Effects of Alcoholic Extracts of *Cinnamomum zeylanicum* and *Origanum Majorana* on Expression of *Hly* Gene in *Escherichia coli*

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Abstract

Escherichia coli isolates isolated from urinary tract infection identified by biochemical test and confirmed by vitek 2 compact system. Minimum inhibitory concentrations of *Cinnamomum zeylanicum* and *Origanum majorana* alcoholic extracts determined by broth macrodilutionassay ,it range (12.5-25)mg/ml for *C. zeylanicum* and 100mg/ml for *O.majorana* leaves. The expression of *hly* gene studied in presence of 16srRNA as reference gene, four isolates of *E.coli* (A1 ,A2,A5 and A6)used to detect the expression of *hly* gene by using Quantitative reverse transcription-PCR(1-StepqRT-PCR) before and after tereatment with plants extracts calibrated with 16srRNA. There was inhibition in *hly* gene expression with *O.majorana*,the fold changes were 1.23 and 2 in isolates A5 and A6respectively while there were induction in *hly* gene expression with *C. zeylanicum*,the fold changes were 137 and 73.5 for isolates A1 and A2 respectively.

Keywords: *Escherichia coli, hemolysin, urinary tract infection, gene expression*

Introduction

Urinary tract infections(UTIs) , the most common bacterial infections affecting high percent of people per year worldwide and more common in women than men.[1] E.coli is the most frequent bacteria followed by Klebsiella and Proteus species . to cause UTI. and other predominant species include Enterococcus, Klebsiella, group B Streptococcus, group B Staphylococcus Citrobacter, Acinetobacter and Pseudomonas species .[2] High resistance to antimicrobial agents and the recent emergence of the resistant made UTI control high costly and difficult .[3] Uropathogenic E. coli (UPEC) is the major cause of UTI. [4] A study on UPEC showed 100% resistance percentage of bacteria against antibiotics like ceftazidime ciprofloxacin, kanamycin and others .[5] Virulence factors of recognized importance in the pathogenesis of UTI contain adhesions,, hemolysin, capsule, and a cytotoxic pore forming toxin .[6]

Origanum vulgare contain essential oil with high percentages of phenolic compounds which gave the antimicrobial properties .[7]

Origanum has anti inflammatory effects, anti microbial action, decreasing cardiovascular disease, boosting cognitive function and reducing danger of colon cancer .[8] *Cinnamomum zeylanicum* essential oil has antibacterial activity against bacteria and its main components against *Paenibacillus larvae* . [9]

Materials and Method

Bacterial isolates:

Eight E. coli isolatesfrom patients suffering from UTIs were identified by biochemical test .[10] and confirmed by Vitek2 compact system.

plants extracts:

Alcoholic bark extract C. *zeylanicum and O. Majorana* alcoholic leaves extract were prepared .[11]

Determination of Minimum Inhibitory Concentrations (MIC) of plants extracts:

Broth macro dilution assay used to determined the MIC of Alcoholic extracts against *E. coli* according to .[12]

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HLY* expression in *E.coli* isolates in presence of alcoholic extracts of *O. majorana and C. zeylanicum

hly expression was studied in *E.coli* in presence of sub MIC concentrations of *C. zeylanicum* and for *O. majorana*

RNA Extraction from bacteria:

The RNA was extracted from the bacteria by using ZR Fungal/Bacterial RNA MiniPrep™ kit, according to the kit protocol as manufactured company

Extermination of RNA Concentration:

1. 200 µl from Tris EDTA (TE) was added to 3,800 from D. water the mix 4000 µl,pull 10 µl ignore it and add 10 µl from dye (RNA Dye).

2. 200 µl of the mix for each sample was Pulled .

3. The series of the following tubes are prepared as follows:

4. The mixture was shacked by vortex for second to mix and then leave on a rank at room temperature.

5. The value was extracted from the device immediately.

Quantitative reverse transcription-PCR

Step 1: Preparation of qPCR master mix

qPCR was prepared , the volume of components was calculated based on the following table 1 and kept on ice during use, and assembled reactions on ice to avoid premature cDNA synthesis .[13] The specific primer of 16srRNA and *hly* genes showed in table 2.

Table (1): Master mix components for 1step-qPCR

Component	20 µL (Final volume)	Final concentration
Sybr green kappa master mix	10 Ml	
Forward primer	0.4 µL	0.2 µM
Reverse primer	0.4 µL	0.2 µM
50 X KAPA RT Mix	0.4 µL	1 X
Nuclease free water	4.2 µL	
RNA sample volume	5 µL	1 pg-100 ng

Table (2) : The specific primer of 16s RNA and *hly* genes

Primer	Sequence	Tm (°C)	GC (%)
Forward (16s RNA)	5'- AGAGTTTGATCCTGGCTCAG- 3'	54.3	50.0
Reverse (16s RNA)	5'- GGTTACCTTGTTACGACTT- 3'	49.4	42.1
Forward (hly)	5'-ACCTTGTCAGGACGGCAGAT - 3'	58.6	55
Reverse (hly)	5'-CCGTGCCATTCTTTTCATCA - 3'	53.5	45

The reaction condition for 16srRNA and hly genes showed in table 3:

Table(3):Reaction condition for qPCR of *16s RNA* and *hly* gene

Step	Temp. (°C)	Time	Cycle	Scaning
Reverse transcription	42 ° CC	10 min	Holdd	
Enzyme activation	95 ° CC	3 min	Holdd	
Denaturation	95.0 ° CC	15 sec	40	
Annealing/Extension	55.0 ° CC	15 sec		

Results and Discussion

Isolation:

Six E. coli isolates from urine samples were identified by biochemical test and confirmed by Vitek2 compact system.

MIC of alcoholic plants extracts:

The MIC of Alcoholic extracts against *E. coli* was determined by broth macro dilution assay.MIC value of bark extract of *C. zeylanicum* was (12.5-25) mg/ml for isolates . MIC value of O. majorana leaves extract was 100 mg/ml.The result of this study demonstrated that the plant extracts had inhibition activity on E. coli isolates and varied in their effect .[14] Evaluate the antibacterial properties of medicinal plants like Ocimum sanctum (Tulsi), Origanum majorana (Ram Tulsi), Cinnamomum zeylanicum (Dalchini), and Xanthoxylum armatum (Timur), for potential antibacterial activity against bacterial strains. The antibacterial activity of ethanol extracts was determined by agar well diffusion method. The plant extracts were more active against Gram-positive bacteria than against Gram-negative bacteria. E.coli were the high resistant bacteria followed by Shigella dysenteriae, Klebsiella pneumoniae and Salmonella typhi. [15] Antibacterial effects of O.majorana essential oil on E.coli may be related to thymol which has phenolic compound distinguish by GC/MS .[16]

RNA Concentration (ng/ µl):

RNA was extracted from 6 isolates of *E. coli* to study the expression of *hly* gene,the results showed that the RNA concentrations for *E.coli* isolates were 71.1 ng/µl,70.3 ng/µl , 6.5 ng/µl , 5 ng/µl, 87.4 ng/µl and 96.5 ng/µl for isolates A1 to A6 respectively.

Effect of *C.zeylanicum* and *O.majorana* alcoholic extracts in expression of *hly* gene in *E.coli*

The expression of gene was detected successfully by using new molecular technique which is Real time PCR (qRT-PCR) with used specific primer (house keeping gene of 16srRNA). The amplification accuracy of gene product was noticed by the value of cycle threshold (Ct) for the triplicate reactions as show figure 1 and 2 .

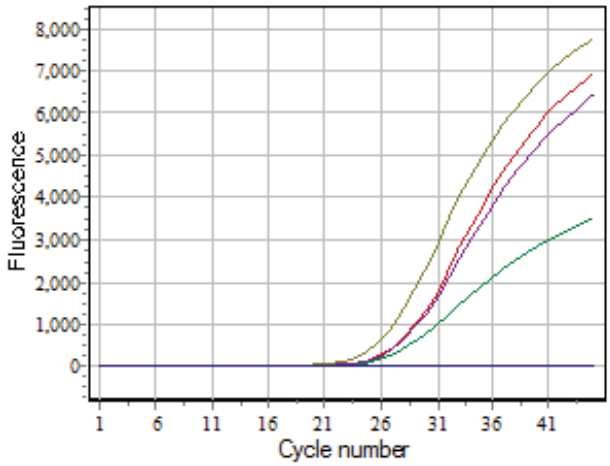
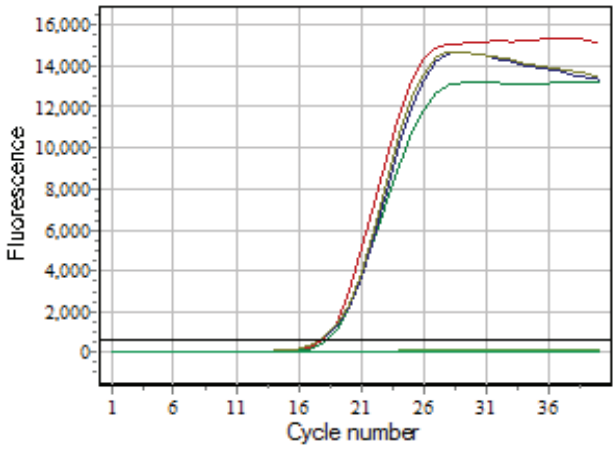


Figure (1):Ct value of *hly* after treated with *O.majorana in E.coli*



Figure(2):Ct value of *16s rRNA* after treated with *O.majorana in E.coli*

Effect of *C.zeylanicum* and *O.majoranum* alcoholic extracts in expression of *hly* gene in *E.coli*

The result showed that there were slightly induction of *hly* expression in *E.coli* isolates in presence of *O.majorana*in sub MIC concentration (50 mg/ml) according to fold change values which were 1.23 and 2 for *E.coli* isolates 5 and 6 respectively , while there were high expression of *hly* gene in presence of *C.zeylanicum* in sub MIC concentration (6mg/ml) , the fold change values were 137 and 73.5 for *E.coli* isolates 1 and 2 respectively (table 4) , this mean that *O.majorana* had an inhibitory effect on *hly* gene in *E.coli* isolates in contrast to *C.zeylanicum* which induced the expression of this gene in isolates.

Table (4):Fold changes in expression of *hly* after treated with *O.majorana and C.zeylanicum in E.coli* isolates , isolates A5 and A6 treated with *O.majorana*, isolates A1 and A2 treated with *C.zeylanicum*

Calibrator		**O.majorana**						
Isolates	**Cthly (mean)**	**Ct16sRNA (mean)**	**ΔCt**	**Ct hly (mean)**	**Ct16sRNA (mean)**	**ΔCt**	**ΔΔCt**	**Fold change**
A5	24.9	17.8	7.1	25.3	18.2	6.8	-0.3	1.23
A6	24.4	18.2	6.2	25.4	18.2	7.2	1	2
A1	24.6	17.8	7.1	0	0	0	-7.1	137
A2	24.4	18.2	6.2	0	0	0	-6.2	73.5

Haemolysin is important virulence factor of *Salmonella*, *E.coli* and other enteric bacteria ,Haemolytic activities of cell extracts of *S.typhi* and *E.coli* grown under stress conditions like oxygen or glucose starvation, either separately or together, were found to be considerably normal growth conditions .[17]

The therapeutic plants, for example, cinnamon, timur, tulsi and origanum are being utilized normally in treatment of irritation and a few diseases. The antimicrobial action has been related to the presence of some important components . Studies refered to the antimicrobial activity of cinnamon was due to their main factors , cinnamaldehyde, which is a natural antioxidant and the animal studies indicate that an extract of cinnamon bark taken orally to diminish stomach ulcer, because Cinnamaldehyde inhibit strains of *Helicobacter pylori.* A significant property of plant extracts and their contituent is their hydrophobicity, which enable them to split membrane lipids and mitochondria of bacterial cell , disturbing the cell composition and increase the permeability. [18]

The antimicrobial activity of many plant extracts can be as a result of different mechanisms, like destruction

of membrane, increase its permeability and polarity, decrease pH of cytoplasm and ATP concentration .[19]

The activity of extracts of *Lippiagr aveolens* and *Haematoxylonbrassiletto*, and carvacrol, brazilin tested by an microdilution method using citral and rifaximin as controls. All products showed bactericidal activity with minimal bactericidal concentrations ranging from 0.08 to 8.1 mg/ml. , These extracts influence *E. coli* growth, motility swarming and expression of virulence gene, sub lethal concentrations had various effects on phenotypic and genotypic character , and expressionn of virulence gene .[20]

Conclusions

The use of natural compounds afford a good way to control the growth of microorganisms. Results obtained in the present study on the antimicrobial effect with *C. zeylanicum* and *O. majorana* denote a down regulation and up regulation of *hly* gene.

Conflict of Interest: The authors declare that they have no conflicts of interest.

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Ethical Clearance: The work was approved by the ethics committee of Biology Department/ College of Science / Baghdad University , Reference No.:BEC/0918/0011

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