JJBS

Volume 1, Number 3, September. 2008
ISSN 1995-6673
Pages 124 - 128

Jordan Journal of Biological Sciences

Effect of *Allium sativum* and *Myrtus communis* on the elimination of antibiotic resistance and swarming of *Proteus mirabilis*.

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Abstract

Proteus mirabilis isolated from clinical sources (urine, wound and burn) in Erbil city hospitals. These isolates were characterized culturally, morphologically, and biochemically. The API20E system was used to support their identification. All isolates were tested for their resistance to twelve different antibiotics with the resistance of six isolates to all of the tested antibiotics (antibiotype A6). However, A1 antibiotype isolates were more sensitive and were resistant to seven of the tested antibiotics. Transformation experiment revealed that the resistant genes of (Chl, Dox, Ery, Gm, Kaf, Lin, and Pen) in P7 isolate and the resistant genes of all tested antibiotics from P23 isolate are not chromosomally coded. Sub-MIC of watery extract of A. sativum eliminated 50 and76% Kaf, and Gm resistance genes in P23 isolate, and reduced Ery resistance genes only 16.6% for P7 isolate, while M. communis eliminated Pen 3.3%, and curing of plasmid confirmed by determining the loss of resistance markers in the cured derivative culture. The Sub-MIC of crude extract of A. sativum habited the swarming of P7 at the concentration of 200 and 250 µg/ml when Amp, Am, Ceh, Chl, Lin, and Pan-c were applied to the nutrient agar plates, and M. communis at the concentration of 200 µg/ml inhibited the swarming when Chl and Pan-c antibiotics are applied. Neither A. sativum nor M. communis extracts were inhibited swarming at any concentrations in P32 isolate.

الملخص

عزلت بكتيريا *Proteus mirabilis* من مصادر طبية مختلفة في مستشفيات مدينة اربيل اعتمادا على الصفات المزرعية، المظهرية و البايوكيميائية والتي ضمنت اختبارAPI20E. اختبرت مقاومة جميع العزلاته تجاه عشر مضادات حيوية. اضهرت نتائج عملية النقل الجيني بان الجينات المسؤلة عن مقاومة المضادات Dox, Ery, Lin, Chl, Pen للعزلة P7 و جميع المضادات المختبرة للعزلة P32 تقع على DNA البلازميدي. عند استخدام Sub-MIC للمستخلص المائي لنبات الثوم اختزلت مقاومة الجينات Gen و Kaf بنسبة50 -76% للعزلة P23 و كذلك Ery جين فقط بنسبة 16و6%، في حين مستخلص نبات المورت (M. communis وان تحييد البلازميدات وجدت من خلال فقد المقاومة للمضاد في المزارع. ثبط المستخلص الخام لكلا النباتين عملية swarming للعزلة P7 بتركيز 200و250 ميكروغرام/ سم^3 عند تواجد المضادات Amp, Amx, Cep, Chl, Lin, Pan-c في الوسط الزرعي، في حين200 مايكرو غرام/سم^3 ثبط عملية swarming للعزلة P7 عند تواجد المضاد الحيوي Chl, Pan-c.

Keywords: Allium sativum, antibiotic resistance, Murtus communis plant extract, Proteus mirabilis.

1. Introduction

It has been well established that the most genes that are responsible for antibiotic resistance are borne on a plasmid DNA in most strains of *P. mirabilis* (Khder, 2006 and Alski, 2002). It found that frequency of loss resulted after growth of some strains at elevated temperature (Al-Safawi, 2001) or after exposure to compound which interfere with deoxyribonucleic acid (DNA) replication Such as acridin dyes(Staner et al, 1984 & Khder, 2002), and ethidium bromide (Villar et al, 1981), SDS (Ahmad, 2002) or by medicinal plant extract (Mawlud, 2006 & Khder, 2006).

Swarming which are multinucleated, nonseptated cells 20-80 mm in length containing even 500 fold more flagella, this phenomena is important in the pathogenicity of bacteria, and is important in ascending urinary tract infection (*UTI*) which are more common in Proteus strains (Alski, 2002).

The aims of this study are studding the effect of A. *sativum* and *M. communis* extracts on elimination of antibiotic resistance and swarming of *P. mirabilis* isolated from different clinical origins in Erbil city hospitals.

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2. Materials and Methodes

Thirty isolates of *p. mirabilis* were isolated from various clinical specimen at Komary, Rizgary and Emergency hospitals in the city of Erbil, Iraq. The reference bacteria *Escherichia. coli* K12JM83 was kindly provided by Dr.Khaled Daham Ahmed- University of Mosul-Iraq. All isolates identified by cultural, morphological and biochemical tests. Moreover the API 20E (Bio Merieux, Marcyl, Etoile, France) system and oxidase test were performed. All isolates were biotype on the basis of susceptibility testing. The isolates and reference bacteria were maintained as frozen stocks at -70 ^{o}C in presence of 80% glycerol and cultured in nutrient broth for 18 hr at 37 ^{o}C, for later analysis.

2.1. Antibiotic Susceptibility Test

Susceptibility to different antibiotics was tested using Muller-Hinton agar medium (Al-Najjar, 1976). Variable commercially and widely used antibiotics were used {Ampicillin (Amp), Amikacin (Am), Cepholothin (Ceh), Chloramphenicol (Chl), Doxicillin (Dox), Erythromycin (Ery), Gentamycin (Gm), Kanamycin (Kan), Kafalexin (Kfa), Lincomycin (Lin), Nalidixic acid (NA) and Penicillin (Pen). These antibiotics were used at final concentrations or plant extracts were added to medium after sterilization and cooling to 50 °C, the medium were mixed and poured into Petri-dishes, and inoculated with isolated bacteria using streaking method. Susceptibility or resistances of Proteus isolates to these antibiotics were recorded after incubation for 24 hr at 37^{o}C.

2.2. Genetic Site Determination of Antibiotic Resistant Genes.

Plasmid DNA from proteus isolates was extracted by following the method that described by (Birnboim and Doly, 1979). Transformation process was performed to determine the location of antibiotic resistance genes of tested isolates using the method of (Mandel and higa, 1970). The isolated plasmid DNA has been transformed to *E. coli* K12JM83 strain which has the following genotype {ara, Δ(lac pro A,B), rpsl, Ø80, lacZ ? M15, JM83 r^{+k} m^{+k} piR}. Competant cells were prepared using the method described by (Mandel and Higa 1970). A culture of *E. coli* K12Jm83 was prepared by inoculation of single colony in a test tube containing 5 ml of nutrient broth, then incubated with shacking (100rpm) for 18-24 hr at 37^{o}C, After growth, 1 ml of this bacterial culture was added to a flask containing 50 ml nutrient broth, then incubated under the above condition until the culture reach to active logarithmic phase with optical density of 0.5 at 600nm. The cells were harvested by centrifugation at 8000 rpm, and then resuspended in 1 ml of cooled transformation buffer (TE buffer pH 8, 50 mM $CaCl_2$ and 10mM Tris-HCl pH8), and the volum was completed to 40 ml using the same buffer. Resuspended cells were left in ice for one hour, and then centrifuged for 15 minutes at the same speed. Finally the pellet was resuspended in 1 ml of cooled transformation buffer.

To increase the efficiency of genetic transformation, the competent cells were kept at 4ºC for 24 hours before adding the plasmid. Transformation of plasmid DNA was performed by following the method of (Lederberg and Cohn, 1974). One hundred µl of prepared plasmid DNA was added to a tube containing 0.2 ml of competent cells, the mixture was placed in ice for 30 minutes, exposed to heat shock at 42ºC for 6 min (Hoekstra et al, 1980), then 1 ml of fresh nutrient broth was added to transformation mixture and then incubated at 37ºC for 60 minutes.

Aliquots of 0.1 ml from the transformation mixture were spread on the surface of 5 nutrient agar plates containing the appropriate antibiotic. Control plates were prepared by spreading of 0.1 ml of competent cells on the surface of nutrient agar containing the same antibiotics used as control. All plates were incubated at 37ºC for 48 hours. Number of transformant colonies were scored and purified several time on plates containing the different tested antibiotics used. The genetic transformation frequency was calculated according to (Puhler and Timmis, 1984).

2.3. Preparation of Plant Extracts

Extraction of *Allium sativum* and *Myrtus communis* was performed using Steam distillation technique as described by (Adler and Irobi, 1993). Twenty gram of plant powder was extracted using 300 ml deionized distilled water for 2-3 hours, with a distillation rate of 150 ml/minute (El- Astal et al, 2004; Rassol, 2004). The extract was dried for 2-3 hr using rotary evaporation (Bibby Re200 U.K.) (Rose et al, 1987), and then the extract was sterilized by membrane filtration using 0.22µ pore size membrane filters (Sharref, 1998).

2.4. Determination of MIC of Plant Extract.

MIC (minimum inhibition concentration) of plant extracts or chemical material (menthol) were performed through addition of different concentrations (25 µg to 750 µg) of the sterilized plant extracts to a sterilized nutrient broth, after incubation time for 24 hr at 37^{o}C with shaking, the turbidity was recorded using spectrophotometer at 600nm (Atlas et al, 1995).

2.5. Curing of Plasmid DNA

Plasmid DNA was cured by using pure menthol or water extracted from the tested plants as described by (Tomoeda et al, 1974). MIC of menthol and plant extracts was determined against the tested bacteria. Nutrient broth containing a range of concentrations (50-800 µg/ml) was prepared and 0.1 ml of fresh grown culture was inoculated in each test tube, then 0.1 ml was distributed on nutrient agar plates, then the plates were incubated at 37 ^{o}C for 24 hr then colonies were counted. The lowest concentration showing no significant viable count compared to the control plates (untreated plates) when plated on nutrient agar mediums was considered as the MIC of that curing agent/or plant extract. A range of sub-MIC concentrations were selected to treat the culture. A freshly grown culture of 0.1 ml was inoculated in each tube containing different concentrations of the curing agent. Control tubes were prepared without addition of curing agents. Tubes were incubated at 37ºC for 18 h. Cell broth was diluted by using a sterile normal saline and then spread on the surface of nutrient agar plates. Plates were incubated at 37ºC over night. Isolate colonies were replica-plated on to nutrient agar plates containing antibiotics to which the test bacterium was resistant. A plate without antibiotic was simultaneously also inoculated as control. Percent of cured colonies were determined by taking (the mean count of the colonies from antibiotic agar plates that did not grow of total mean colonies tested) X100.

3. Results

Clinical isolates of *Proteus mirabilis* isolated from different patients in three hospitals in the city of Erbil-Iraq were identified culturally, morphologically and biochemically. The API20E system was also used to support the identification process. All isolates were tested for their resistance profiles to twelve antibiotics representing different groups. The resistance pattern is shown in table (1), with the resistance of six isolates (P1, P23, P24, P26, P15, and P21) to all of the tested antibiotics (antibiotype A6). Isolates (P7, P11, P12, P29, and P30) of A1 antibiotype were more sensitive and were resisting to seven of the tested antibiotics.

To determine if the antibiotic resistance is encoded by plsmid or chromosomal DNA transformation process was performed to the most sensitive or resistant isolates of *Proteus mirabilis* and *E. coli*K12JM83 strain Table (2). Extracted plsmid DNA from P7 and P23 transferred successfully to JM83 strain, this derives us to conclude that the (Ch, Dox, Ery,Gm, Kaf, Lin, and Pen) in P7 and the resistant genes of all tested antibiotics in P23 are not chromosomally coded.

Table 1: Antibiogram groups, and numbers for *P. mirabilis* isolates.

Antibiogram	Amp	Amx	Ceh	Chl	Dox	Ery	Kan	Lin	Nal	Pen	Isolate No.	No.of antibiic resist
A1	-	-	-	+	+	+	-	+	-	+	7, 11, 12, 29, 30	5
A2	+	+	-	+	+	+	-	+	-	+	6, 13, 14	7
A3	+	+	+	+	+	+	-	+	-	+	8, 9	8
A4	+	+	-	+	+	+	+	+	-	+	3, 5,	8
A5	+	+	-	+	+	+	+	+	+	+	16, 17, 18, 19, 20, 22	9
A6	+	+	+	+	+	+	+	+	+	+	1, 23, 24, 26, 15, 21	10

Table 2: Number of transformation colonies and transformation frequency for P7 & P23 isolates of P. mirabilis.

Isolat	Am	Amx	Cep	Chl	Dox	Ery	Kan	Lin	Nal	Pen	Transformation frequency
P7 A1	S*	S	S	30	30	30	S	30	S	6	
P23 A6	18	20	30	30	30	30	30	30	21	20	

*: The bacteria are sensitive

Table 3: Curing % of *P. mirabilis* P23 andP7 isolates after treating with sMIC of *A. sativum* and *M. comunis* extract.

P23	sMIC µg/ml	Am	Amx	Cep	Chl	Dox	Ery	Gm	Kan	Kaf	Lin	Nal	Pen	Curing frequency
A. sativim	700	0.0	0.0	0.0	0.0	0.0	0.0	76.6	0.0	50	0.0	0.0	0.0	
M. comunis	700	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.3	0.23
P7	sMIC µg/ml	Chl	Dox	Ery	Kan	Lin	Nal	Pen	-	-	-	-	-	
A. sativum	700	0.0	0.0	0.0	0.0	0.0	16.6	0.0	-	-	-	-	-	1.15
M. comunis	700	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	-	-	-	0.0
Menthol														
For P23	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
For P7	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	-	-	-	0.0

Table 4: Swarming of *P. mirabilis* after treating with *A. sativum* and *M.communis* extracts.

Isolate No.	Plant extract µg/ml	Antibiotics used at final concenterations										
		Amp	Amx	Ceh	Chl	Dox	Kan	Lin	Pen	Pan-c	St	Tri
P7 P23	Garlic 200	No* Sw**	No Sw	No Sw	No Sw	Sw Sw	Sw Sw	No Sw	Sw Sw	No Sw	Sw Sw	Sw Sw
P7 P23	Garlic 250	No Sw	No Sw	No Sw	No Sw	Sw Sw	Sw Sw	No Sw	Sw Sw	No Sw	Sw Sw	Sw Sw

P7 P23	Murts 200	No Sw	No Sw	No Sw	No Sw	Sw Sw	Sw Sw	No Sw	Sw Sw	No Sw	Sw Sw	Sw Sw
P7 P23	Murts 250	Sw Sw	Sw Sw	Sw Sw	No Sw	Sw Sw	Sw Sw	Sw Sw	Sw Sw	No Sw	Sw Sw	Sw Sw

*No: No swarming,**Sw: Swarming.

In table (2) MIC of watery extract for A. sativum and M. communis were determined (750µg/ml) and sub MIC (700µg/ml) was used as curing agent against multidrug resistance (was resist to all tested antibiotics) P23 and more sensitive isolate P7 (was resist to seven antibiotics). Antimicrobial activity was exhibited when plant extract used irrespective to drug resistance pattern of tested bacteria table (3). A. satevum eliminated 50 and 76% Kaf and Gm resistance genes in P23 respectively, and reduce Ery resistance genes only 16.6% for P7 isolate, while *M. communis* 3.3% eliminated Pen gene.

The effect of SMIC of both plant extracts on swarming of P7 & P23 isolates was determined, and the results were combined table (4). Swarming was inhibited in P7 only when 200 and 250 µg/ml of A. sativum or 200 µg/ml M. communis was applied when Amp, Amx, Ceh, Chl, Lin and Pan-c was applied to culture media, while no swarming was inhibited when 250 µg/ml M. communis was applied for P7, except with present of Chl and Pan-c swarming was inhibited. Swarming was not eliminated either using 200 or 250 µg/ml A. sativum extract or 200 and 250 µg/ml M. communis extract when applied for P23 isolate.

4. Discussion

Emergence of multidrug resistance in pathogenic bacteria has created immense clinical problem in the treatment of infectious disease. Elimination of plasmid DNA mediated antibiotic resistance in pathogenic bacteria is great practical significance both in chemotherapy of bacteria and in microbial genetics. Elimination of plasmid DNA can be performed using different chemical materials including plant extracts (Reuter and Sendel, 1994). Sulfur amino acids that is present in large quantity in garlic clove in which they named alliin, which was found to be the stable percussive that is converted to allicin by the active of enzyme termed allinase, which is also present in cloves of A. sativum (Mawlud, 2006). Allin is considered as a broad spectrum antibacterial for G^+ & G^- bacteria (Lawson, 1996; Sharref, 1998 and Feldberg et al, 1988). The main antimicrobial effects of Allicin is due to its interaction with important thiol containing enzymes, also it is believed to exert its primary antimicrobial effect through the inhibition of RNA synthesis via inhibition of RNA polymerase which seen in E. coli, and protein synthesis are also inhibited by allicin in S. typhimarium, and suggested that RNA polymerase be target for allicin (Feldberg et al, 1988; Ozolin and Kaxazolova, 1990 and Sharref, 1998), and other inhibited enzyme thiol-containing enzyme, alcohol dehydrogenase, and thioredoxin reductase (Ankir et al, 1997). Ajoen is antimicrobial which present in garlic cloves, and antimicrobial of ajoen is through additional microbe-specific enzymes may also target for allicin and wide spectrum antimicrobial effects of allicin and ajoene are due to the multiple inhibition effects they may have on vireos thiol-depending enzymatic.

The inhibition effect of M.communis towards proteus isolates may refer to the phenolic and polyphenolic they contain. These compounds were found to denature proteins and block enzymes and subsequently the bacteria losses its activity (Al- Asady, 1988). the phenolic activity of M. communis is refered to the hydroxyl phenol group (phenolic OH) that form a in hydrogen bond with the active sit of enzymes (Black, 1985). More over Myrtucomlone A&B are considered as two new acylphloraglucinols identified in the leaves and fruits of M. communis and shown a significant antimicrobial activity against. The inhibition activity may be regard to present of Tannin via producing hydrogen bonds with proteins, which converted its structure and lead to block the protein synthesis , and tannins considered as a phenolic compounds of plants which have ant oxidative effects (Makoto et al, 1995).

It is clear from this study that SMIC of tested plant extracts eliminated the resistance of Proteus isolates to certain antibiotics when applied with final concentrations, however the swarming of Proteus bacteria that is important in the pathogenesis of bacteria is reduced when these plant extracts are applied with some antibiotics, and the later being particularly important in ascending urinary tract infections, which are more common on proteus strains, one of the virulence factors and properties of proteus sp. mediating infectious process are swarming phenomenon.

References

Alder I & Irobi ON. 1993 Antimicrobial activity of cured leaf extracts of *A.wilkensiana.*.J. of Ethnopharmacol. 39,171-174.

Ahmad I. 2002 Effect of *Plumbago zeylanica* extract and certain curing agents on multidrug resistant bacteria of clinical origin. World J. of Microbi. and Biotech. 16(8-9):841-844.

Al-Asady JG. 1988. Studies on the 1 Biochemical effects of some compounds of *Myrtus communis* L. (Myrtacea). M.Sc. Thesis Univ. of Mousl, Iraq.,

Al- Najjar AR. 1976 Studies on the effect of surfactants on the antibimicribial activity of several antibiotics. Ph.D. Thesis. Manchester Univ., UK.

Al- Safawi NT. 2001. Removal of the resistance of S. aureus bacteria isolated from various human infections to antibiotics by using chemical material and physical factors. MSc thesis. Mousl Univ., Iraq.

Alski AR. 2002 Rczoeci bakterii z rodzaju Proteus Molekularne podstawy chorobotw molecular basis of the pathogenicity of Proteus bacteria. Adv. Clinic. Exp. Med. 11, 1, 3-8.

Ankir S, Miron T, Rabincov A, Wilchek M, & Mirelman D. 1997. Alicin for garlic strongly inhibits cysteine propenases and

cytopathic effects of *Entamoeba histolytica*. Antimicrobial Agent Chemotherapy, 10: 2286-2288.

Atlas RM, AE Brown & LC Parks. 1995. Laboratory manual exoerimental microbiology. Mosby-year Book, Inc. St. Louis.

Black E. 1985. The chemistry of garlic and Onion. Scientific American J.,252: 94-99.

Birnboim HC., Doly J. 1979. A raped alkaline extraction procedure for screening recombination plasmid DNA. Nuclic acid Res. 7: 1513-1524.

El- Astal ZY., Ashour AA, & Kerrit AA. 2004. Antimicrobial activity of some Medical plant extract in Palestine. Pak. J. Medic. Sci., 21: 2: 187-193.

Feldberg RS, ChangS C, Kotik AN, Nadler M, Neuwirth Z, Sundstrom DC, & Thompson N.H. 1988. In vitro mechanism of inhibition of bacteria cell growth by allicin. Antimicrobials Agents Chemotherapy, 32:1763-1768.

Hoekstra WPM, Bergmans HEN, & Zuidweg EM. 1980. Transformation in *E. coli* chromosm during curing by acridin orange. J. of molecular Biolo. 45: 51-64.

Khder AK. 2002. Studies on antibiotic resistance by plasmids of *Pseudomonas aeruginosa*. Ph.D. Thesis, College of Education, Salahadeen Univ., Erbil- Iraq.

Khder AK. 2006. Effect of *Thymus serpyllum* and *Mentha spicata* extract and some chemical materials on multidrug resistant *Proreus mirabilis*.4th Int. Con. Biol. Sci. Tanta Univ. 161-165.

Lederberg EM & Cohn SN.1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribo nuclic acid. J. of Bacterio. 119: 1072-1074.

Lawson LD. 1996. The composition and chemistry of garlic cloves and processed garlic, in: Koch, H. P. and L. D. Lawson (Eds.), Garlic: The science and theroptic application of *Allium sativum* L. Willams an Wilkins, Baltimor, pp: 37-108.

Makoto I, Suzuki R, Sakaguchi NLZ, Takeda T, Ogohara Y, Jiang BY, and Chen Y. 1995. Selective induction of cell death in cancer cells by garlic acid. Biological Pharmaceutical Bull, (11):1526-1530.

Mandel M and Higa A . 1970. Calcium- dependent bacteriophage DNA infection. J. of Molec. Biol. 53: 159-161.

Mawlud SQ. 2006. The effect of some medicinal plant extract on curing plasmids of *Klebsella pneumoniae* isolated from different environment. MS.C. thesis. College of Science Education. Salahadeen Univ., Iraq.

Ozolin ON, and kaxazolova SG. 1990. Specific modification of the alpha-subunit of *Eschericia coli* RNAs polymerase by monomercurial derivative of flouorescein mercuric acetate. Molecular Biol. J. (MOSK), 24: 1057-1066.

Puhler A, and Timmis N K. 1984. Advanced in Molecular Genetics spring-verlarg Berlin Heidelberg. New York.

Rassol AA. 2004. Estimition of some plant products in *Asphodelus microcarpus*, *Colchicum koschyi,* and *Thymus sp.* Naturally grow in Iraqi Kurdistan, and their Antibacterial Activities. Ph.D.thesis, Univ. of Sulaimani, Iraq.

Reuter HD, and Sendel A. 1994. *Allium sativum* and *Allium ursinum*: Chemistry, Pharmacology and medicinal application. London Academic Press, 6: 55-113.

Rose JL, Recio MC, and Villar A. 1987. Antimicr activity of selected plants emploed in the Spanish Mediterrnanean area. Ethopharm Ecolo.,21: 139-152.

Sharref AY. 1998. The molecular effect of some plant extract on the growth and metabolism of some gram positive and gram negative bacteria.Ph.D. Thesis, college of Science, Univ. of Mousl, Iraq.

Staner RY, Adelberg EA, and Ingraham JL. 1984. General Microbiology.4thed. The Macmailian Press LTD London & Basingstoke.

Tomoeda M, Inuzuka M, Anto S, and Konishi M. 1974. Curing action of sodiumdodecyl sulphate on a *Proteus mirabilis* R strain. J. of Bacteriol. 120 :1158-1163.

Villar CJ, Medoza MC, and Hardisson C. 1981. Characteristics of two resistance plasmids from a clinical isolates of Sarratia mercescens.Microbiol. Lett. 18: 87-96.