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IN VITRO ANTIBACTERIAL ACTIVITY OF AQUEOUS EXTRACT OF CRANBERRY (VACCINIUM MACROCARPON) AGAINST E. COLI ISOLATED FROM URINARY TRACT INFECTION

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Abstract: The present study was carried out to investigate in-vitro antibacterial activity of aqueous extract of Cranberry (Vaccinium macrocarpon) fruit against Escherichia coli isolated from urinary tract infection. The present study included two experiment; the first one is isolate and identify of E. coli; the second experiment include study in-vitro antibacterial activity of aqueous extract of Cranberry, consisting two steps, the first step involved collection of Cranberry fruit then they were identified and then squeezed by an electric mixer to obtain the aqueous extract. The fruit extract then dried. The second step was to study the in vitro antibacterial activity of Cranberry aqueous extract including minimum inhibitory concentration (MIC) against E. coli used as reference antibiotics. The yielding percentage of Cranberry (Vaccinium macrocarpon) fruit extract was (30%). The in-vitro antibacterial study showed the Cranberry aqueous extract was more effective than Gentamicin against E.coli, this organism was quite resistant to Ciprofloxacin. It could be concluded however that Cranberry aqueous extract was more effective in comparison with the standard antibacterial agent, and that the antibacterial activity of Cranberry aqueous extracts against urinary tract infection may be attributed to its essential particularly the important phenolic compound (Proanthocyanidin).

Keywords: -

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Introduction

Urinary tract infections (UTIs) are represent one of the most common clinical infections in the world that affecting the human and animal. Approximately 150 million people worldwide develop UTI each year, with high social costs (Flores-Mireles et al., 2015). It is estimated that 40% of women develop at least one UTI during their lifetime (Micali et al., 2014). Pathogenic urinary tract infections occur in about 14% of dogs throughout their life. Furthermore, the significant rate of morbidity and mortality caused by multidrug-resistant (MDR) in intensive care units has resulted in an immense challenge to public health globally (Clark et al., 2016).

E. coli is the most common bacterium causing 80–90% of community- acquired UTIs and 30–50% of nosocomially acquired UTIs (Ejrnaes, 2011). In Egypt, the prevalence of UTI cases with MDR bacteria among pregnant women ranged from 22 to 35% in 2017 and increased to 53.5% in 2018 (Mohamed et al., 2017; El-Kashif and Eaid, 2018).

The infections with multidrug-resistant (MDR) uropathogens have become significantly challenging due to their high resistance to commonly used antibiotics. the high prevalence of MDR isolates and their resistance to most common antibiotics could be due to the inappropriate, inadvertent, intensive and excessive use of these antibiotics in treating UTI, these bacterial isolates have acquired multidrug resistance and it has become much tougher than ever to treat these infections (Laxminarayan et al., 2013; Paphitou, 2013). The rates of UTIs with multidrug-resistant E. coli in Iraq and Egypt, have dramatically increased and the treatment of these infections with monotherapy such as imipenem, ciprofloxacin and tobramycin, β-lactams, fluoroquinolones, trimethoprim–sulfamethoxazole, became limited or ineffective (Zhanel et al., 2006; El- Kashif and Eaid, 2018; Agyepong et al., 2018: Odoki et al., 2019; Mahde et al., 2021).

Urinary tract bacterial infections are common in women because they have a shorter urethra than men (Todar, 2007). The most common cause of UTI is Gram negative bacteria that belong to the family Enterobacteriaceae. Members of this family mostly include E.coli, Klebsiella, Enterobacter and Proteus. Also Gram positive Staphylococcus spp. play a role in the infection (Kunin, 1997). A UTI is common in dogs, cats, cows and horses, especially females (Joseph, 2007).

Escherichia coli is one of the most common bacteria capable of causing infection in humans and animals, particularly urinary tract infections (Iroha et al., 2009). One of the most important strains of Escherichia coli is O157:H7, it is an entero-hemorrhagic strain of the bacterium Escherichia coli and a cause of food borne illness. Infection often leads to hemorrhagic diarrhea, and occasionally to kidney failure, especially in young children and elderly. E. coli remains the predominant uropathogen (80%) isolated in acute community-acquired uncomplicated infections (Ronald, 2003) and it is the most prevalent pathogen associated with UTIs in young children (Sakran et al., 2003). At the first step of developing infections, bacteria must bind to the host cells and tissues, in most cases uroepithelial cells. For uropathogenic E.coli, Type1 fimbriae (Bahrani et al., 2002) and P-fimbriae are proteinaceous macromolecules that facilitate the adhesion of E. coli to uroepithelial cells (Gunther et al., 2001; Mulvey, 2002) due to continuing concern over antibiotic resistance in numerous types of infections (Wilson and Gaido, 2004).

At this time, resistance to the antimicrobial agents is recognized as a major global public health problem, (Iwu et al., 1999), it is possible that anti-microbial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have a clinical value in treatment of resistant strains (Cox and Balick, 1994). As resistance becomes more common there becomes a greater need for alternative treatments (Goossens et al., 2005).

Cranberry (Vaccinium macrocarpon) fruits and juice have been used to prevent urinary tract infection (UTI) and to protect humans against oxidative stress (Klein, 2005). Cranberries contain mainly vitamin C, dietary fiber, glucose and fructose, flavonoids [flavonols, anthocyanins, and proanthocyanidins (condensed tannins)], and gallic, benzoic, citric, and oxalic acids. The medicinal effectiveness and safety of cranberry juice/pills have been critically evaluated (Jepson, 2004). Cranberries seem to be the most effective in preventing the adhesion of E. coli to uroepithelial cells, which is responsible for 85% of UTI.

Therefore, due to the adverse effects of drugs, Thus there is a need for more effective and safe antibacterial agents. Therefore herbal medicines are considered safer alternatives because of natural ingredients with no side effects. However, plant extracts are the most important sources of herbal medicine and new drug development which produce efficient results in treatment of UTIs (Mohd et al., 2016).

This study was presented to find an alternative source to the use of antibiotics and among of medicinal plants, as well as avoiding the side effects of antibiotics and their use in treatment of recurrent urinary tract infections. These aims may accomplish by studying the in-vitro antibacterial activity of Cranberry aqueous extract E.coli O157:H7.

Methods

Table (2-1) Chemical Solutions used in the study

Chemicals and solutions	Company	Manufacture origin
Barium chloride	Merck	UK
Distilled Water	Biochemistry laboratory	C.V.M
Normal Saline 9.0 %	Fischer	Germany
Phosphate buffer saline	BDH	England
Sulfuric acid	Merck	UK

Table (2-2) Culture media used in the study

Culture media	Company	Manufacture origin
Brain heart infusion agar	Oxoid	England
Brain heart infusion broth	Oxoid	England
Sorbitol MacConkey agar	Himedia	India
Chrome Agar	Himedia	India
Eosin methylene blue agar	Oxoid	England
MacConkey Agar	Himedia	India
Muller- Hinton agar	Oxoid	England
Muller- Hinton broth	Oxoid	England

Table (2-3) Antibiotic discs that used in vitro study

Antibiotics	Company	Origin
Amoxicillin/Clavulanic acid	Bioanalyse	Turkey
Ampicillin	Bioanalyse	Turkey
Cefixime	Bioanalyse	Turkey
Cefuroxime	Bioanalyse	Turkey
Ciprofloxacin	Bioanalyse	Turkey
Fosfomycin	Bioanalyse	Turkey
Gentamycin	Bioanalyse	Turkey
Methicillin	Bioanalyse	Turkey
Norfloxacin	Bioanalyse	Turkey
Sulfomethoxazole	Bioanalyse	Turkey

2.1. Laboratory Apparatus and Equipment

Table (3-4): General laboratory equipment used in laboratory experiments

Apparatus	Company	Origin
Autoclave	Hirayama	Japan
Beakers 100,250,500	HBG	England
Burner	Himedia	India
Centrifuge	MPW	Poland
Cylinders 50 ,100, 250 ml	HBG	England
Digital balance	Etec	China
Digital thermometer	Honda	China
Electric oven	Memmert	Germany
Flask 250 , 500 ml	HBG	England
Freezer	SIEMENS	Germany
Hood	Electrothermal	England
Incubator	Gallekamp	Germany
Loop	Himedia	India
Micropipette	Slamed	Germany
Petri dishes (plastic)	Iraq-local market	Iraq
Pipette tibs	Gilson	France
Plastic rack	Meheco	China
Refrigerator	LG	S.Korea
Sensitive balance	Sartorius	Germany
Soxhlet apparatus	Electrothemol	China
Spectrophotometer	Apple	Japan
Transport swab / media (Amies media)		Jordan
Test tubes	Exceller	England
Vortex mixer	ADAM	India
Water bath	Electrothermal	England
Water distillatory	Kottermann	Germany

2.2. Preparation of Chemical Solutions:

2.2.1. Phosphate Buffer Saline (PBS, pH: 7.2):

It was prepared by suspending these following components; 8.5 gram NaCl, (1.15gm) Na2HPO4, (0.2gm) KH2PO4, (0.2gm) KCl in amount of double distilled water (dDW) with gentle stirring until the material dissolved completely. Then, the volume was completed to one liter and put in sterile bottle then the pH adjusted equal to 7.2 and filtration by using membrane filter 0.22µm, autoclaving at 15 Pounds (lbs)/Inch2 pressure with 121oC for 15 minutes then cooled and stored in refrigerator at 4oC (Simbert, 1981).

2.2.2. McFarland Turbidity Standards Tubes

The McFarland solution prepared by mixing of 0.05ml of barium chloride dehydrate 1.175%, with 9.95ml of sulfuric acid 1% to obtain McFarland standard No. 0.5 by (equivalent to 1.5 x 108 CFU/ml) (Quinn et al., 2004; Shakeel et al., 2015).

2.2.4. Preparation of Normal Physiological Saline

This solution was prepared by adding 8.85g of sodium chloride in 1000 ml of deionized water, and then was sterilized by the autoclave, cooled and stored in a refrigerator at 4oC until used (Hoorn, 2017).

2.3. Preparation of Cultural Media

Cultures media were prepared according to the manufacture's instructions, and sterilized by autoclaving at 121 Co, 1.25kg/cm2 for 15 min.

D- Brain Heart Infusion Agar

It was prepared by dissolving 52 g of Brain Heart Infusion agar in 1000 ml of distilled water, pH was adjusted to 7-7.2 .The media was autoclaved at 121 Co, 1.25kg/cm2 for 15 min.

H- Eosin Methylene Blue Agar

It was prepared by dissolving 36 g of Eosin Methylene Blue agar in 1000 ml of distilled water, pH was adjusted to 7-7.2 .The media was autoclaved at 121 Co, 1.25kg/cm2 for 15 min.

I- Mueller-Hinton Agar

It was prepared by dissolving 38g of Mueller-Hinton Agar in 1000 ml of distilled water, pH was adjusted to 7-7.2 .The media was autoclaved at 121 Co , 1.25kg/cm2 for 15 min.

2.5.1. Sterilization Methods

1- Sterilization by Autoclave: Culture media and solution used in the study were sterilized in autoclave for 15 minutes at 121 Co and pressure 1.25kg/cm2.

2- Oven sterilization (Dry heat): This method was used for sterilization glassware for caps used in the study, commonly; a temperature of 160 Co is maintained for 3 hours.

3- Direct flaming (heating) : Inoculating wire, point of forceps and spatulas were sterilized by holding them on the flame of benzen burner until they were seen to be red hot (Quinn et al.,2004).

2.4. Isolation and identification of E. coli

2.4.1. Samples Collection

Urine sample was collected during 2/2021 from Al_yarmook hospital laboratories /Baghdad province. The urine was obtained from a patient suffering from urinary tract infection (UTIs) signs including dysuria, flank pain, urinary frequency or urgency, and fever. The sample was obtained sterilely in cup using an aseptic technique. Within 30 minutes of collection, the specimen was transferred to the laboratory by cooled box.

2.4.2. Bacteriological Examination

2.4.2.1. Culturing

urine sample was subjected to bacterial culturing on nutrient agar by spreading 0.1 ml of precipitated urine following centrifugation at 3000 rpm for 15 minutes then were incubated at 37 ºC up to 24 hrs. and based on morphological characters (shape, color, and size) the suspected colonies of E. coli were re-inoculated on MacConkey agar for to obtain pure culture, also on selective media (Eosin Methylene blue, Sorbitol MacConkey agar and Chromogenic agar O157 (OIE, 2000).

2.4.2.2. Activation and Maintenance of Bacterial Isolates

One loop from pure culture was streaked on brain heart infusion slant then incubated at 37 ºC for 24 hrs. Slants kept in 4 ºC and was sub-cultured once every two-weeks (Quinn et al., 2004).

2.5. Preparation of standard bacterial suspension

The bacterial suspension of E. coli was prepared by taking 1 ml from brain heart infusion broth (BHIB) overnight culture of E. coli suspension mixing with 9 ml of peptone water and standardized by matching to the (0.5) Standard McFarland solution.

2.6. Sensitivity test by using Agar well diffusion method

The agar well diffusion method was adopted according to (Grove and Randall, 1955 and Kavanagh, 1972), for assessing the antibacterial activity of the prepared extract. 5 ml of standardized bacterial stock suspensions (1.5 ×108 CFU/ml) of E. coli O157:H7 was thoroughly mixed to each 500 ml of sterile Mueller Hinton agar. 25 ml of the inoculated Mueller Hinton agar was distributed into sterile Petri dishes of each.

The agar was left to setile for 10 minutes to allow solidifition the agar, and in each of these plates 6 well, 6 mm in diameter were cut using a sterile Pasteur pipette and the agar discs were removed by a sterile forceps, the wells were filled with 0.1ml of each concentration of 10, 20, 40, 60, 80 and 100 mg/ml of Vaccinium macrocarpon extract using microtiter-pipette, that allowed to diffuse at room temperature for two hours.

The plates were then incubated in the upright position at 37 Co for 24 hours. Three replicates were carried out for each concentration extract and the activity of plant extract was determined by measuring the diameter of inhibition zone around each well by millimeter against the tested organism. The results and standard errors means values were tabulated Table (3-8).

Table (3-8): Zone diameter Value (mm) used to indicate susceptible, intermediate and resistance bacteria (NCCLS, 2000).

	MIC (μg/ml)	Zone Diameter (mm)
Susceptible	≤ 4	≥ 20
Intermediate	8-16	15-19
Resistant	≥ 32	≤ 14

" Susceptible breakpoint " is 4μg/ml or 20 mm.
" Resistant breakpoint " is 32 μg/ml or 14 mm

2.7. In-vitro Antibacterial Test of Ciprofloxacin and Gentamycin

Ciprofloxacin and Gentamycin were used as a reference antibiotics to determine sensitivity of E. coli (Okwu and Igara, 2009 ; Sharma and Patel, 2009).The same technique which was used for Cranberry antibacterial sensitivity was used for determination of Ciprofloxacin activity by using the concentrations of 10,20,40,60,80 and 100 μg/ml., 0.1 ml of sterilized distilled water was served as a control.

2.8. Plant Materials

Fresh cranberry (Vaccinium macrocarpon) was collected from field at Duhock city (North of Iraq). Later these plant fruit were washed under tap water. The fruits were squeezed to a juice by electrical scramble.

2.8.1 Preparation of Crude Aqueous Extract Vaccinium macrocarpon Plant

Aqueous extraction of the Vaccinium macrocarpon was carried out according to (Fehri. et al., 1994), by taken (50 g) of the fruits of plant cranberries and then squeezed by using an electric mixer and then put in a sterile beaker glass and filtrate by filter paper (Whitman No. 1), then collect filtrate collected in glass beakers were placed in the

electric oven of (40Co). The dried extract was placed in an incubator under 40 Ċ for complete dryness. The final dry extract was kept frozen at -20 Co until use.

2.8.2. In vitro Antibacterial activity of Aqueous Extract of Vaccinium macrocarpon

2.8.2.1. Preparation of Different Concentration of Aqueous extract

Stock solutions were prepared by mixing 1 g. of dried fruit extract with 10 ml of distal water (D.W) that was sterilized with Millipore membrane filter (0.20μm). Then concentrations of 10, 20, 40, 60, 80 and 100 mg/ml were prepared by mixing known volume from the stock solution with D.W.

2.9. In vitro Antibacterial Activity of Standard Antibiotics

Gentamicin, and Ciprofloxacin were used as a reference antibiotics to determine sensitivity of bacterial species tested (Okwu and Igara, 2009; Sharma and Patel, 2009). The same technique which was used for Vaccinium macrocarpon antibacterial sensitivity was used for determination of Standard antibiotic activity by using the concentrations of 10, 20, 40, 60, 80 and 100 μg/ml., 0.1 ml of sterilized distilled water was served as a control

Result and Discussion

3.1. Extraction of Vaccinium macrocarpon

Aqueous extraction of Vaccinium macrocarpon fruit gave a deep red colored extract with yield of 30%; this was determined by using the followingequation:

Percentage yield of the extract = weight of extract (gm) /weight of Cranberry fruit (gm) × 100

(Banso and Adeyemo, 2006).

=15 (gm) / 50 (gm) × 100 =30%

This result is nearly similar to the results of Howell et al. (1998) who found that the yield extract was 24g from 100g of fresh fruits. Deep red crude extract color is similar with what found by Deborah et al. (2010).

3.2. Isolation and identification of E. coli

The results of current study showed that the colony of positive isolates of E. coli appeared pink in color on MacConkey agar Figure (4-1-A), this occurs due to the ability of the bacteria to fermenting the lactose and produce pink color colonies. In Eosin Methylene Blue (EMB) agar was applied for selection and differentiation purposes and it was considered as a rapid method for distinguishing E. coli from other gram-negative bacteria, the colonies appeared as green metallic sheen and that indicated a vigorous fermentation of lactose, and acid production which precipitated the green metallic pigment Figure (4-1-B). These results agreed with Quinn et al., (2004) and Oyewole et al., (2019).

Figure (3-1): Colonies of E. coli on MacConkey agar and Eosine methelene blue (EMB)

4.4. In-vitro Antibacterial activity of Aqueous Extract of Vaccinium macrocarpon

Different concentrations of aqueous Cranberry extract that were used in agar diffusion assay caused different degrees of zones of inhibition against E.coli O157:H7. The sizes of inhibition zones were different according to concentration of extract (Table 4-3). The results showed that E. coli was more sensitive to aqueous extract of cranberry than Ciprofloxacin in all the concentrations using in this study. In all concentration that used there was a significant increase ($P<0.05$) in diameter of zone of inhibition in E.coli growth, while it was resistance to Ciprofloxacin (Figure 3-3).

The results of Cranberry antibacterial activity against E.coli growth was in agreement with Magaarinos et al., (2008) who studied the antibacterial activity of cranberry extract against pathogenic microorganisms including E.coli, Salmonella spp, Listeria monocytogenes P. aeruginosa and S. aureus.

Table (4-3): Antibacterial activity of cranberry against E. coli O157 H7.

Concentration (mg/ml) / Zone of Inhibition (mm)	10mg/ml	20mg/ml	40mg/ml	60mg/ml	80mg/ml	100mg/ml
Aqueous Extract of Cranberry	8.83±0.31 F a	11.83±0.32 E a	13.50±0.34 D a	18.33±0.21 C a	21.50±0.41 B a	23.83±0.31 A a
Distilled water	0.0±0.0 A b	0.0±0.0 A b	0.0±0.0 A b	0.0±0.0 A b	0.0±0.0 A b	0.0±0.0 A b

Values represent mean ±S.E

Different capital letters mean significant ($P< 0.05$) results between different concentrations. Different small letters mean significant ($P< 0.05$) results between solvent and extract.

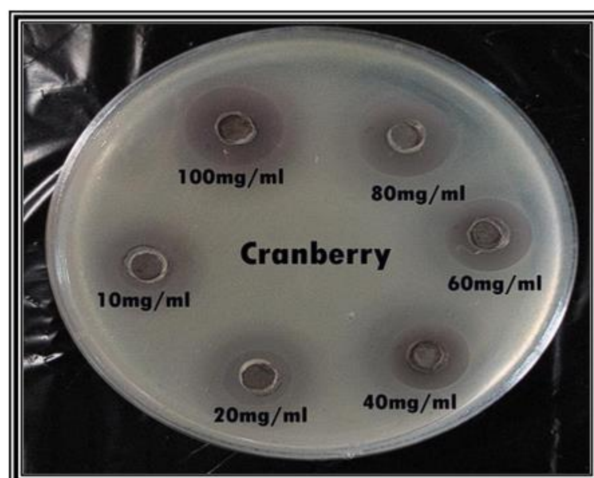


Figure (3-3): Sensitivity of E. coli to aqueous extract of Vaccinium macrocarpon (mg/ml).

4.4.1. In vitro Antibacterial Activity of Ciprofloxacin and Gentamicin

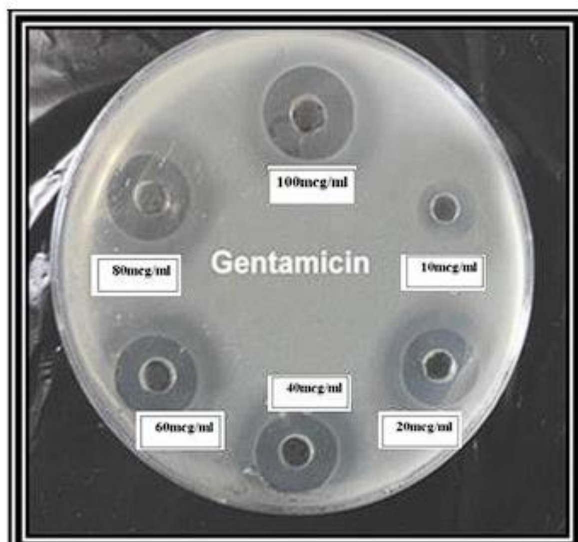
Ciprofloxacin and Gentamicin was used as a reference antibiotic E. coli was sensitive significantly (P<0.05) to Gentamicin in dose dependent concentration1 (100, 80, 60, 40, 20 and 10 µg/ml), Gentamicin wasbactericidal against susceptible organisms by irreversibly binding to the bacterial ribosome and inhibiting protein synthesis. Once inside the nucleus ofthe cell, the Gentamicin irreversibly binds to the 30S subunit of the bacterial ribosome, disrupts protein synthesis, and eventually causes cell death through leakage of essential bacterial constituents. Gentamicin active against some Gram-positive and many Gram- negative organisms and also uses for treatment of urinary tract infections. The results of the current study were agreed with Badaruddin (2007) who stated that E.coli was predominant isolate from UTI patients and treated with Gentamicin is appearing good quality response against the isolates of Escherichia spp (80%). While E. coli was resistance to Ciprofloxacin in all the concentrations using in this study. Evidence obtained from laboratory and epidemiology studies indicated thatthe persistence of resistance bacteria was related to the persistence of antimicrobial drug use (Hirsch and Lundquist, 2009). If an antimicrobial drug is used, continuously, the persistence of resistant organism will go on. Thus E.coli has often higher degrees of antimicrobial resistance which have a long history of use. In contrast, the results of the current study were agree with Ahmed et al., (1997) they state that Ciprofloxacin was resistance for E. coli that isolated from patients infected with UTI ,so this drug considered as a drug of choice in UTI infections .

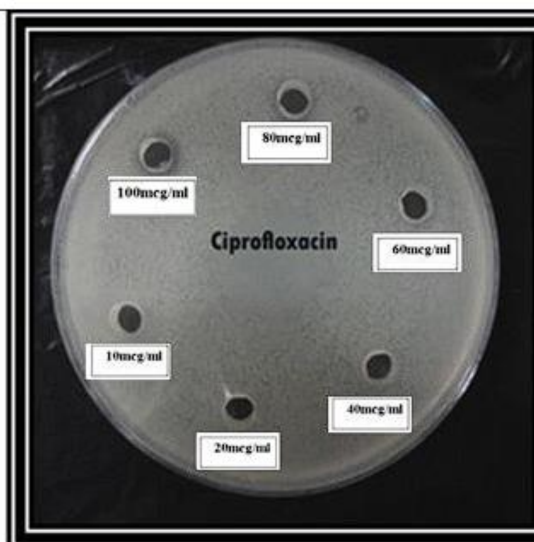
Resistant E. coli isolates in the study of Ciprofloxacin used may be due to a change in the target site for a link counter to the enzyme, aschange occurs in (GyrA), one of the building blocks of the enzyme (DNA gyrase), as it happens mutagenesis of the gene (parC) which encodes for (parC), which is one of the structural units of the enzyme (Topoisomerase IV)(Brisse et al., 1999; Fluit et al., 2001). Table (3-4), and (Figure 3-3)

Table (3-4): Antibacterial activity of reference antibiotics (Gentamicin and Ciprofloxacin) used against E. coli

Concentration (µg/ml) / Zone of Inhibition (mm)	10µg/ml	20µg/ml	40µg/ml	60µg/ml	80µg/ml	100µg/ml
Gentamicin	**9.67±0.33 F a**	**11.23±0.36 E a**	**12.03±0.067 D a**	**13.83±0.40 C a**	**18.17±0.31E B a**	**20.33±0.49F A a**
Ciprofloxacin	**0.0±0.0 A b**	**0.0±0.0 A b**	**0.0±0.0 A b**	**0.0±0.0 A b**	**0.0±0.0 A b**	**0.0±0.0 A b**

- Values represent mean ±S.E
- Different capital letters mean significant (P< 0.05) results between different concentrations.Different small letters mean significant (P< 0.05) results between different antibiotics.





Figure(3-5): Sensitivity of *E. coli* to **Figure (4-4): Sensitivity of *E.coli* Gentamicin. to Ciprofloxacin.**

Conclusion

From the previous results, the following can be concluded:

1- The aqueous extract of cranberry has highly antibacterial activity especially against pathogenic E. coli for UTI and low MIC.
2- The pathogenic bacteria of E. coli are resistant to Ciprofloxacin and sensitive to Gentamicin.

Recommendations

1- Extraction of cranberry (Vaccinium macrocarpon) fruit and other plant parts by solvent (ethanol and methanol) and different techniques.
2- Study the antibacterial activity of the extract on other gram negative and gram positive pathogenic bacteria of V.M extracts by using different parts of the plant.
3- Study the antifungal activity of V.M extract on fungus by using differentparts of the plant.

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