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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(1):77-85. **OPEN ACCESS**

Antimicrobial activity of two alcoholic plant extracts against uro-pathogenic *Escherichia coli*

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The *Escherichia coli* isolated from UTIs,were identified with biochemical tests and genotyping test with 16S rRNA. The ethanolic extracts of *Cinnamomum zeylanicum* bark, and *Origanum majorana* leaves were tested against *Escherichia coli* by using the well agar diffusion test, the alcoholic extracts were possessed antimicrobial activity against tested microorganism. At 200 mg/ml, and 100 mg/ml concentrations *Cinnamomum zeylanicum* bark was gave diameter of inhibition zone rang from (18-26mm), (14-16mm) respectively, when the diameter of inhibition zone was ranged from (7-9mm), (5-6mm) subsequently for *Origanum majorana* leaves, and these results compared with antibiotics sensitivity test were used by discs diffusion method against *Escherichia coli*, they were resisted to all antibiotics used in this study. Minimum inhibitory concentration was estimated for plant extracts, and was ranged versus *Escherichia coli* (12.5-25) mg/ml for *Cinnamomum zeylanicum* alcoholic bark extract, while *Origanum majorana* leaves extract also had antimicrobial activity but in high concentrations only. It could be concluded that alcoholic extract of *Cinnamomum zeylanicum* bark had a good antimicrobial effects, and may be able to use in low concentrations for treatment UTIs cause by *Escherichia coli*.

Keywords: antimicrobial activity,Esherichia coli, *Cinnamomum zeylanicum*, *Origanum majorana*)missing

INTRODUCTION

UTIs are caused by both Gram-negative and Gram-positive bacteria, as well as by certain fungi. The most common causative agent for both uncomplicated and complicated UTIs is uro-pathogenic *Escherichia coli* (UPEC) (Foxman, 2014). The most common type of infection due to ExPEC is urinary tract infection (UTI); 70–95% of UTIs are caused by ExPEC (Stamm and Hooton, 1993; Hooton and Stamm, 1997). *E. coli* is the common cause of many infections such as: urinary tract infections, diarrhea and bacteremia. *E. coli* can cause these infections because it have many virulence factors like adhesion ,iron uptake, toxins, capsular polysaccharides , and proteins(Oliveira et al.,2011) .(O'Hanley et al.,1991) showed that the hemolysin contributed to renal parenchymal damage in a murine model of urinary tract infections (UTIs) but it did not influence colonization levels in the kidney.

Moreover, cinnamon has also been shown to prevent the production of COX-2, a pro-inflammatory agent. Therefore, cinnamon has antibacterial and anti-inflammatory properties. The chemistry of cinnamon bark oil primary contains a cinnamaldehyde that is different from that found in cinnamon leaf oil (eugenol)(Wijesekera,1978).*Origanom vulgare* and *O. majorana* are rich in the essential oil characterized for high amount of phenolic compounds which are believed to be

responsible for their antimicrobial property (Skandamis, 2002).. In addition, they have been used in the folk medicine to treat several illnesses as spasmodic, antimicrobial, digestive, expectorant and aromatic for the whooping and convulsive coughs (Novak, 2003).

MATERIALS AND METHODS

Isolation of bacteria:

Sixty samples of urine were collected from Al-Yarmuk, and Al-Karama hospitals from patients suffering from UTIs.

Identification of bacteria by biochemical tests:

The isolated bacteria were identified with morphological characters and biochemical tests.The MacConkey agar (Himedia/India) was used for primary identification of *E. coli* and other gram negative bacteria; Blood agar was used in identification of isolated bacteria, and for the detection of hemolytic activity and the kind of hemolysis, EMB media used also in identification. Indole and Simmons citrate tests to identify *E. coli* from other Enterbacteriacae (Collee, 1996)1996).

Identification of bacteria by genotyping test:

The diagnosis of the isolates was done also by PCR depending on 16S rRNA amplification genes.

RNA amplification genes.

DNA Extraction from bacteria:

The DNA was extracted from the bacteria *E. coli*, G- spin DNA extraction kit, Intron biotechnology, and cat. No. 1704, and according to the kit protocol as manufactured company as following:

Transfer 2 ml cultured cell into 2 ml tube. Pellet of cell by centrifugation for1 min at 13,000 rpm, and discard supernatant. Resuspend completely the cell pellet with remnant supernatant by tapping or vigorously vortexing. Add 200 µl Buffer CL, 20 µl Proteinase K and 5 µl RNase. A Solution into sample tube and mix vortexing vigorously. Incubate lysate at 56 °C (preheated heat block or water bath) for 30 min. After lysis completely, add 200 µl of Buffer BL into upper sample tube and mix thoroughly. Then incubate the mixture at 70 °C for5min. Centrifuge the sample tube at 13,000 rpm for 5 min to remove un-lyses tissue particles. Then carefully transfer 350 µl of the supernatant into a new1.5 ml tube. Add 200 µl of absolute ethanol into the lysate, and mix well by gently inverting 5 times or by pipette. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from inside of the lid. Carefully apply the mixture to the Spin Column (in a 2 ml Collection Tube) without wetting the rim, close the cap, and centrifuge at 13,000 rpm for 1 min. Discard the filtrate and place the Spin Column in a 2 ml Collection Tube . Add 700 µl of Buffer WA to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and reuse the Collection Tube. Add 700 µl of Buffer WB to the Spin Column without wetting the rim, and centrifuge for 1 min at13,000 rpm. Discard the flow-through and place the Column into a 2.0 ml Collection Tube, Then again centrifuge for additionally 1 min to dry the membrane. Discard the flow-through and Collection Tube altogether. Place the Spin Column into a new 1.5 ml tube, and 100 µl of Buffer CE directly onto the membrane. Incubate for 1 min at room temperature and then centrifuge for 1 min at 13,000 rpm to elute.

PCR Amplification:

PCR assay was performed in a monoplex pattern in order to amplify16S r RNAgene as showing in table (1), and table (2).

Sequencing of *E. coli* PCR product:

The samples of (bacteria) were sent to Microgen /Korea, for gene sequencing. Using genetic analyzer (Applied Biosystem) and homology search was performed and also using (BLAST) program online blast n and blast x algorithms at NCBI. With Primer was designed specific for this study as showing in table (1); Annealing temperature was 52^{o}C in table (2).

Agarose gel electrophoresis of DNA

Electrophoresis has been done to determine DNA pieces after the process of extraction or to detect the result of the interaction of PCR during the presence of the standard DNA to distinguish the bundle size of the outcome of the interaction of PCR on the Agarose gel as shows in Figure (1).

Preparations of plants extracts:

The bark of *C. zeylanicum* and *leaves* of *O. majorana* was used in current study was bought from stores of plants in Baghdad city. Bark was washed with water and then dried in an air oven at 380° C. Then they were grinded and stored until they used.

Table (1): The primer 16s rRNA of gene used for *E. coli* identification:

Product size	GC (%)	Tm (°C)	Sequence	Primer
1250.bp	50.0	54.3	'AGAGTTTGATCCTGGCTCAG- 3 -'5	Forward
	42.1	49.4	'GGTTACCTTGTTACGACTT- 3 -'5	Reverse

Table (2): Program used to amplify the 16s rRNA of gene used for *E. coli* identification

No. of cycle	Time	Tm (°C)	Phase	No
40 cycle	3 min	95°C	Initial denaturation	1
	45sec	95°C	2-Denaturation	2
	45sec	52°C	Annealing	3
	50sec	72°C	1-Extension	4
	10 min	72°C	2-Extension	5

10 g of each plant powder was extracted with 200 ml of 80% (v/v) aqueous ethanol by Soxhlet apparatus used for 8 hrs, then the extract was dried in oven with 40° C, and the powder of plant extract was kept in a refrigerator to further use (Ahmed et al., 1998).

Antibacterial activity of plants extracts:

Antimicrobial activity of bark *C. zeylanicum*, and *O. majorana* leaves ethanol extract was tested with agar well diffusion method by (Das *et al*., 2010), with using the plates of Mueller Hinton Agar (Himedia /India). The pathogenic strains of *E. coli* were spreading on the surface of agar as described in sensitivity test with discs. Wells of 8 mm in diameter were cut into agar plates and extract were prepared as concentrations (200, 100, 50, 25, 12.5, and 6.25) mg/ml then placed into each well. The plates were incubated at 37°C for 24 hrs, and antibacterial activity was evaluated by measuring the diameter of the inhibition zone around the wells in millimeter by ruler.

Antibiotics sensitivity test:

In vitro sensitivity test was done according to Kirby-Bauer (Kirby *et al*.,1966);after dilution to the standard turbidity of McFarland tube no. 0.5. With a sterile swab dip it into the broth culture of organism and then were inoculated on Mueller Hinton agar plates. After the streaking is complete, allow the plate to dry for 5 minutes. Antibiotic discs can be placed on the top of agar by using after dilution to the standard turbidity of McFarland tube no. 0.5. With a sterile swab dip it into the broth culture of organism and then were inoculated on Mueller Hinton agar plates. After the streaking is complete, allow the plate to dry for 5 minutes. Antibiotic discs can be placed on the top of agar by using sterilized forceps.

The inoculated plates incubate over night at 37°C.In this study eleven antibiotics discs (Bioanalyse/Turkey) were used they are: Ceftazidime(30µg), Cefotaxime(10µg), Amikacin (10µg), Amoxicillin (10µg), Ciprofloxacin(5µg),Trimthoprim(30µg),Kanamycin(25µg),Norfloxacillin(10 µg), and Cefalexine (10µg), Tetracycline(5 µg), Doxycillin (5µg).Then measured the diameter of inhibition zone in millimeter by ruler for antibiotics usedCompare the measurement obtained with the standard table on NCCLS to determine the sensitivity zone (CLSI, 2015).

Minimum inhibitory concentrations (MIC) determination of plants extracts:

The MIC of crude extracts was determined by broth macro-dilution assay. A set of test tubes with concentrations of plant extract (200, 100, 50, 25, 12.5, and 6.25) mg/ml. Tubes were inoculated with bacteria. After incubation, tubes were examined for changes in turbidity as an indicator of growth. The first test tube that appeared clear was considered as MIC of *C. zeylanicum* bark extract, and *O. majorana* leaves extract against *E. coli* (Das et al., 2010), with used positive and negative control for Comparison.

RESULTS

Isolation:

Sixty samples of urine were collected from Al-Yarmuk, and Al-Karama hospitals in Baghdad from patients suffering from UTIs from September 2017 to January2018. Only twenty five samples were gave growth for bacteria. Two isolates belong to *E. coli*.

Identification of bacteria by biochemical tests:

Sixty urine samples were collected from patients with UTIs. The samples were cultured on MacConkey agar, the bacteria gram negative

were appeared pink colonies because lactose ferments in table (3). IMViC tests were used in identification. Indole test was positive for *E. coli* with red ring because the bacteria can split amino acid tryptophan to form the compound indole. Methyl red (MR) was positive (red), while Voges-Proskauer (VP) was negative (yellow). And Simmons Citrate test the bacteria cannot utilize the citrate; therefore, the media was remained green with growth of bacteria. And on blood agar the types of haemolysis was gamma or alpha haemolysis, on EMB *E. coli* gave green metallic sheen phenomena.

The PCR products were separated on a 2% agarose gel electrophoresis and visualized by exposure to ultra violate light (302nm) after Red Stain staining. The PCR products of ***E. coli*** isolates was compared with DNA ladder (100), and the amplified size was 1250bp as shows in table (1).The isolated bacteria were showed identity 99%, Figure (1).The Polymerase Chain Reaction (PCR) is a molecular method, which can be used for detection of pathogenic microbes from food, water and other environmental samples. (Duarte et al., 2009).

Antibacterial activity of Cinnamomum zeylanicum and Origanum majorana:

The results showed an inhibitory effect of *C. zeylanicum* bark extract at a concentration of 200 mg/ml (18-26mm), in concentration 100 mg/ml (14-16mm) against bacteria, while the other concentrations 50 mg/ml appear inhibitory activity against the isolates of *E. coli* (11-12mm), and also in 25mg/ml (9-11mm).While in concentration 12.5mg/ml the diameter of inhibition zone was ranged(8-10mm).and according to these results was found the effect of plant extract decreases as its concentration decreases, on the other hand the *O. majorana* had a very weak antibacterial activity against *E. coli* with high concentrations 200,and 100 mg/ml only and not active in lower concentrations; when the diameter of inhibition zone was ranged from(7-9 mm),(5-6mm) subsequently, as shows in table (4).

The results of this study were showed that extract of bark with ethanol(80%) *C. zeylanicum* were given that extract in concentrations (200, 100, 50,25, and 12.5) mg/ml, were showed antibacterial activity against *E. coli*, but in low concentrations had not given any activity. And these results agree with the results of (Mandal et al., 2011), who were improved, the antibacterial activity was determined with diameters of inhibition and MIC values at different times of incubation. Another study said that the antibacterial activities of *C. zeylanicum* bark extracts, extracted with used different organic solvents, as ethyl acetate, acetone and methanol, were tested in vitro against gram negative(*Klebsiella pneumonia*e, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter cloacae*),and gram positive(S*taphylococcus aureus*, *Bacillus megaterium*, *Streptococcus faecalis*) by the discs diffusion method. The results showed that the antibacterial activity, appear as inhibition zone, ranges from (7–18) mm in concentration 30 µL, the author suggested a high antibacterial activity (Seukep et al., 2013).Also other results showed that the antibacterial activity of the plant extracts against Gram negative bacteria was more than of Gram positive bacteria and this might be relevant because the differences in the chemical structure of the bacterial cell wall of these bacteria (Muchuweti et al.,2007).The main compounds isolated and identified in cinnamon (*C. zeylanicum*) belong to two chemical classes: polyphenols and volatile phenols. Among polyphenols, cinnamon contains mainly vanillic, caffeic, gallic, protocatechuic, *p*-coumaric, and ferulic acids(Burt and Reinders,2003).Also another study was showed that alcoholic bark extract from (200-12.5)mg/ml possessed antimicrobial activity against*K. pneumoniae*(Sweedan E.,2018).Myrcene, γ-terpinene, α-terpinene, p-cimene, borneol, thymol, carvacrol, β- pinene, β-cariophyllene, limonene, α-pinene, linalool and sabinene are some compounds found in *O. vulgare* and *O. majorana* essential oils (Novak et al.,2003; Chun et al., 2005).Thymol and carvacrol, which have been known as major compounds of these essential oils (Lambert et al.,2001; Marino et al.,2001),are able to increase the microbial cytoplasm membrane permeability, probably because their capability of dissolving into the phospholipid bilayer aligning between the fatty acid chains and causing a distortion of the membrane physical structure (Ultee and Smid ,2001).

Table (3): the biochemical tests for *E. coli* identification

The test name	results
Microscopically	Gram negative
Indole	positive
MR	positive
VP	negative
Simmons Citrate utilization	negative
Mac Conkey agar	Pink colonies
Blood agar	Alpha or gamma haemolysis
EMB agar	Green metallic sheen phenomena

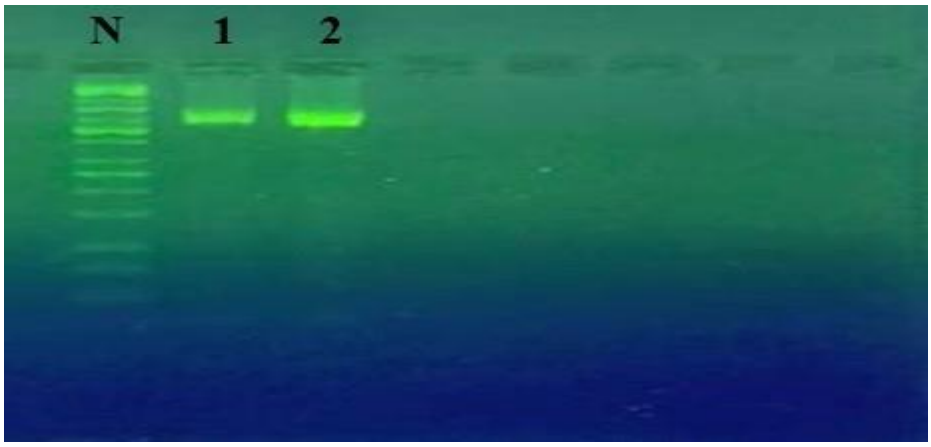

Figure (1): gel analysis of PCR product the band size 1250 bp. The product was electrophoresis on 2% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100)
1. *E. coli* isolate 1 2. *E. coli* isolate 2

Table (4): Range diameter of inhibition zone in millimeter of the plant extracts *Cinnamomumzeylanicum and Origanummajorana*on *E. coli*

Concentrations of plant extract in mg/ml	200	100	50	25	12.5	6.25	Control
Cinnamomum zeylanicum	18-26	14-16	11-12	9-11	8-10	-	-
Origanum majorana	7-9	5-6	-	-	-	-	-

Table (5): inhibition zone diameter by discs sensitivity test on *E. coli*:

Antibiotics discs name	Range of inhibition zone diameter in mm of *E. coli* isolates	Percent of resistant (%)
Ceftazidime (CAZ)	0mm	100%
Norfloxacin(NOR)	0 mm	100%
Amoxicillin(AMC)	0mm	100%
Cefotaxime (CTX)	0mm	100%
Doxycillin(DA)	0mm	100%
Cefalexin (CN)	0mm	100%
Trimthoprim (TMP)	0mm	100%
Tetracycline(TE)	0mm	100%
Ciprofloxacin (CIP)	0mm	100%
Amikacin(AK)	13mm	100%
Kanamycin(KN)	0mm	100%

Sensitivity test for antibiotics and compared with plant extract antimicrobial activity:

The sensitivity test of isolates *E. coli* was tested to a number of antibiotics used to treat some of the infections caused by this species in humans.100% of isolates were resistant to all antibiotics used in this study as shows in table (5). A study in Nigeria was determined the prevalence and antimicrobial susceptibility of *E. coli* from clinical sources.A total of 32 samples were analyzed for isolation and identification of bacteria and antimicrobial susceptibility testing. *Escherichia coli* were isolated from 14(43.75%) samples.The *E. coli* - infection was highly implicated in urine specimen (21.42%).The *E. coli* isolates showed resistance to ciprofloxacin (92.86%), cotrimozazole (92.86%) and ceftriazone (78.58%). Lower susceptibility was observed with oflaxacin (28.57%).Indiscriminate use of antibiotics should be discouraged. Regular hygiene methods should be advocated among community dwellers and hospital personnel .

The β-lactams, fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole are often used to treat community and hospital infections associated with *E. coli* (Joseph et al., 2017).

Table (5) shows *E. coli* which isolated from UTIs were resisted to all antibiotics used in this study, and this disagree with other study that *E. coli* were resistant to commonly used antibiotics but sensitive to Nitrofurantoin, Amikacin and Cefotaxime are considered appropriate for empirical treatment of E. coli in the study area of (Acar and Rostel ,2001). Microorganisms causing UTI vary in their susceptibility to antimicrobials agents from region to other; while the extracts of plants were very active against these isolates. Only Amikacin was showed inhibition zone with diameter 13 mm, and this diameter was less than diameter of plants extracts with high concentrations used in this study, shows in table (4).Because of the increasing in drug resistant of strains of *E. coli* especially that which isolated from UTI, the increasing the frequent in discriminate use of antibiotics (Punnam , 2016).

When comparing the inhibitory effect of antibiotics with the inhibitory effect of the *C. zeylanicum* extract in table (4) ,and (5) ;it was observed that the *C. zeylanicum* at concentration (200mg/ml) and inhibition diameter was (18-26mm) for isolates were near potent the efficacy of antibiotics such as Norfloxacin and concentration (100mg/ml) in inhibition diameter is (14-16mm) was less potent than the efficacy of antibiotics such as Norfloxacin inhibition diameter zone was (24mm-30mm), and Cefotaxime with inhibition diameter of (26-27mm) and this same potent the efficacy of antibiotics such as Cefotaxime and according current results may can use *C. zeylanicum*bark to treat UTI which caused by *E. coli* because it had a good antimicrobial activity against this bacteria with less side effects. While *O. majorana was showed a diameter of inhibition zone less than the used antibiotics in current study, therefore; cannot use for treatment of this bacteria.*

Minimum inhibitory concentrations (MIC) determination of plants extracts:

The MIC of crude extracts was determined by broth macro-dilution assay .Aset of test tubes with different concentrations of two plants extracts

(200, 100,50, 25, 12.5, and 6.25) mg/ml. And after incubation over night we was found that the first clear tube with the low concentration of extract was determined as MIC value for bark extract of *C. zeylanicum* and MIC value was ranged (12.5-25) mg/ml for all isolates. And these results agree with results of (Alsalim et al., 2016); their laboratory tests of antibacterial activity showed that *Staphylococcus aureus* was the most affected by the extracts under study then followed with *Enterococcus faecalis, Streptococcus pneumoniae, Escherichia coli and Klebsiella pneumoniae* respectively. Aqueous extract showed highest values in MIC (400 µg/ml) for *S aureus*, followed by chloroform (350 µg/ml, MIC), then methanol (335 µg/ml, MIC) , while the lowest values were recorded for *K. pneumoniae*(MIC 175, 200 and 250µg/ml, respectively), while in this study the MIC value of *O. majorana* leaves extract was 100 mg/ml and this result not agree with Ramadan and his group who was mentioned that *O. majorana* was showed a moderate antibacterial activity of the ethanol extract with MIC value 25 mg/ml against Gram negative bacteria *E. coli* (Ramdan et al.,2018) ,and for that might can use bark extract of *C. zeylanicum* to treat UTIs with *E. coli* because it has little side effect for human health less than other chemical drugs used in the treatment of UTIs.

On the other hand *O. majorana* leaves extract was had a moderate antibacterial activity against *E. coli* isolated from UTIs, therefore; it is not best treatment use in the treatment of these infections.

CONCLUSION

Alcoholic bark extract C*. zeylanicum* had a good antibacterial effect on *E. coli* in low concentrations, while *O. majorana* alcoholic leaves extract had weak antibacterial activity with high concentrations. On the other hands the isolates of *E. coli* were resisted to all antibiotics used in this study, therefore; these medicinal plants may can use for treating UTIs were caused by *E. coli*.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEGEMENT

This work was supported by Biology department, College of Science, University of Baghdad.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the experiments. MTF designed and performleed the experiments and reviewed the manuscript . EGS performleed the experiments and also wrote the manuscript ,and data analysis . LMS performleed the experiments, and data analysis . All authors read and approved the final version.

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