University of Al-Qadisiyah

College of pharmacy

Antibiotic resistance of *E. coli* isolates from patients in Al-Diwaniyah hospital with UTIs and the effect of alcoholic grape seed extract on the bacterial isolate.

By :

MurtadhaSalih

Noor Nabeel

Supervised By:

Lec. Saba Abood Ali

Dedication

To the fountain of patience, optimism and hope ,

To each of the following in the presence of god and his messenger my mother dear.

To those who have demonstrated to me what is the most beautiful of my Brothers life,

To the big heart my Father dear.

To the people who paved our way of science and knowledge All our teachers Distinguished

To the taste of the most beautiful moments with my

I guide this research .....

بسم الله الرحمن الرحيم

(وقل ربي زدني علما)

صدق الله العظيم

List of Subjects

Abstract

Background:Urinary tract infections (UTIs) are among the most common conditions requiring medical treatment and the resistance to antibiotics arising in *Escherichia coli*species isolates may complicate therapeutic management of urinary tract infection(UTI) by this organism, which make athreattomankindhascausedanurgentneedforthesearchofinnovativewaystocontrolbacterial pathogens.Hence,naturalantibioticsareinprocessofbeingdiscoveredasalternativetosyntheticproducts.Grapeseedsusedinherbalmedicineandasdietarysupplementsareproposedtoprovideantimicrobialactivity

Aim: The aim of this study was to assess antibiotic activity against UTI isolates of *E.coli* in Al-diwaniyahgovernorate, and the effect of grape seed extract as an inhibitor.

Result: Susceptibility of the isolates to antibiotics was tested by standard methods. Highest rate ofresistance (97%) was found toampicillin , while alarming resistant to cephalosporin and ciprodar(55%).

The results of the alcoholic extract of grape seed showed that the concentrations(100% and75%) have inhibitory effect on the bacterial isolate, while concentrations of (25% and 50%)did not show any inhibitory effect on the bacterial isolation.

Conclusion:The present study concluded that *E.coli* resistance to multiple antibiotic were recognized.Also, the alcoholic extract of grape seeds have inhibitory effect on the bacterial isolate.

Keywords: Urinary tract infection, *Escherichia coli* ,Resistance to antibiotics, grape seed extract .

مقاومة المضادات الحياتيةلعزلاتالايشريشيا المعوية من المرضى المصابين بالتهاب المجاري البولية في مستشفى الديوانية مع تاثير المستخلص الكحولي لبذور العنب الاسود على العزلة البكتيرية.

1.Introduction

Urinary Tract Infections (UTIs) are one of the most prevalent bacterial infections. Nowadays, it represents one of the most common diseases affecting people of all ages from the neonate to the geriatric age group [1]. Worldwide, about 150 million people are diagnosed with UTI each year [2].

Most infections are caused by retrograde ascent of bacteria from the faecal flora via the urethra to the bladder and kidney especially in the females who have a shorter and wider urethra and are more readily transferred by microorganisms [3]. The structure of the females urethra and vagina makes it susceptible to trauma during sexual intercourse as well as bacteria being massaged up the urethra and into the bladder during pregnancy and/or child birth [4,5].

Majority of UTIs are not life threatening and do not cause any irreversible damage. However, when the kidneys are involved, there is a risk of irreparable tissue damage with an increased risk of bacteremia [6]. The emergence of highly drug-resistant bacteria are turning up in urinary-tract infections which is a serious public health issue, particularly in the developing world where apart from high level of poverty, ignorance and poor hygienic practices.

E.coli is the most commonly isolated organism from the UTI patients. Others include *Proteus mirabilis* and *Klebsiella species* which may be quite resistant to antimicrobials. *Staphylococcus saprophyticus* can account for up to 10% of UTI's in sexually active women.

In the last decades interest on the antimicrobial plant extracts has been growing in all over the world including Iraq; The medical values of plants depend on some chemical substances that produce a definite physiological action on the human body. The most important of thesebioactive constituents of plants arealkaloids, tannins, flavonoids, and phenoliccompounds.[7]

The grape contains various nutrient elements such as: vitamins, minerals, carbohydrates, edible fibers and phytochemicals. Polyphenols are the most important phytochemicals in grape because they possessmany biological activities and health promoting benefits.[8]

The research aims to determine the resistance of pathogenic *E.coli*to antibiotics, and the antibacterial activity of black grape seed extracts against *E.coli* isolate.

2. Literature review:-

2.1 urinary tract infection

UTI is one of the most common infections occurring in all the age groups from neonates to old age. It is more common in females as compared to males, especially females of reproductive age group(from 15-50 years).

Urine in the human bladder is normally sterile. UTIs refer to the presence of microbial pathogens within the urinary tract and it is usually classified by the infection site: bladder (cystitis), kidney (pyelonephritis), or urine (bacteriuria)[9]

.2.2 symptoms of urinary tract infection

Diagnosis of UTI in young healthy patients can usually be made clinically. Patients with cystitis typically present with any or all of the following: cloudy urine; abnormal urine odor; dysuria; urinary frequency, hesitancy, or urgency; suprapubic discomfort; gross hematuria. More severe symptoms are more predictive of UTI than milder ones, as is the presence of combinations of symptoms rather than just a single symptom.[10]

2.3 Causative agents

Urine is generally considered to be sterile and is believed to begerm free. Any source of possible infection occurs through urethrawhich initiates the incidence of the infection. The predominantpathogen responsible for UTI is *E. coli* which constitutes up to80-85% and is followed by *Staphylococcus saprophyticus* whichaccounts to 5-10%. The occurrence of the infection due to viralor fungal agents is a rare phenomenon. In addition to the abovementioned bacterial species, *Klebsiella, Proteus, Pseudomonas* and *Enterobacter*are associated with UTI. The bacteria enter thebladder through urethra and the infection can also occur through blood and lymph[11].

These strains are found in the normal flora of the intestinal tract, skin, and vagina. However, under individual predisposing conditions they can multiplyrapidly and are capable of adhering to uroepithelial cells, producing infection[12].

2.3.1 *Escherichia coli*

E. coli strains in particular are responsible for 80% of the UTI cases These strains are found in the normal flora of the intestinal tract, skin, and vagina. However, under individual predisposing conditions they can multiply rapidly and are capable of adhering to uroepithelial cells, producing infection[13]

I. Bacterial Characteristics

Escherichia coli is a gram-negative, non-spore forming, , rod-shaped, coliform bacterium that is commonly found in the lower intestine of human and animal; it's a member of the family *Enterobacteriaceae*of the genus *Escherichia*. they grow on peptone or meat extract media without the addition of sodium chloride or other supplements; grow well on MacConkey agar; grow aerobically and anaerobically (are facultative anaerobes); ferment rather than oxidize glucose, often with gas production; they are catalase positive, and oxidase negative**[**14**].**

II. Virulence factors

Bacterial virulence factors play a significant role in determining whether an organism willinvade the urinary tract and the level of infection acquired. Uropathogenic E. coli (UPEC) ispresent within bowel flora and pathogenic strains of this microorganism can infect the urinary tract by expressing specific virulence factors that permit adherence and colonization of the lower urinary tract.Adhesins found on the surface of the bacterial membrane are responsible for initial attachment onto urinary tract tissues [15].

After binding to the epithelial surface the adhesins migrate towards deeper urothelial layers and penetrate the cell membrane (Mulvey et al. 2000). Once the uropathogen is intracellular the invasive process continues as bacteria proliferate within the cytosol to form clusters.

2.4 pathogenesis of UTI

UTIs occur as a result of interactions between theuropathogen and host and their pathogenesis involves several processes. Initially the uropathogen attaches to the epithelial surface; it subsequently colonises and disseminates throughout the mucosa causing tissue damage. After the initial colonization period,pathogens can ascend into the urinary bladder resulting in symptomatic or asymptomatic bacteriuria. Further progression may lead to pyelonephritis and renal Impairment. Specific virulence factors

residing on the uropathogen's membrane are responsible for bacterial resistance to the normally effective defence mechanisms of the host. Recently, bacterial adhesins and their associated epithelial binding sites have been identified[16].

2.5 treatment of UTI

the treatment process starts after the diagnosis of the infection but these initial attempts of treating the disease can lead to problematic consequences as the treatment has to be made after the confirmation of the etiological agent.

The initial treatment efforts involve the employment a variety of antimicrobial agents and this could in turn make the pathogen resistant to commonly employed drugs. Such kind of treatmentis referred to as empirical treatment. Therefore, a sporadic assessment of the causative pathogens against the antimicrobial agents is necessary.

Studies have also provided sufficient evidences to confirm the property of antibiotic resistance exhibited by certain pathogens against amoxicillin and ampicillin which in turn has limited their usage[17].Though studies demonstrate the efficacy of nitrofurantoin, it is ineffective against Proteus species[18].

2.6 Medicinal plants

The Medicinal plants have been used from prehistoric times.The earliest historical records of herbs are found from the Sumerian civilization, where hundreds of medicinal plants are listed on clay tablets[19].

In the last decades the scientist community is trying to make drugs and antimicrobial systems for limiting the growth of antibiotic-resistant pathogenic bacterial species and toxin producing molds. The polyphenols extract from plant origin gained more attention of researchers for their use against drug-resistant food borne pathogens[20]. the

antimicrobials from these sources have been found to work more efficiently with fewer side effects and less cost of production[21,22].

2.6.1 The grape(*Vitisvinifera*)

❖ **Origin of grape**

It is believed that the origin of the grapes grown in Europe is in the Caspian Sea region. The seed dispersal by birds, wind and water spread the plant westward to the Asian shores of the Mediterranean.

❖ Content and Active ingredients of *Vitisvinifera*

Black grape (Vitisvinifera) is a great source of phenolic compounds. Grape polyphenols contain from simple compounds (monomers) to complex tannin type substances (oligomers and polymers). There are many classes of negatively charged polyphenols have been identified in grapes, such as phenolic acids (benzoic and hydroxycinnamic acids), stilbene derivatives (resveratrol), flavan-3-ols (catechin, epicatechin), flavonols (kaempferol, quercetin, myricetin), anthocyanins, etc.These polyphenols possesses many beneficial effects on human health such as inhibition of free radical damage, antibacterial, antifungal, decreasing the risk of cardiovascular diseases, anticarcinogenic, anti-inflammatory, etc.

As an antimicrobial agent, these polyphenols can penetrate the semi permeable cell membrane where they react with the cytoplasm or cellular proteins[23].

3. Material and method

3.1 Study Population

A total number of 100 patients clinically suspected of having urinary tract infectionseeking a medical advice in AL-Diwaniyah Teaching Hospital were involved.

The studygroup comprised of males and females with age range from 15 years to 79 years, during a period since (15/August to 15/November 2016).

3.2 Collection of Samples

Specimen of urine was collected from each patient (under aseptic conditions Clean Catch Mid-Stream), and transported to microbiological laboratory in hospital for further processing, with the information of each patient (the name, age and sex).

3.3 Culturing of the samples

The urine sample was centrifuge and a loop full of the sedimentwas cultured on Blood agar and MacConkey agar media.

After allowing the urine to be absorbed into the agar, the plates were then inverted andincubated at 37°C for 18-24 hrs.

3.4 Identification

After positive of growth were appear , the sample were identified with Gram stain and biochemical test.

3.4.1 Biochemical test:

Gram negative rod isolates were identified by standard biochemical tests[24].

The IMVIC tests (indole production, methyl red, Vogas-Proskauer and citrate utilization) were used to identify the bacterial isolates as it shown in table(1-1).

IMVIC Test for *E.coli*	RESULT
Indole	**(+)**
Methyl red	**(+)**
Voges_proskauer	**(-)**
Citrate	**(-)**

Table (3-1) IMVIC Testsresultsof*E.coli*

3.5 Evaluation of UTI by Antibiotic Sensitivity Pattern:

3.5.1Preparation of bacterial suspension

A sterile swab was used to transfer a sufficient number of colonies of an overnight growth culture of *E. coli*to suspend in 3.0 mL of sterile saline tube. The turbidityis adjusted accordingly and measured using a turbidity meter called the Densi-Chek, the turbidity adjusted to 0.5McFarland standard.

3.5.2 Antibiotic sensitivity test (disc diffusion method)

Bacterial suspension was spread on Muller-Hinton agar plates with the help of sterile cotton swab uniformly and finally antibiotics were placed at regular distance after 5 minutes.

The plates were incubated at 37˚C for 18-24 hours. Zone diameter was measured in millimeters. The size of zone inhibition was interrupted by referring to the CLSI (Clinical Laboratory Standard Institute) guidelines and organism was labeled susceptible, intermediate, or resistance accordingly[25].

Antibiotic sensitivity test was performed for each isolate utilizing the method of Kirby-Bauer (disc diffusion method) [26]. This was performed with the following antibiotic discs (Ampicillin AMP 10µg, Amikacin AK 30µg, sulfamethaxole-trimethprim SXT 5µg, Cefotaxime CTX 30µg, Ceftazidim CAZ 30µg, Ceftraxon CTR 30µg, Ciprofloxacin CIP 5µg, Chloramphenicol C 30µg, Gentamicin CN 10µg, Nalidixic acid NA 30µg, Nitrofurantoin NIT 300µg, Oxacillin OX 1µg). Sensitivity was read after incubation for 24 hrs. at 35ºC. The bacteria isolates were regarded as sensitive or resistant according to CLSI criteria.

3.6 Preparation of ethanolicgrape seed extract

3.6.1 grape seed

The seed of the black grape were grounded to powder using a coffee blender. The ethanol extract was prepared by mixing 50.0gm of dry powder of the seed added to 95% ethanol at final volume 100ml and kept at room temperature for 3 days in a round bottom flask with occasional shaking. After 3 days period, the extract was then filtered through a muslin cloth for coarse residue and finally filtration was done through whatman No.1 filter paper then sterilized by membrane filter unit 0.4µ and finally stored in airtight bottle at 4°C until use. The stock solution was prepared by adding 1 g of extract in 10 ml of sterilized distilled water(100mg/ml), then the three dilutions(25,50 and75mg/ml)were preparedaccording to[27]

3.6.2 Agar well diffusion method

the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface.Then, a hole is punched aseptically with the back of a sterile Pasteur pipette, and a volume (10 µL) of the extract solution at desired concentration is introduced into the well. Then, agar plates are incubated at 37 ^{o}C.

4. The Results

The present study included a collection of 100urine samples from AL-Diwaniyah-Teaching Hospital during the period from (15-August to 15-November 2016). The females were 66 (66%) while the males were 34 (34%).

4.1 The prevalence of positive culture among age group

Out of the 100 cultured samples; 82 (82%) showed significant positive bacterial culture; which agreed with Al-Jumaily (2005).the females were more infected than males with percentage 55 (67%) out of the 82 positive cultures and the males were 27 (32.9%).

Table(4-1) showed that42 (80%) femalesof age group (15-45) gave a positive bacterial culture out of 52 female patients. Also the females in age group (46-75) find out 13(92%) from 14 positive culture; while in male the age group (15-45) showed 17

(77%)from 22 positive result, and the age group(46-75) showed 10 (83.33%) from 12 positive result.

Table(4-1): Distribution of 100 patient according to age and sex

Age groups	**female**	**result**		**%infection**	**male**	**result**		**%infection**
		+	-			+	-	
15-45	***52***	***42***	***10***	***80***	***22***	***17***	***5***	**77**
46-75	***14***	***13***	***1***	***92***	***12***	***10***	***2***	**83.33**
total	***66***	***55***	***11***	***83.33***	**34**	**27**	**7**	**79.4**

4.2 the prevalence of *E.coli*

E.coli was the predominant microorganism among other bacterial isolates, which confirms the results obtained by other researchers in iraq.[28,29].

Out of the 82 positive cultures (90) bacterial isolates were obtained. The isolates were as follow: 48(53.33%) isolates of *E.coli*, 29 (32.22%) isolates of *Klebsiella*spp, 8(8.88%) isolate *Proteus* spp. and 5(5.55%)of *Pseudomonas* spp. As it showed in (Table 4-2)

Bacteria	Number of isolate	Percent
Escherichia coli	48	53.33%
Klebsiellaspp	29	32.22%
Proteus spp	8	8.88%
Pseudomonas spp	5	5.55%
total	90	99.99%

4.3 The results of the grape seed extract

The effects of the ethanol extract of the grape seedsshowed an inhibitory effect (17and 22mm) against the bacterial isolate with the concentrations (75% and 100%) respectively; which agreed with the results of Kandasamy M[30].(Figure 4-1).while the concentrations (25% and 50 %) did not show any inhibitory effect. (Figure 4-2).

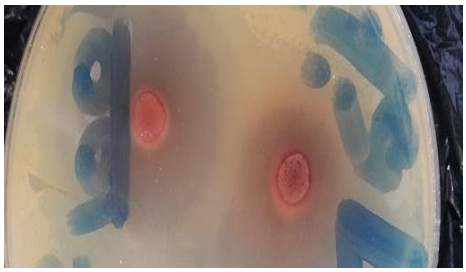

Figure 4-1 the inhibitory effects of the grape seed extract concentrations(75% and 100%) on the *E.coli* isolate

Figure 4-2 the inhibitory effects of the grape seed extract concentrations(25% and 50%) on the *E.coli* isolate.

4.4 Antimicrobial Sensitivity Tests results

The frequency of antibiotic resistance of the *E. coli* isolates were found to be (97%) resistant to ampicillin. (94.7%) of *E. coli* isolates were highly resistant to oxacillin, and alarming resistant to cephalosporin, ceftriaxone55%,cefotaxime(58.5%),ceftazidime(59.8%)ciprofloxacin55%, gentamycin(47.8), trimethoprim (46.5%), nitrofurantoin (44.1%), nalidxic acid (37.2%), and chloramphenicol (27.9%).

On the other hand, all *E. coli* isolates were susceptible to imipenem, meropenem Among these antimicrobial drugs tested, the sensitivity rate was Amikacin (94%) Meropenem (96%) imepenem95%). Figure(4-1).

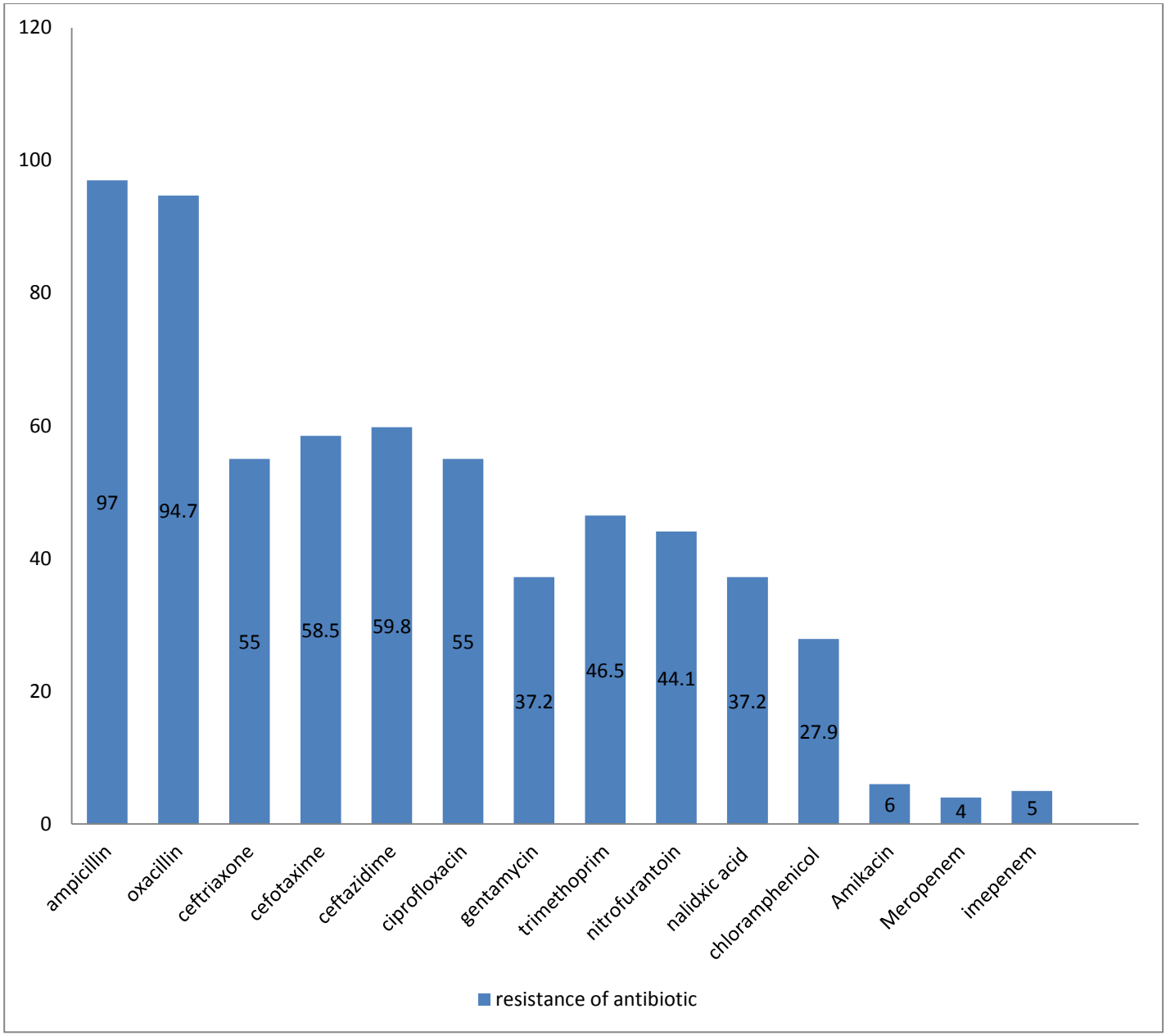

Fig. (4-1): the percentage of *E.coli* isolates resistance of antibiotics

5.DISCUSSION

Urinary tract infections are the most common type of the clinical disease produce by the *E.coli*. The results showed a high incidence of UTI in females than males. which might be due to variety of factors, such as the close proximity of the female urethral meatus to the anus [31], and alternations in vaginal microflora that play a critical role in encouraging vaginal colonization with coliforms which may lead to UTI [32, 33, 34] Reported that the bladder infections are 14-times more common in females than males by virtue of the shortened urethra. Moreover, [35] showed that UTI are more common in females, 40% of women have an episode in their lifetime when they are sexually active.

Urinary tract infections are one of the most commonly diagnosed infections in our hospital and probably in every hospital. Microorganisms causing UTI vary in their susceptibility to antimicrobials agents. The percent positivity for urinary cultures was found to be 82% (82 out of 100 samples). The possible explanation for isolation rate in highly suspected cases by clinical base may be due to prior use of antibiotics by those patients and the possible technical error during collection, transportation and processing of samples.

Numerous in vitro studies have shown the *E. coli* and *Klebsiellaspp.* as the most common causes of UTI [36]. The development of antibiotics resistance in these isolates is often related to the overuse and misuse of the antibiotics prescribed. Iraq is one of the developing countries where antibiotics sold over the counter, an attitude that encourages self-medication.

The reason of β-lactam resistance of *E. coli* isolates is probably due to the production of TEM β-lactamases, which may be genetically localized on the chromosome or on a plasmid. The TEM-1 is the most commonly encountered β-lactamase in Gram-negative bacteria; up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 [37]

Bacterial resistance to antibiotics is now widespread and possesses serious clinical threats. The organisms develop resistance to antibiotics by any of the following mechanisms: selection, mutation, phage transduction, and transference. Microbial resistance can be either hereditary in the organism or acquired through the environment[38]. The high resistance in the present study may be due to antibiotic abuse which leads to development of resistant isolates in Iraq. In agreement with the present study, [39]found that 56.8% of clinical *E. coli* isolates in Al-Diwaniyah were resistant to more than five antimicrobial agents.

The possible explanation behind the resistance showed to these antibiotics, may be because these antibiotics have been in use for a long period of time and must have been abused and as a result the micro- organisms have developed mechanisms of circumventing their mode of action. Alarming finding seen in the study was that, the substantial resistance shown to third generation cephalosporins (Ceftazidime and Ceftriaxone) which can be explained by the frequent and improper use of these antibiotics.

The bactericidal effect of grape seed extract is accountedfor the presence of Stigmasterol, a sterol molecule whichcause degradation of bacterial components by surfaceinteraction and pore formation in the bacterial cell wall. Itmight also be related to the presence of tannins which hasthe ability to inactive microbial adhesions, enzymes andcell envelope transport proteins, their complexity withpolysaccharide and their ability to modify themorphology of microorganisms.5 Therefore, thisobservation is suggestive of the antibacterial effect ofgrape seed extract.[40]

Conclusions:

The present study concluded that *E.coli* resistance to multiple antibiotic were recognized,and confirms that black grape seed extract had potential effect against *E.coli*

isolate, so it canbe used as natural alternative microbial inhibitor either in the food industry or as treatment for humaninfections, but further studies needs to establish its effect in vivo and in vitro.

Recommendations

1 . Public awareness of manifestation of UTI and proper use of antibiotics.

2. Continuous statistical records of antibiotics sensitivity to help physician for choosing appropriate antibiotics.

3. Syndicate of Iraqi pharmacists to help in instruction of antibiotics

4. Further studies needs to establish the effect of grape seed extract in vivo and in vitro and use different extraction methoods and compared the antimicrobial effect between group component.