella pneumoniae, Allium sativum, Mentha spicata, Myrtus communis, Thymus vulgaris*

1- Introduction

Klebsiella pneumoniae is an opportunistic pathogen involved in the outbreaks of nosocomial infections including lower respiratory, urinary tract, burn and wound infections (Rasool *et al*., 2003). In fact, nosocomial infections associated with *Klebsiella* spp., have shown an increase in most parts of the world, and it exhibits simultaneous resistance to multiple drugs (Gutman *et al*., 1985).

In recent years, the escalation of multidrug resistance in bacteria has gained worldwide attention due to the high impact on public health. Increased usage of antimicrobial agents to treat bacterial infections has lead to the emergence of multi drug resistant (MDR) strains (Bonnet, 2004), the increasing of MDR incidence in the genetic and mechanisms of resistance evolved by bacteria, as such information could lead to strategies for counter acting the effect of antimicrobial resistance.

Studies from different geographical areas, especially rarely studied areas such as the Middle East, urgently needed, because antimicrobials are available to the public and with different origins and low qualities are distributed. Therefore the aim of this study was to evaluate the effects of aqueous extracts of *Allium sativum, Mentha spicata, Myrtus communis and Thymus*

vulgaris as antimicrobials and elimination of antibiotic resistance in isolated *K. pneumoniae* from different sources in Erbil hospitals.

2 Methodology

2.1 Bacterial isolates

Eighty six isolates of *K. pneumoniae* were randomly collected from several hospitals in Erbil city, Iraq. These isolates were recovered from various specimens; Urine, wound, ear, stool, and sputum were considered to be the causative agent of the patient's illness in most cases during July 2004 and December 2004. The study conducted in Biology Department, college of Education/Scientific Departments, University of Salahaddin, Erbil, Iraq. Data concerning antibiotic usage against these isolates are not available. All isolates were identified by conventional techniques (Murray *et al.*, 1995), confirmed where necessary by the API20E system (Biomerieux, Marcy-I' Etoile, France) and stored in nutrient broth containing 20% glycerol at -20^{o}C until further analysis.

2.2 Bacterial strains

The standard strain of *E. coli* JM83 serotype obtained from George M. Wenstok, Department of Biochemistry and Molecular Biology, University of Texas USA, was used in this study. The organism was maintained on nutrient agar slants at 4^{o}C.

2.3 Antimicrobial sensitivity test

The antimicrobial sensitivity phenotypes of all bacterial isolates were determined using dilution method in nutrient agar plates (Oxoid, Cambridge Uk) according to (Atlas *et al.*, 1995). The antimicrobials purchased from (sigma company, Germany), are Amoxicillin (Amo), Amikacin (Amk), Ampicillin (Amp), Cefotaxime (Cef), Cephalothen (Cep), Ciprofloxacin (Cip), Chloramphenicol (Chl), Doxycyclin (Dox), Gentamycin (Gen), Kaflex (Kaf), Kanamycine (Kan), Lincomycine (Lin), Moxifloxacin (Mox), Nalidixic acid (Nal), Nitrofuration (Nif), Norfloxacin (Nor), Pan-cloxacillin (Pan), Penicillin (Pen), Rifampicin (Rif), Streptomycin (Stm), Tetraciclin (Tet), Trimethoprim (Tri). These antimicrobials were used at final concentrations or plant extracts were added to the medium after sterilization and cooling to 50^{o}C, the medium was mixed and poured into Petri-dishes, then inoculated with isolated bacteria using streaking method, susceptibility or resistance were recorded after incubation for 24 hr at 37^{o}C.

2.4 Determination of in Vitro antimicrobial effects

The aqueous extracts of *Allium sativum, Mentha spicata, Myrtus communis and Thymus vulgaris* were tested against two clinical isolates of *K. pneumoniae* K11 and K32 from patients with wound infection in Erbil hospitals.

2.5 Determination of Minimum Inhibition Concentration (MIC)

The MIC of plant extracts was determined by a broth dilution method in test tubes. Standard inoculums contain 10^{6} CFU ml^{-1}depending on standard curve was added to a series of tubes containing increasing concentration of plant extracts being tested, and

then incubated at 37^{o}C for 24 hr. The MIC is quoted as the lowest concentration of plant extracts which inhibit the viable growth of microorganisms (Bauman, 2007).

2.6. Selection of medicinal plants

Four medicinal plants *A. sativum, M. communis, M. spicata and T. vulgaris* were selected. The seeds or plant leaves were authenticated at the Herbarium of the Department of Biology,College of Education/Scientific Departments, Salahaddin University, Erbil, Iraq.

2.7 Preparation of plant extracts

Leaves of *A. sativum, M. communis, M. spicata and T. vulgaris* were ground into slightly coarse powder using electric blender. Extracts were prepared by soaking leaves portions 20 gm of the dried powders with 300 ml of double distilled water were extracted at the rate of 150 ml min^{-1} by steam distillation for 2-3 hr (Vinatoru *et al.,* 1997), then the extract was evaporated to dryness by Rota vapor. The organic extract was kept overnight under vacuum fame hood to obtain a constant dry weight. The extract weighed and stored in closed vessel at 4^{o}C in refrigerator for further use.

2.8 Elimination of antibiotic resistance

A small inoculum 10^4 bacteria ml^{-1} was grown overnight at 37^{o}C in nutrient broth containing sub inhibitory concentration of medical plant extract, giving in complete inhibition. The cultures were plated on agar, and isolated colonies tested for antibiotic resistance.

2.9 Plasmid extraction

Large-scale plasmid DNA preparation from *K. pneumoniae* was performed followed the method of Birnboim and Doly, 1979.

2.10 Preparation of competent cells

A modified method of Sambrook *et al.,* 1989 was used for preparation of competent cells for transformation. *E. coli* JM83 cells. An overnight culture of *E. coli* was prepared by suspending a colony from a fresh nutrient agar plate in 100 ml nutrient broth. The culture was incubated in a shaking incubator at 100 rpm at 37^{o}C for 24 hr. 10 ml of this culture was then suspended in 90 ml fresh nutrient broth and grow for 90 minutes to an OD 600nm of approximately 0.3. Ten ml aliquots were centrifuged at 4000 X g for 10 min to pellet the cells. The supernatant was discarded and the cells were washed in 5 ml ice-cold 10mM NaCl and re-countergauged. The supernatant was discarded and cells were resuspended in $CaCl_2$ and recenterifuged, twice. Following a third washing with 5 ml 30 mM $CaCl_2$, cells were then recenterfuged and resuspended in 1 ml of ice-cold 30 mM $CaCl_2$ +15% glycerol. The cells could be used immediately for transformation or stored at -20^{o}C.

2.11 Transformation process

Two hundred µl of *E. coli* JM83 competent cells were mixed with 2 µl of extracted plasmid, the tubes then left on ice for 30 min then incubated for 30 seconds at 42^{o}C in

a water bath and placed on ice for a further 5 minutes. 800 µl of nutrient broth was added and the tubes were incubated at 37^{o}C, then the cells were centrifuged at 6000 X G for 1 min. the pellet was resuspended in 200 µl of nutrient broth, 100 µl of the cells were plated out on nutrient agar containing appropriate antibiotic markers. Post incubation, the plates were screened for recombination, which were selected and streaked onto fresh agar plates for plasmid purification (Ausabel *et al*., 2003).

2.12 Preparation of agarose gel electrophoresis

The method of Sambrook *et al.,* 1989 was used for electrophoresis by using 0.75% agarose, 10 µl of ethedium bromide at final concentration 0.5 µg/ml was added, the mixture was poured onto gel tank surrounded by a gel former, the comb was inserted and the gel was allowed to set, the comb and surrounded cover removed and the gel smoked in a gel tank contain Tris-borate buffer. 15 µl of plasmid DNA samples were mixed with 3 µl of loading buffer dye finally 10 µl of this mixture were added to the wells, and then the gel tank was covered by lid. Gel was run at 60 volt for 2 h, the gel examined by ultraviolet illumination and photographed by Digital Camera.

3. Results

A total of eighty six clinical isolates of *K. pneumoniae* were isolated from hospitals in Erbil city-Iraq. All isolates were screened to 22 antibiotics. All isolates 100% were resistant to Amp, Amo. Cph, Chm, Dox, Gen, Kan, Lin, Pac, Pen and Trm, the lowest resistance recorded toward Rif 38.37%. All isolates 100% were sensitive to Cip, Mox, Nit, and Nor table (1). The MIC values of aqueous extracts were 400, 300, 250, and 300 µg ml^{-1} for *Allium sativum, Mentha spicata, Myrtus communis and Thymus vulgaris* respectively Table (2).

Table 1: Susceptibility of *K. pneumoniae* isolates to different antibiotics.

Antibiotics	Number of resistant isolates	Resistant %
Amk	6	69.7
Amp	86	100
Amo	86	100
Cef	67	77.9
Cph	86	100
Chm	86	100
Cip	0.0	0.0
Dox	86	100
Gem	86	100
Kaf	85	98.8
Kan	86	100
Lin	86	100
Mox	0.0	0.0
Nal	61	70.9
Nit	0.0	0.0
Nor	0.0	0.0
Pac	86	100
Pen	86	100
Rif	33	38.37
Stm	82	95.34
Tec	65	75.58
Trm	86	100

Transformation process proved that *E. coli* JM83 had the ability to receive purified plasmid DNA from *K. pneumoniae* K11 and K32 isolates, and transferred successfully. All genes conforming resistance to tested antibiotics used are located on plasmid DNA except Doxycycline gene seems to be chromosomally located.

Aqueous extracts of these plants were tested for curing of antimicrobial resistance against two multidrug resistances clinical *K. pneumoniae* K11 and K32 isolates, and the preliminary assessment of the curing or elimination of antibiotic resistance of these two isolates (table 3 and 4), reveled that *A. sativum* extract at 350 µg $ml^{-1}$was more effective and cured 100% resistance of Amp, Amo, Cef, Kan, Kaf, and Pen and 33.3% for Chl resistance genes in K11 isolate, while for K32 isolate reduced 100% the resistance for Amk, Amp, Amo, Cph, Kaf, Pen, Rif and Tec, and 67-95% for Stm, Gme and Kan. The spontaneous curing for these two isolates were zero table (3). *M. specata* at sub-MIC 300 µg ml^{-1} influenced the genes responsible for Amo, Cph, Chm, Gem, Kan, Kaf, Pen and Stm 16.60 to 50% for K11 isolate, while for K32 isolate reduced resistance for Amk, Amp, Amo, Cph, Cef, Gen, Kaf, Pen, Rif, and Tec 3.33 to 94.8%. *M. communis* at 200 µg ml^{-1} affected Amp, Cph, Gen, Kaf, Pen, and Stm resistance genes and reduced resistance 33.3 to 100% for K11 isolate, and 45.5 to 100% for Amk, Amp, Cef, Gen, Kan, Kaf, Pen, Stm, and Tec in K32 isolate.

Table 2: Optical density reading at 600 nm of *K. pneumoniae* isolates for different concentrations (µg/ml) and different plant extracts.

Treatments	Concentration of different plant extracts (µg/ml)										
	25	50	100	150	200	250	300	350	400	450	500
T.vulgaris+K11	1.74	1.62	1.51	1.14	1.11	0.45	0.27	0.02	0.0	0.0	0.0
T.vulgaris+K32	1.74	1.62	1.59	1.41	1.24	0.58	0.36	0.09	0.0	0.0	0.0
A. sativum+K11	0.21	0.16	0.15	0.14	0.12	0.11	0.09	0.08	0.04	0.02	0.0
A. sativum+K32	0.23	0.18	0.16	0.14	0.12	0.10	0.08	0.05	0.04	0.03	0.0
M. specata+K11	0.11	0.06	0.03	0.01	0.002	0.0	0.0	0.0	0.0	0.0	0.0
M. specata+K32	0.12	0.07	0.04	0.02	0.01	0.0	0.0	0.0	0.0	0.0	0.0
M. communis+K11	0.15	0.14	0.08	0.07	0.04	0.0	0.0	0.0	0.0	0.0	0.0
M. communis+K32	0.15	0.14	0.09	0.05	0.01	0.0	0.0	0.0	0.0	0.0	0.0
Nutrient broth+K11	2.31										
Nutrient broth+K32	2.46										

When 300 µg ml^{-1} *T. vulgaris* extract was treated against K11 isolate affected Amp, Amo, Cph, Gen, Kan, Kaf, Pen, Str, Trm, and reduced resistance 6.66 to 96.6%, while for K32 isolate affected Amk, Amp, Amo, Cph, Cef, Gen, Kan, Kaf, Nal, Pen, Rif. Str, and Tec genes and reduced resistance 6.66 to 96.6%.It is interesting to note that sub-MIC of treated plant extracts apparently cured the plasmids from *K. pneumonia*e K11 isolate treated cells (fig 1), sub-MIC of *A. sativum* aqueous extract cured two plasmids from K11, and three plasmids

from K32 isolate fig. 1. *M. specata* and *T. vulgaris* eliminated 2 and 3 plasmids from K11 and K32 isolates fig. 2 and 3, while *M. communis* reduced two plasmids from K11 isolate and four plasmids from K32 isolate fig 4. Curing of plasmid from *K. pneumoniae* isolates was confirmed by determining the loss of resistance markers in cured cultures e.g. aqueous extract of *A. sativum* at sub-MIC losses resistance to seven antibiotics from 22 antibiotic markers table (3).

4. Discussion

The clinical isolates of *K. pneumoniae* showed resistance phenotypes to thirteen or more antibiotic agents were detected in all these isolates in Erbil city hospitals, this may be due to widespread and discriminate use, and the antibiotics are available to the public, or the use of antibiotics in animal feeds is another factor to increasing antibiotic resistance. In this study, we screened and characterized antimicrobial resistance plasmids of *K. pneumoniae* isolates in Erbil hospitals. Similar studies have been reported from Italy (Mugnaioli *et al*., 2006), Brazil (Minarini *et al.*, 2008), Purtugal (Machado *et al*., 2007) and Canada (pitout *et al*., 2008), where the highest proportion were *K. pneumoniae* isolates. Hospital-acquired infections were mainly caused by *K. pneumoniae* and other Enterobacteriaceae isolates (Coque *et al*., 2008). Antibiotic resistance phenotypic profiles were noticed in previous studies, especially with bacterial isolates that showed close genetic determinants in other countries (Machado *et al*., 2006; pitout *et al*., 2008 and Minarini *et al*., 2008).

Table 3: Curing percent of plasmid DNA from *K. pneumoniae* K11 and K32 isolates by *A. sativium*, T. vulgaris, *M. specata and M. communis*

Treatments	Antibiotics (µg/ml)																	
	Amk	Amp	Amo	Chp	Cef	Chm	Dox	Gem	Kan	Kaf	Nal	Lin	Pen	Pan	Rif	Stm	Tec	Trm
A. sativum+ K11	N	100	100	100	N	33	0	100	100	100	N	0	100	0	N	90	N	o
A. sativum+ K32	100	100	100	100	0	0	0	87	95	100	0	0	100	0	100	67	100	00
T.vulgaris+K11	N	31	7	75	N	0	0	53	27	87	N	0	7	0	N	97	N	190
T.vulgaris+K32	70	7	33	33	30	0	0	70	47	30	20	0	33	0	54	97	73	0
M. specata+ K11		30	19	37		30	0	47	23	50		0	17	0		7		0
M. specata+ K32	93	95	3	27	17	0	0	33	0	13	0	0	10	0	20	0	50	0
M. communis+K11		100	0	43.3		0	0	100	0	33.3		0	70.6	0		33.6		0
M. communis+K11	100	100	0	0	100	0	0	100	53.3	88.7	0	0	93.4	0	0	45.5	63.3	0
Spontaneous Curing for K11	N	0	0	0	N	0	0	0	0	0	N	0	0	0	N	0	N	0
Spontaneous Curing for K32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

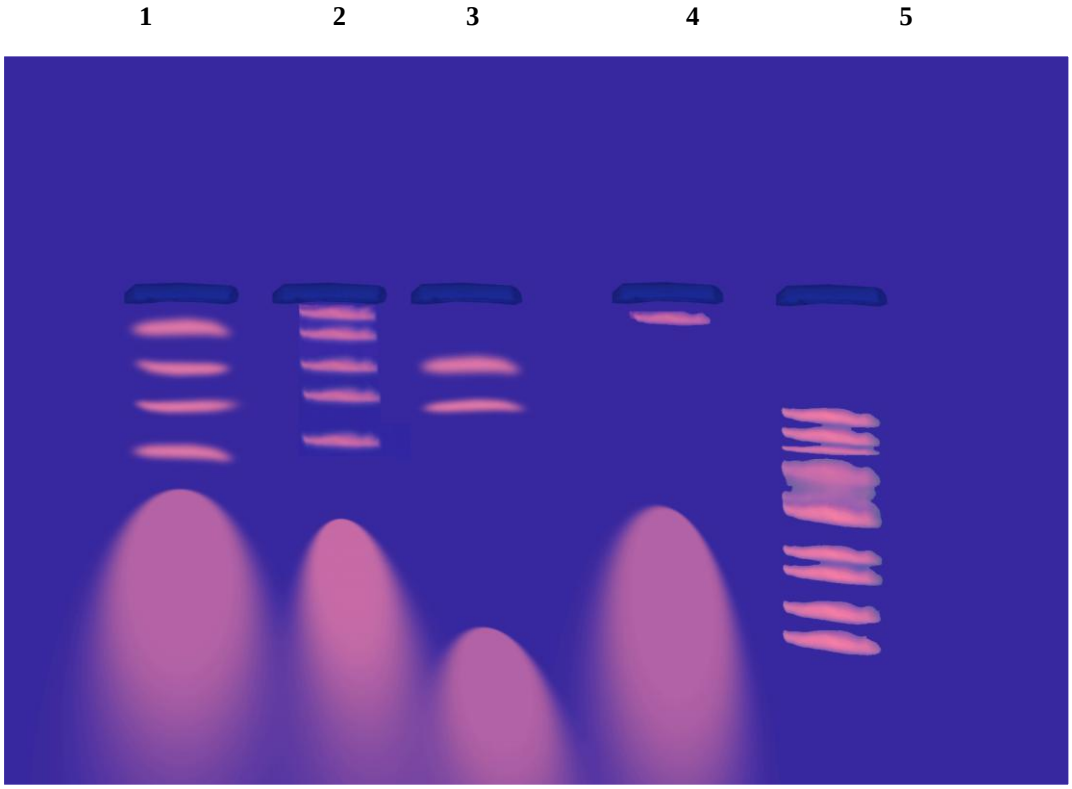

Figure 1. Plasmid DNA profile of the cured bacterial cells of *K. pneumoniae* K11 and K32 isolates by *A. sativum* extract at 350µg/ml:
Lan (1): K11 (*K. pneumoniae* isolate).
Lan (2): K32 (*K. pneumoniae* isolate).
Lan (3): K11 (*K. pneumoniae* isolate after treating with extract).
Lan (4): K32 (*K. pneumoniae* isolate after treating with extract).
Lan (5): plasmid DNA marker.

Plasmid DNA location of 21 resistance genes out of 22 tested antibiotics, explains high resistance of tested isolates, due to easily transferring resistance genes among bacteria by transformation, Conjugation or transduction.

Broad spectrum activity of sub-MIC of these plant extracts on *K. pneumoniae* K11 and K32 isolates Table 3, e.g. where the sub-MIC of aqueous extract for studied plants reduced resistance of these two isolates for most antibiotics except for Dox, Lin, Pan, and Tri when *A. sativum* was treated, Dox, Lin, Pan, Tri, and Chm when *T. vulgaris* and *M. communies* were treated, Dox and Pan when *M. specata* extract was treated, this finding is an apparent indication that the plant extract might have a different mode of action than commonly used antibiotics. Antimicrobial activity of these plants, and active constituents were reported by other researchers, main constituents found in steam distillation of garlic clove termed Allicin which is considered as broad spectrum antimicrobial for gram negative and gram positive bacteria, the main antimicrobial of Allicin is due to its interaction with important thiol containing enzymes, through inhibition of RNA synthesis via inhibition of RNA polymerase present in bacterial DNA, and protein synthesis are also partially inhibited by Allicin in *Salmonella typhimurum*, they suggest that RNA polymerase be a target for Allicin (Feldberg *et al.*, 1988, Shareef, 1998). Allicin also specifically inhibit other bacterial enzymes such as acetyl co A- forming system consisting of acetate kinase and phosphotransacetyl-co A

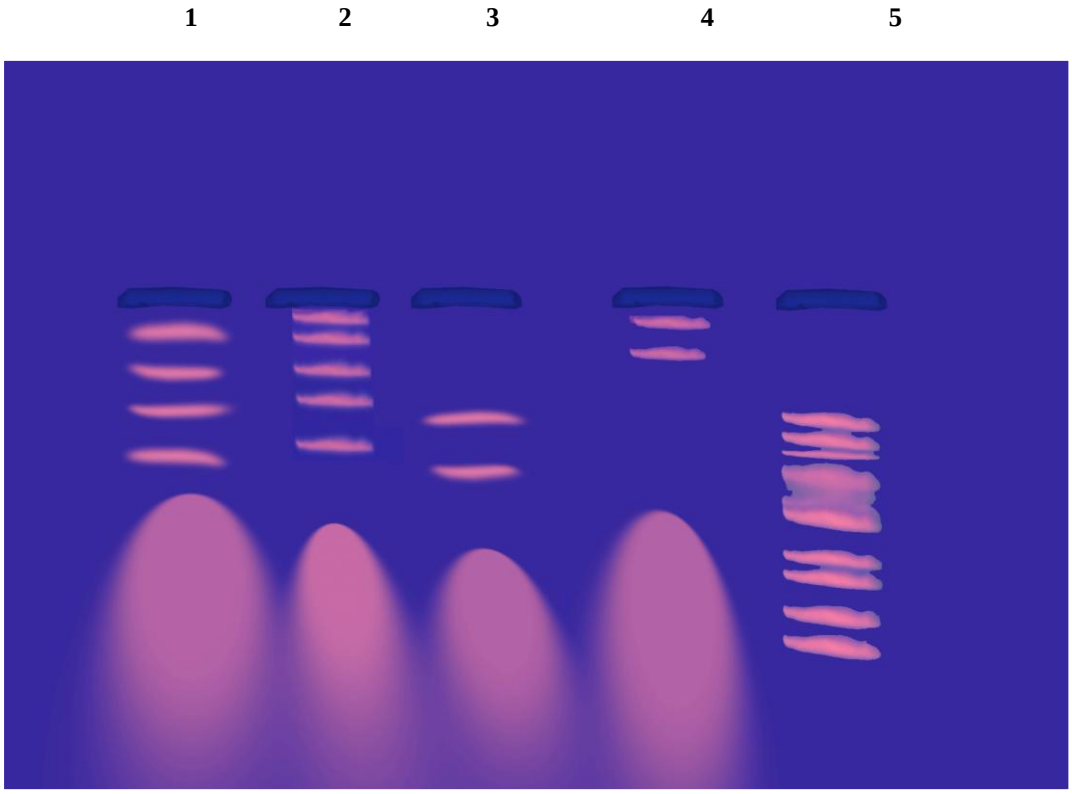

Figure 2. the plasmid DNA profile of the cured bacterial cells of *K. pneumoniae* K11 and K32 isolates by *T. vulgaris* extract at 300µg/ml:
Lane (1): K11 (*K. pneumoniae* isolate).
Lane (2): K32 (*K. pneumoniae* isolate).
Lane (3): K11 after treating with T. vulgaris extract.
Lane (4): K32 after treating with T. vulgaris extract.
Lane (5): plasmid DNA marker.

synthetase (Ficke *et al.*, 1990). Agoene present in garlic clove is oxygenated group, (Klepser, 1998) showed that Ajoene has an inhibition action on different bacteria including *K. pneumoniae*, and found that the concentration of 152 µg ml^{-1} act as MIC on *K. pneumoniae*. The effect of thyme extract in inhibition of bacterial isolates regarding its chemical consistituents which composed chiefly from thymol, carvacrol and p-cymen (Schulz *et al*., 2005 and Wooten, 2006), the mechanisms though to be responsible for toxicity to microorganisms including enzyme inhibition, the oxidized compounds, through reaction with non-specific interactions with the proyrin (Mason and Wasserman, 1987), or act in the damage to cytoplasmic membrain through the loss in the membrane ability to act as a permeability barrier (Matkowski and Wolniak, 2005). The antimicrobial effects of thymol and menthol are regarded to perturbation of the lipid fraction of bacterial plasmamembrain resulting in alteration of membrain permeability and in leakage of intracellular materials (Trombetta *et al*., 2005).

The inhibitory action of *M. communis* is due to presence of Tannin which is reported to have inhibitory activity against bacteria by forming H bonds with proteins, and converting their structures leading to blockage in protein synthesis (Appendino *et al*., 2002).

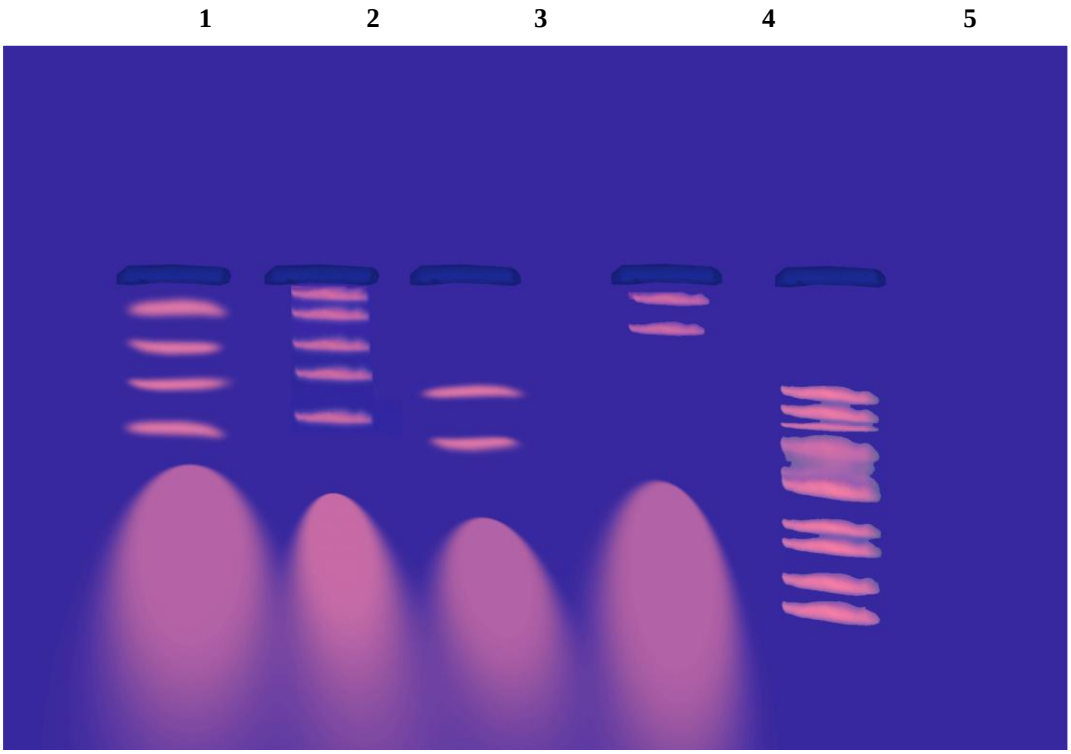

Figure 3. Plasmid DNA profile of the cured bacterial cells of *K. pneumoniae* K11 and K32 isolates by *M. spicata* extract at 200µg/ml:

Lane (1): K11 (*K. pneumoniae* isolate).
Lane (2):K32(*K. pneumoniae* isolate).
Lane (3): K11 after treating with extract.
Lane (4): K32 after treating with extract.
Lane (5): plasmid DNA marker

5. Conclusions

The clinical isolates of *K. pneumoniae* appeared resistance to more than 13 antibiotics out of 22. Transformation process revealed that antibiotic resistance genes of tested isolates are located on plasmid DNA, except Doxycilin was chromosomally location. Sub-MIC of *Allium sativum, Mentha spicata, Myrtus communis and Thymus vulgaris* proved to have antimicrobial activity, and acting as curing agent of decreasing antibiotic resistance in *K. pneumonia* K11 and K32 isolates, through curing of plasmids bearing such genes.
Further activity-guided fractionation of crude extracts is needed to determine the effective fractions, on the other hand data on *in vivo* toxicity of these plants extracts, stability of active components and protection against infections caused by drug-resistant bacteria are to be generated to determine the theraptic potential of these plant extracts.

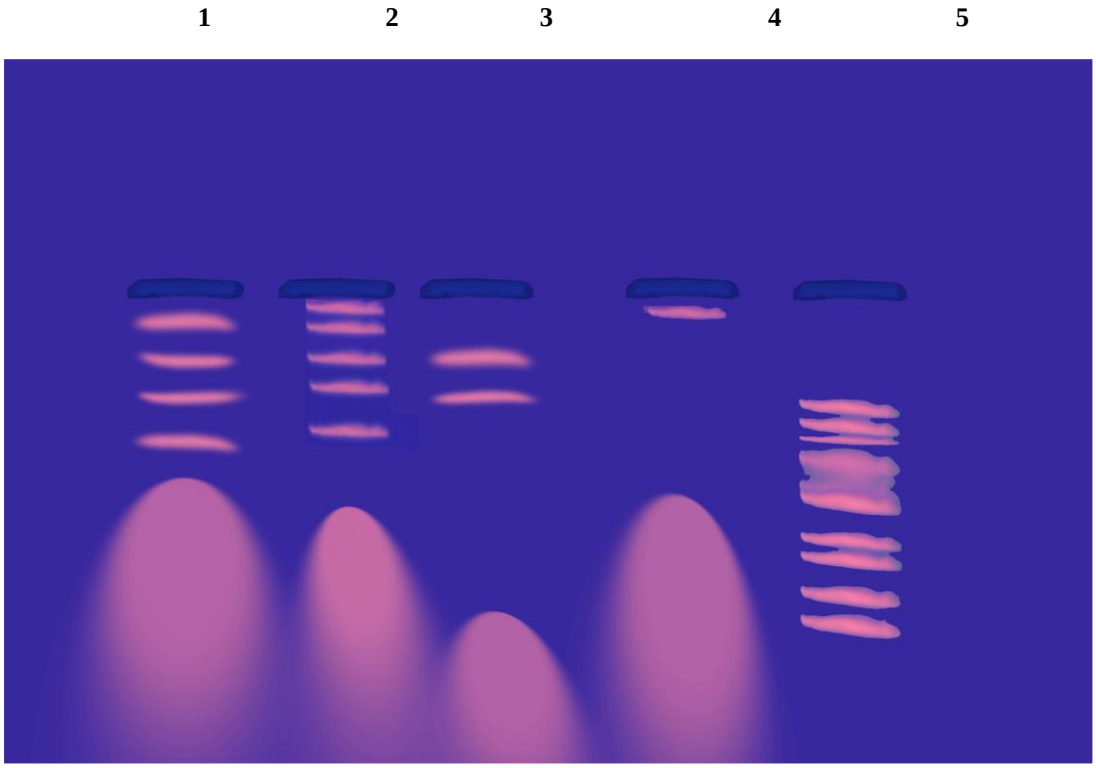

Figure 4. Plasmid DNA profile of the cured bacterial cells of *K. pneumoniae* K11 and K32 isolates by *M. communis* extract at 200µg/ml:

Lane (1): K11 (*K. pneumoniae* isolate before treating)
Lane (2): K32 (*K. pneumoniae* isolate before treating)
Lane (3): K11 (*K. pneumoniae* isolate after treating with plant extract)
Lane (4): K32 (*K. pneumoniae* isolate after treating with plant extract)
Lane (5): Plasmid DNA marker

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