Evaluate the antibacterial effect of Garlic (*Allium sativum*) and antimicrobial susceptibility on *Pseudomonas aeruginosa* isolated from otitis media

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الخلاصة:

في هذه الدراسة تم أخذ عشرين عينة من مسحات مرضى مصابين بألتهاب الأذن الوسطى وزرعت على اوساط بكتيرية مختلفة. شخصت العزلات المختلفة بالاعتماد على الفحوصات المظهرية والكيميائية ووجد ان 50% منها تعود لبكتيريا *P.sudomonas aeruginosa* . وجد من خلال الدراسة أن نسبة اصابة الرجال في مرحلة المراهقة اكثر من اصابة النساء.

اختبرت حساسية خمسة عزلات لمضادات الحياة بإستخدام مجموعة من المضادات وأظهرت معظم العزلات مقاومتها للبنسلين والتترا سكلين وسيفوتكسين. بالإعتماد على نتائج اختبار الحساسية للمضادات الحياتية اختيرت عزلتين لتحديد التركيز المثبط الادنى(MIC) لبعض النضادات حيث بينت النتائج ان العزلات P1,P2 لها القابلية على النمو بتركيز 128 مايكروغرام/مل لمضادات الكلور وامفنيكول والسيفوتكسيم يليها تركيز 64 لمضاد الامبسلين بالنسبة للعزلة P1 بينما العزلة P2 فكان تركيز 128 ماكروغرام/مل هذا بالاضافة للاموكسلين اذ وجد انه اقل تركيز 16 مايكروغرام/مل للعزلة P1 و 64 مايكروغرام/مل للعزلة P2 .

تم اختبار الفعالية التثبيطية لمستخلص الثوم حيث وجد قابلية مستخلص الثوم على تثبط بكتيريا *P.sudomonosa aeruginosa* بتركيز 8 مايكروغرام/مل للعزلة P1 و64 مايكروغرام/مل للعزلة P2 بعد 24 ساعة من تحضير المستخلص. بينما وجدت التراكيز التالية (128,32,64) مايكروغرام/مل أكثر تأثيراً على العزلتين بعد 72 ساعة من تحضير المستخلص . بينما أضهرت النتائج لا يوجد اي تأثير لمديات الرقم الهيدروجيني عل مستخلص الثوم.

Abstract:

Twenty swab samples were taken from patients suffering from otitis infection; these samples were cultured on different agar media (blood agar base, macConky and cetramide). Several microscopical, morphological and biochemical tests were performed and results showed that 50% of isolated colony were belonged to *Psudomonas aeruginosa*. In addition it was found that males were more susceptible to infection with otitis than female especially at teenager aged.

Five isolates were subject to sensitivity test against several antibiotic groups. Result showed that all five isolates were resistance to pencilline, tetracycline, Cefotaxtin (100%) and (60%) of them showed resistance to cefotaxime and (40%) to chloroemphenicol and (20%) to amoxicillin while all isolates were sensitive to ciprofloxacin. The minimum inhibitory concentration (MIC) test was performed for two multiple drugs resistance isolates (P1 and P2) result declared that both P1and P2 showed high-level of resistance to Cefotaxime and chloraemphenicol with MIC (128µg\ml), followed by (64µg\ml) to ampicillin while P2 was (128 µg\ml), as well as, the amoxicillin showed the lowest MIC (16 µg\ml) forP1 and (64 µg\ml) to P2.

No pH effect was observed on the activity of garlic when antibacterial effect of garlic extract against *Pseudomonas aeruginosa* was investigated at different PH. The MIC of garlic extract on *Pseudomonas aeruginosa* at different incubation periods was determined. Results showed that after 24hrs MIC of extract was greater than 8µg/ml for P1 isolate and 16µg/ml for P2 isolate. While, after 72rs it was found that the MIC at concentrations (32, 64 and 128 µg/ml) were more effective in inhibition growth of (P1, P2) while in (8 µg/ml) that indicated heavy growth of *P. aeruginosa*

Introduction:

Otitis media (Latin for "Middle otitis") is inflammation of the middle ear, or middle ear infection. Otitis media occurs in the area between the ear drum (the end of the outer ear) and the inner ear, including a duct known as the Eustachian tube.[1]

Middle ear infection is mainly a problem in children, although it also occurs in adults. [2] declared that otitis media is one of most common disease of children which is the leading cause hearing loss in children and the most frequent indication for antimicrobial or therapy in children.

Pseudomonas spp. were the most commonly identified etiologic agents causing dermatitis, conjunctivitis, or otitis. In humans, *P. aeruginosa* is the second most frequent gram-negative nosocomial pathogen in hospitals.[3] *P.aeruginosa* is frequently found in the normal ear and is the predominant bacterial pathogen in some cases of external otitis . The bacterium can cause a more serious ear infection in elderly patients, possibly leading to hearing problems, facial paralysis, or even death. Ear infection of *P. aeruginosa* can cause infections in the external ear canal-- so- called swimmer's ear"-- that usually disappear without treatment. [4]

There is extensive literature on the antibacterial effects of fresh garlic juice, aqueous and alcoholic extracts, lyophilized powders, steam distilled oil and other commercial preparations of garlic. It is a broad spectrum antibiotic, killing a wide variety of bacteria. Many pharmaceutical antibiotics kill only a narrow range of these germs while garlic has the broadest spectrum of any antimicrobial substance that otitis know due to antibacterial, antifungal,

antiparasitic, antiprotozoan and antiviral. This property belongs to the garlic constituent allicin, which is released when cutting garlic clove. [5] Moreover, garlic extracts exhibited activity against both gram negative (*E. coli, Salmonella* sp. and *Citrobacter, enterobacter,* Pseudomona and *Klebsiella*) and gram positive (*S. aureus, S. pneumonia* Group A *Streptococcus* and *Bacillus anthrcis*) all of which are cause of morbidity worldwide .[6] The present study tested an aqueous extract of dried garlic *in vitro* for its antibacterial activity against *Pseudomonas aeruginosa* isolates isolated from otitis patients with determination their susceptibility to antimicrobial agents.

Materials and Methods:

Bacterial isolation:

Twenty swab samples were obtained from Al-Yarmook Teaching Hospital in Baghdad from the periods (1/9/2009-1/10/2009) from patients suffering from otitis disease. Specimens were obtained by sterile cotton swab these were processed for direct examination and cultivation on blood agar media, MacConkeys agar and cetramide agar media (Banagalore, India) and were identified initially as *Pesudomonas* species according to their morphological, physiological and biochemical properties as indicated by [7,8] depending on Gram stain, colony shape, oxidase , catalase, growth on cetramide medium, production of pyocynine, growth at 4 and 42 ^{o}C.

Antibiotic susceptibility

The use of antimicrobial sensitivity test is essential for the selection of an appropriate drug for treatment of otitis infection. The disc diffusion method was used in this study against 5 isolates of *Pseudomonas aeruginosa* depending on the Kirby-Bauer diffusion method [9]. Up to 7 different groups of discs of the available antimicrobial agents were used in this study.

Minimum inhibitory concentration:

Broth microdilution method was performed, in this experiment twofold dilutions of antibiotics were done in broth media and broth was inoculated with 105 CFU/ml of the tested organisms. After incubation for 18-24 hrs, MIC was described as concentration in which no visible growth was observed. [8]

Preparation of garlic extract:

The garlic bulbs were washed thoroughly under tap running water aseptically cut into small pieces with a knife and then kept in the shade for 1-3 days at 32-35ºC. The semi-dried pieces were then crushed using pestle and mortar, and left to dry in the shade at room temperature for further two days. The dried garlic materials were further ground to powdery form with a Kenwood blender. Two hundred gram (200.0 g) of garlic powder was extracted with 500 ml of solvents distilled water, for 24 h by using Soxhlet apparatus. The extract were concentrated using a rotary evaporator at 40ºC. Clearly prepared garlic powder was thoroughly mixed with distilled water and the concentration was determined with varying amounts of crude preparation of garlic to give the final

concentration of 8, 16, 32, 64 and 128 mg/ml of brain heart infusion broth media and the final volume of 10 m l.[10]

Results and Discussion:

Isolation of *Pseudomonas* Species:

Twenty swab samples were collected from patients suffering from middle ear infection(otitis) (male and female) from Al-Yarmook teaching Hospital belongs to variety ages from (5-25) years. Results in table (3) showed that males were more susceptible to infection with otitis than female especially at teenager aged the reason for that is male have hospitalization rate ratio (HRR) for admission due to a respiratory tract infection more than female. Hospitalizations for otitis media by *pseudomonas, pneumonia, influenza*, and other acute respiratory tract infections of hospitalized patients differed by age and gender. The male have hospitalization rate ratio (HRR) for admission due to a respiratory tract infection more than female. [4]

In humans, *P. aeruginosa* is the most commonly isolated bacterial pathogen in adults and children with a clinical history of chronic, suppurative otitis media, and a major cause of otitis media in neonates [11]. This is in contrast to studies which demonstrated that *P. aeruginosa* is not a normal inhabitant of the external auditory canal, having been recovered from that site in only 1% of 1,377 healthy volunteers [12].

Age	Male	%	Female	%
5-10	2	18	3	30
10-15	5	45	1	18
15-20	1	10	2	22
20-25	3	27	3	30
Total	11		9	

Table-1: Distribution of otitis media patients according to different ages and sex.

Isolation and Identification of bacteria:

Twenty Clinical swab specimens taken from middle ear infection patients were cultured on cetramide agar plates, ten isolates which may be belonged to be *Pseudomonas* were further identified according to morphological characteristic and biochemical tests.

There have been reported cases of clinical inner ear disease in mice affecting the vestibular apparatus, attributed to natural *P. aeruginosa* infection[13,14]. These were characterized clinically by either circling [15] or rolling [16]. Similar signs of spinning/circling had also been experimentally produced by intravenous inoculation of mice with *P. aeruginosa*.[17].

For the former, colonies of each isolate was plated on nutrient agar showed different morphological characteristics of mucoidal growth, smooth in shape with flat edges and elevated center, whitish or creamy in color, have fruity oder, all of them were pyocynine producers, and the shape of the colony appears like a fried egg shape, these result are reasonable with the result demonstrated by [7, 18]. Microscopically examination of each isolate showed that they were all motile, non –spore forming, gram negative and rod shape. Furthermore, six isolates that were suspected to be belongs to *Pseudomonas* sp. were subjected to a number of biochemical tests. Results indicated in table-2 showed that these isolates gave a positive result for oxidase and catalase and were able to growth on cetramide medium which indicated that these isolates belong to *Pseudomonas* sp. [19] indicated that only *Pseudomonas* species were able to grow on cetramide medium. Glucose hydrolysis test was also performed to characterize *P.aeruginosa* from other species. On the other hand, these isolates differ in growth at 42°C and 4°C, five isolates (P1, P2, P3, P4 and P5) were able to grow at 41°C but they can not grow at 4°C. It was found that five isolates were belonging to *P. aeruginosa*. Our results were in agreement to result obtained by [19].

Tests	Result
Colony color	Green
Growth on cetramide medium	+ve
Cell shape	Rod
Gram stain	-ve
Catalaseoduction Pr	+ve
Oxidase production	+ve
Growth at 4^0 C	-ve
Growth at 42 ^{0}C	+ve

Table-2: Some biochemical tests and morphological examination to identify *Pseudomonas* Sp.

Antibiotic sensitivity test:

The development of antibiotic resistance is considered a major therapeutic problem that can be explained by some hypothesis such as, the influence of excessive and /or inappropriate antibiotic use [20].

Standard disk diffusion test has been performed for detection of susceptibility of pathogenic *P. aeruginosa* five isolates for several antibiotic disks, decision for considering an isolate as resistant or sensitive was taken in comparison of the diameter of inhibition zone with that of standard value of [21]. As shown in table-3.

Isolate No.	CTX (10)µg	AMX (10) µg	C (30) µg	CIP (5) µg	FOX (30) µg	TE (30)µg	P (10) µg
P1	R	R	R	S(25mm)	R	R	R
P2	S(25mm)	R	R	S(20mm)	R	R	R
P3	S(25mm)	R	S(15mm)	S(22mm)	R	R	R
P4	R	S(15mm)	R	S(30mm)	R	R	R
P5	S(12mm)	R	S(23mm)	S(23mm)	R	R	R

Table-3: Antibiogram of of *P. aeruginosa* isolates isolated from otitis patients
S=Sensitive, R=Resistant, CTX=Cefotaxime, AMX=Amoxicillin, C=Chloramphinicol, CIP= Ciprofloxacin, FOX=cefoxitin, TE=tetracycline and P=Penicillin

Result shown in table-3 indicated that resistance to antibiotics was widely distributed among isolates, however, they varies according to nature of the isolate and kind of antibiotics when all isolates (P1, P2, P3, P4 and P5) showed resistance for (Tetracyclin, Pencilline, Cefoxitin) and to (Amoxicilline) except P4 also three isolates showed resistance to Chloraemphicol (P1, P2 andP4) which were sensitive to it except(P3, P5) while all isolates showed sensitivity to Ciprofloxacin and cefotaxime except (P1, P4) which were resistance to the former one.

From these results it was concluded that *P. aeruginosa* is probably has the ability to produce more than one enzyme among them β-lactamases. Bacterial resistance to beta-lactam antibiotics can be achieved by any of three strategies: the production of beta-lactam-hydrolyzing beta-lactamase enzymes, the utilization of beta-lactam-insensitive cell wall transpeptidases, and the active expulsion of beta-lactam molecules from Gram-negative cells by way of efflux pumps. [22] The greater sensitivity observed with Ciprofloxacin which gives the largest zone of inhibition compared with other antibiotics. This result was in agreement with result obtained by [23] who found that ciprofloxacin was effective in the treatment of the virulent gram negative bacteria including *P.aeruginosa*.

The mechanism of resistance for ciprofloxacine included effective suction pump of the antibiotic from inside to outside to escape its effect and prevent the accumulation of antibiotic inside bacterial cell.[24]. From this result of table-3 one could conclude that ciprofloxacin remain the first choice when all isolates were sensitive to it.

The percentage of *P. aeruginosa* resistance isolates to each antibiotics were shown in figure-2, when all isolates were resistance to pencilline,

tetracycline, Cefotaxtin (100%) and (60%) of them showed resistance to cefotaxime and (40%) to chloraemphenicol and (20%) to amoxicillin while all isolates were sensitive to ciprofloxacin .

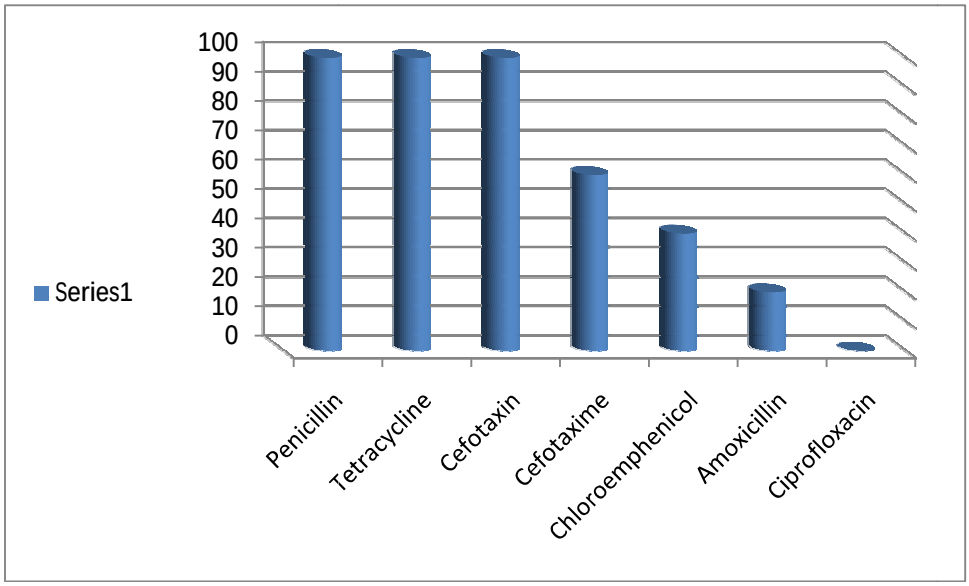

Figure-1: Percentage resistant of *P. aeruginosa* isolates

The number of multiple antibiotic resistance strains has been increasing since resistance is mainly mediated by R- plasmids which determined beta-lactamase in Gram negative bacilli[20] all the R-plasmids carried the markers of resistance to chloraemphenicol, tetracycline, ampicillin, gentamycin and streptomycin [21].

In the case of ciprofloxacin none of *P. aeruginosa* isolates were resistance to it because ciprofloxacin is a fluoroquinone antibiotic with broad spectrum bacterial activity. Ciprofloxacin inhibit bacterial DNA gyrease, so preventing thr supercoliong of DNA, a process that is necessary for compacting chromosomes into the bacterial cell [22,25].

Minimal Inhibitory Concentration (MIC) Test:

P. earuginosa infections are more difficult to treat due to the organisms high intrinsic resistance to many antimicrobial agents, this resistance is partly due to its relatively low outer membrane permeability [26]. However there are other mechanisms that included decreasing the passage into or increasing the efflux of drug from bacterial cell and modification of the target site.

Tow isolates were selected due to multiple antibiotic resistance, therapy, these isolates were selected in order to evaluate bacteria susceptibility to antibiotic that revealed earlier by disk diffusion test when examined against antibiotics. MIC was defined as lowest concentration of antibiotic in microgram per milliliter that prevents the in *vitro* growth of bacteria. The MIC test was performed to determine their minimal inhibition concentration as in table-4, both P1and P2 showed high-level of resistance to Cefotaxime and chlorempheniсol with MIC (128µg\ml), followed by (64µg\ml) to ampicillin while P2 was (128

µg\ml), as well as, the amoxicillin showed the lowest MIC (16 µg\ml) forP1 and (64 µg\ml) to P2 .

Isolate No.	C µg\ml	AMX µg\ml	AM µg\ml	CTX µg\ml
P1	128	16	64	128
P2	128	64	128	128

Table-4: The MIC value of some antibiotics for *P. aeruginosa* (P1, P2) isolates. C=Chloramphenicol, AMX=Amoxicillin, AM=Ampicillin and CTX=Cefotaxime.

P.aeruginosa has an outer membrane with a low permeability and most antimicrobial agents were not easly diffused through. Active efflux to be especially effective mechanisms of antibiotic resistance in this bacterium[27] indicated that MIC values are greatly affected by environmental conitions including the pressure of CO2 cocentration causing acidification of the test media.

Antibacterial effect of garlic on *Pseudomonas aeruginosa*:

The phytoconstituents of garlic have longed been known and its antimicrobial properties have been widely reported [28]. The antimicrobial activities of plant extracts including garlic have been linked to the presence of some bioactive compounds. These secondary metabolites also serve to protect the plants themselves against bacterial, fungal and viral infections [29]. These bioactive compounds are known to work synergistically to produce various effects on the human and animal subjects [30]. However, most reports on the activity of garlic have focused mainly on the commensally microflora and community acquired infections, while informations on its activity against hospital based pathogens is scanty.

The pH of each of the garlic solution was 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8. The activity of the garlic was tested in these different pH levels and has no pH effect on the garlic activity been observed. This was similar to the observation of[31].

The MIC of garlic extract on *Pseudomonas aeruginosa* at different incubation periods was determined. Table-5 showed that after 24hrsMIC of extract was greater than 8µg/ml for P1 isolates and 16µg/ml for P2 isolates which is almost similar with the work of [31]. The lower concentration of garlic had no antibacterial effect in this work, however; it may effective as [31]. This is may be due the species difference or the garlic difference in different biologic condition.

No .of isolate	Concentration of garlic µg\ml				
	8 µg\ml	16 µg\ml	32 µg\ml	64 µg\ml	128 µg\ml
P1	+	+	+	-	-
P2	+	+	+	-	-

Table (5) The MIC value for Garlic extract on *P. aeruginosa* (P1, P2) after 24hrs.
+=Indicates growth of *P.aeruginosa* and – = no growth

No .of isolate	Concentration of garlic µg\ml				
	8 µg\ml	16 µg\ml	32 µg\ml	64 µg\ml	128 µg\ml
P1	+	+	-	-	-
P2	+	-	-	-	-

Table (6): The MIC value for Garlic extract on *P. aeruginosa* (P1, P2) after 72hrs.
+ = Indicates growth of *P.aeruginosa* and – = no growth

In the table (6) the MIC of garlic extract on *P. aeruginosa* was determined after 72rs and it was found that at concentrations (32, 64 and 128 µg/ml) were more effective in inhibition growth of (P1, P2) while in (8 µg/ml) that indicated heavy growth of *P. aeruginosa* .

It has shown that dilute solutions of garlic can completely inhibit the growth of *P.aeruginosa* at the concentration of more than16 µg/ml. This could be due to the action of biological active ingredient of allicin exhibits its antimicrobial activity mainly by immediate and total inhibition of RNA synthesis, although DNA and protein syntheses are also partially inhibited, suggesting that RNA is the primary target of allicin action.[32]. From our study it was concluded that *Psudomonas aeruginosa* were highly prevalence among teenager age of otitis patients and High prevalence of antibiotic resistance was observed among *Psudomonas aeruginosa* isolates especially to penicillin cefotaxin and tetracycline and it was found that garlic extract has considerable inhibitory effects against the tested *P.aeruginosa* isolates

References:

1- Hedstrom, R. C.; Funk, C, R.; Kaper J. B.; Pavlovskis, O. R. and Galloway, D. R. (1986). Cloning of Gene involved in Regulation of Exotoxine A Expression in *Pseudomonas aeruginosa*. Infect. Immun.51(1): 37 _42 .
2- Brook, G. F.; Bulet, J. S. and Arnston, L. N. 1995. Jawetz, Melnick and Adelbergs Medical Microbiology. 20 (ed).
3- Botzenhart, K., and Doring, G. (1993). Ecology and epidemiology of *Pseudomonas aeruginosa*. IN:Campa, M., *etal*(Eds). *Pseudomonas aeruginosa* as an Opportunistic Infection. New York: PlenumPress, pp. 1-18.

4- Gates, G. A. (1998). Acute Otitis Media and Otitis Media with Effusion. In Cumming, C. W., Fredrikson, J. M., Harker, L. A., Krause, C. J. and Schuller, D. E. Otolaryngology Head and Neek Surgery. 3 ed. St. Louis,C.V .Mosby. P.2808_ 2822.

5- Farbman KS, Barnett ED, Boldiro GR, Klein JO 1993. Antibacterial activity of garlic and onions: a historic perspective. Paediat. Infect. Dis. 12: 613-614.

6- Jaber, M.A.and Al-Mossawi, A. (2007). Susceptibility of some multiple resistant bacteria to garlic extract. Afr. J. Biotechnol. 6 (6): 771-776

7- Collee, J. G.; Fraser, A. G.; Marmion, B. P. and Simmons, A. (1996). Pseudomonas, Stenotophanas, Burkhldria, Practical Medical Microbiology.14th ed.

8- Atlas, R.; Parks, L. and Brown, A. (1995). Laboratory Manual of Microbiology . 1st (Ed). Mosby,Inc. Missori

9- Bauer A.W.; Kirby W.M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 43: 493-496.

10- Abubakar, E.M. (2009). Efficacy of crude extracts of garlic (*Allium sativum* Linn.) against nosocomial *Escherichia coli, Staphylococcus aureus, Streptococcus pneumonea and Pseudomonas aeruginosa*. Journal of Medicinal Plants Research Vol. 3(4), pp. 179-185, April.

11- Brook, I., and Finegold, S.M. (1979). Bacteriology of chronic otitis media. J.A.M.A. 241:487-488.

12- Singer, D.E. et al.(1952). Ann. Otol. Rhinol. Laryngol. 61(2):317-330.

13- Ediger, R.D.; M.M. Rabstein, and L.D. Olson. (1971). Circling in mice caused by *Pseudomonas aeruginosa*. Lab. Anim. Sci. 21:845-848.

14- Kohn,D.F., and W.F. MacKenzie. (1980). Inner ear disease characterized by rolling in C3H mice. J. Am. Vet. Med. Assoc. 177: 815-817

15- Olson, L.D. and R.D. Ediger. (1972). Histopathologic study of the heads of circling mice infected with *Pseudomonas aeruginosa*. Lab. Anim. Sci. 22:522-527.

16- Gorrill, R. H. (1956). Spinning disease of mice. J. Pathol. Bacteriol. 71:353-358.

17- Gorrill, R.H. (1952). Bacterial localization in the kidney with particular reference to *Psuedomonas aeruginosa*. J. Pathol. Bacteriol. 64:857-864.

18- Holt, J. G.; Krieg, N.R.; Sneath, P.H.A.; Staley, J.T. and Williams, S.T. (1994). Bergeys manual of determinative bacteriology 9th ed Baltimore. pp: 42-43.

19- Weyant, R.S.; Moss, C.W.Weaver, R.E.; Hollis, D.C.Jordan,J.G. and Daneshver, M. I.(1995). Identification of anusual pathogenic Gram negative and facultative anaerobic bacteria. P.318-319, 340-341, and 470-503. The Williams and Wilkins. Co. Balttimore,Md.

20- Scott, Y.E.M.; Liwan, A.and TariqV. N.(1999). Development of resistance and across- resistance in *Psuedomonas aeruginosa* exposed to subinhibitory bantibiotic concentration.APMIS.107:585-592.
21- National Committee for Clinical Laboratory Standards. (1999). Performance standards for antimicrobial susceptibility testing: Ninth informational supplement. NCCLs document M100-S9. National committee for clinical laboratory standards, Villanova.
22- Hooper, D.C.(1999). Mode of action of fluroquinolones.Drugs.58 suppl.2:6-20 .
23- Muller, M.; Brunner, M.; Hollenstein, U.; Joukhadar, C.; Schmid, R.; Minar and Eicher ,H. G. (1999) . Penetration of ciprofloxacine into intersitiial space of inflamed foot lesions in non- insulin- dependent diabetes mellitus patient. Antimicrobs. Agents Chemother
24- Wigley, S.M.;Rigg, G.P.; Kavari, M;Webb, A.K.; Matthews, R.C. Burine,J.P.(2002).identification of an immunodomenant drug efflux pump in *P.aeruginosa*. J. antimicrob.chemother. (JAC) 49: 619-624.
25- Reese, R. E.; Betts, R.F. and Gumustop,B. (2000).Handbook of antibiotics. 3rd-ed.Lippincott Williams and Wilkins.
26- Martinez-Martinez, L.; Pascual, A. and Perea E. J. (1991). Effect of *P.aeruginosa* preincubation of an subinhibitory cocetrations of Amikacin, Ceftazidime and Ciprofloxacin on adherence to plastic catheters.J.Chemother.37:62-65.
27- Owlia, p.; Behzadiyan-Nejad, Q.; Souri, E. and Saderi, H. (2007). Microscopic study the effect of subinhibitory concentrations of gentamycin on capsules production of P.aeroginosa. Depart. microbial.Shahad university, Tahran, Iran(personal communication).
28- Roy, J,; Shakaya, D. M. ;Callery, P. S. and Thomas, J. G. (2006). Chemical constituents and antimicrbila activity of a traditional herbal medicine containing garlic and black cumen. Afr. J. Trad. CAM 3 (20): 1-7
29- El-Mahmood, A.M.and Amey, J.M. (2007). *In vitro* antibacterial activity of *Parkia biglobosa* (Jacq) root bark extract against some microorganisms associated with urinary infections. Afr. J. Biotechnol.6 (11): 1272-1275
30- Amagase, H. (2006). Clarifying the real bioactive constituents of garlic. J. Nutr. 136:716-725.
31- Shokrzadeh, M. and A.G. Ebadi, (2006). Antibacterial effect of Garlic (*Allium sativum*) on *Staphylococcus*. Pak. J. Biol. Sci., 9(8): 1577-1579.
32- Farbman, K.S; Barnett, E.D.; Boldiro, G.R.and Klein, J.O. (1993). Antibacterialactivity of garlic and onions: a historic perspective. Paediat. Infect. Dis.12: 613-614.