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Suppression the activity of *hpmA* gene expression in *Proteus vulgaris* by ethanol leaves extract from *Catharanthus roseus*

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Abstract

Proteus vulgaris is one of the most common bacterial infection in humans is a urinary tract infection. More than 20% of all cases of infections acquired outside hospitals are due to urinary tract infection. The purpose of the current study was to investigate the activity of the ethanol extract of *C. roseus* leaves on *hpmA* gene expression in this bacteria. The urine samples were obtained from patients with urinary tract infection. Different concentrations of plant extract (10, 25, 50, 75, 100 mg/ml) were used to test the antibacterial growth. The bacterial DNA was extracted and the *hpmA* gene was detected and analyzed for expression activity with plant extract using convention PCR. The leaves extract of *C. roseus* have been shown there are significant differences between the groups treated with concentrations (50,75,100,mg/ml) and (control,10,25 mg/ml). It has been showed that the expression level of *hpmA* gene was decreased at concentration(75%,50% mg /ml) and completely stop at concentration 100mg/ ml compared to control. In this study, we have confirmed that *C. roseus* has activity against *Proteus vulgaris*, an important pathogen infecting the urinary tract by investigating its effect on virulence factor expression (hemolysin).

Keywords: Hemolysin, *hpmA,* Gene expression, *Proteus vulgaris*.

INTRODUCTION

Proteus vulgaris is one of the gram-negative bacteria classified in Enterobacteriaceae family, it found as a normal flora but treated as opportunistic pathogens. The important characteristic feature which distinguishes proteus bacteria is swarming phenomenon [1]. This bacterium play important role in urinary tract infection (UTI) and defined as an opportunistic etiological cause in infection of the respiratory tract , wound , skin, burns, eye, ears, nose, and throat, in addition, the gastroenteritis resulting from the ingestion of polluted food or meat [2, 3].

Proteus vulgaris possesses virulent factors included: fimbriae, flagella, enzymes such as (urease, proteases, and amino acid deaminases) and toxins (hemolysins and endotoxin) which work alone or through combined infection [4]. Hemolysin is more critical exotoxins and its activity in *Proteus* spp. is related to *hpmA* gene. Chiefly the prevalent hemolysin *hpmA* is responsible for tissue damage which is activated when its N-terminal peptide is cleaved [5].

The strains of *Proteus vulgaris* are usually resistant to many of antibiotics such as penicillins and many of cephalosporins, and *P.vulgaris* is more resistance than *P. mirabilis* [6]. Therefore using of medicinal plants open the way for a great source of natural drugs, which have the properties of antibacterial, anti-fungal, anti-cancer, anti-inflammatory, anti-diabetic, anti-oxidant, and anti-hypertensive [7].

Plants established natural metabolites, develops more popular through the world nowadays , such as alkaloids, flavonoids, saponins, terpenoids, tannins, and glycosides are derivative from different parts of plants like bark, leaves, flowers, seeds, roots, fruits [8]. *Catharanthus roseus* is one of the medicinal plants classified in Apocynaceae family. It has anti-bacterial, anti-fungal, anti-diabetic, anti-cancer, due to the presence of indole alkaloids and other biologically active compounds like vinblastine and vincristine [9]. The current study was aimed to investigate the effect of ethanol leaves extract for *C. roseus* as a blocking factor on *hpmA* gene expression activity.

MATERIALS AND METHODS

Bacterial samples preparation, isolation, and identification:

Mid-stream urine (MSU) specimens were collected under a septic condition in asterial tubes (5-10 ml) were collected from Children and delivery's Hospital and Al-Hussien Teaching Hospital in the Samawa city, from1/11/2016 to 23/04/2017, and then these samples were transferred by brain heart infusion broth to the laboratory of the microbiology in the College of Science/ Muthanna University at which the samples were processed. Bacterial isolates were identified according to Bergey's manual using different morphologicand and biochemical tests [10]. The samples were inoculated in Brain heart infusion broth, incubated at 37 $^{\circ}$C for 24h. The growing bacteria on the medium were subcultivated on MacConkey plates agar and Blood plates agar and incubated under (aerobic and anaerobic condition) at 37 $^{\circ}$C for 24h.

Preparation of *Catharanthus roseus* ethanol extract:

The extract was prepared according to a method presented by [11]. According to this method, the dry leaves about 10 g was powdered by the electrical blender. Volume of 250 ml (70% ethanol) was used for the extraction of the Soxhlet apparatus. The plant material was loaded into the inner tube of the Soxhlet apparatus and then fitted into around bottomed flask containing 70% ethanol. The solvent was boiled gently 40℃ over a heating mantle using the adjustable rheostat. The extraction was continued for 8 hr. The solvent was dried at 40℃ and stored at 4℃ until the preparation of required concentrations.

Determination the antibacterial activity of *C. roseus* leaves extract:

To determine the antibacterial activity, various concentrations (10,25,50,75,100 mg/ml) of *C. roseus* extract was add (50 µl) in tubes containing 1ml of bacterial culture (brain heart broth). All tubes were incubated at 37 ℃ for 24 hrs. The optical density of grown bacteria was measured by spectrophotometer at a wavelength of 600 nm. All readings were taken in three replicates [12].

RNA extraction from *P. vulgaris* bacteria:

The extraction of bacterial RNA was carried out using the SV Total RNA Isolation System (Promega / USA). The RNA sample converted to cDNA by reverse transcriptase according to manufacturers` instructions of (One Taq® RT-PCR Kit, Bio Labs).

Detection of *hpmA* gene using PCR:

PCR primers F-5 ′ GCGTAGTGGCTATGGGCTAA 3′ and R -5′ GGGGTGGCTTACAAAAGAAT 3′were designed for detection of *hpmA* (2233 bp in size) using the Primer3 software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

PCR reaction mixtures prepared in a 0.2 ml Eppendorf tube with 25µl full reaction volumes, which contained the following: 12.5µl One Taq Hot Start 2X Master Mix , 0.5µl of Forward primer, 0.5µl of Reverse primer, 5µl of Diluted cDNA, 6.5µl of Nuclease-free H_2O. The reaction mixture was prepared by mixing the components in sterile Eppendorf of tubes (0.5 ml), These components was mixed gently in a PCR tube on ice. For product detection, 5 µl of the PCR mixture was subjected to electrophoresis in a 1.5 % agarose gel.

The effect of *C.roseus* leaves extract on *hpmA* gene expression:

A volume of 50µl from *C. roseus* extract at different concentrations (100%, 75%, 50%, 25%, and 10% mg /ml) were

add separately to 1ml tubes containing bacterial culture. The cultures were incubated for 24h at 37°C then the mRNA was extracted. The activity of *hpmA* gene expression was detected using conventional PCR. The results of gene expression was confirmed by DNA migration electrophoresis on agarose gel in a concentration 1.5 % at 70 volts for 45 minutes.

Statistical analysis. A statistical test was used to find the significant differences among bacterial culture treated with *C. roseus* extract concentrations and absorbance reading. All the results present the average of three independent experiments of absorbance reading. The data were presented as mean and analyzed by one-way analysis of variance (ANOVA) with a less significant difference (L.S.D.) at P <0.05.

RESULTS AND DISCUSSION

Determination the antibacterial activity of *C. roseus*

The effect of *C. roseus* leaves extract on the growth of *P. vulgaris* was detected by observing the turbidity and the absorbance reading of the suspension post-incubation using spectrophotometer (O.D 600). It was observed that concentrations (0, 10, 25, 50, 75,100 mg/ml) of the ethanolic extract of *C. roseus* leaves extract inhibit the growth of bacteria (1.6, 1.5, 1.2, 0.8, 0.5, 0.3 O.D at 600nm, respectively) at significant level $P<0.05$ fig (1). The leaf extract contained many indole alkaloids, and some phenolic compounds, the phenolic compounds are known for their antimicrobial properties [13]. This result supported with previous studies showed the antibacterial effects of plants extracts against *Staphylococcus aureus* [14, 15].

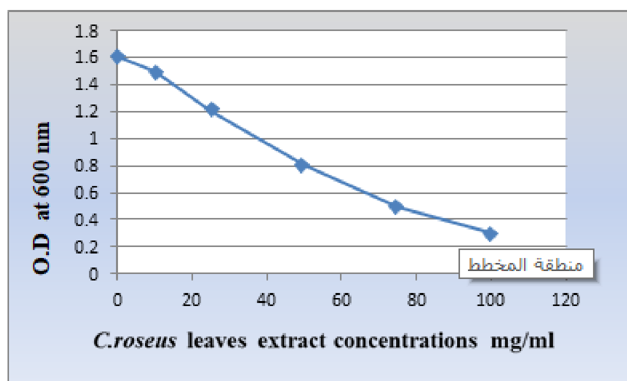

Figure (1): standard curve of the effect of *C.roseus* extract on the growth of *P.vulgaris* at OD600 nm in presence of different concentrations of *C.roseus* extract (0, 10, 25, 50, 75,100µg/ml). The data represent the average of three independent experiments. There is significant difference between concentrations (control,10,25) and (50,75,100) in P-value <0.05.

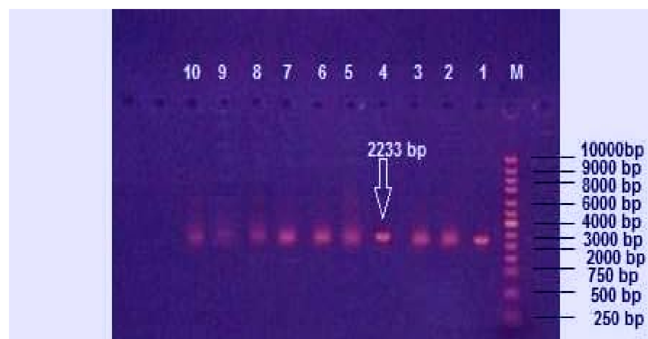

Figure (2): Ethidium bromide-stained agarose gel of PCR amplified products from extracted *P.vulgaris* DNA amplified with primers *hpmA* F and *hpmA* R.Lane (M) DNA molecular size marker(10000-bp ladder), Lane(1-10) show positive results with *hpmA* gene.

Figure (3): Ethidium bromide-stained agarose gel of expression of *hpmA* gene in *P. vulgaris* by RT-PCR after exposed to *C. roseus* extract at different concentrations (0,10,25,50,75,100).

Detection of *hpmA* gene from *Proteus vulgaris*

The extracted RNA from bacteria was converted to cDNA for the purpose of gene detection and conformation. The results showed hpmA gene bands (2233 bp in size) fig (2). The *hpmA* gene is an important gene in detection *proteus ssp* bacteria. There is evidence that hemolysins lead to increased virulence in Proteus infections [16]. It was reported that the *hpmA* gene acts as a potent cytolysin against HRPTEC kidney cells (human renal proximal tubular epithelial cells). Meanwhile, it was referred to the *hpmA* gene responsible for degradation the erythrocyte membrane and lead to damage of tissue [17], and out that, the hemolysin although it is not considered essential during the initial infection stages, it is important in the final stages when the bacteria are already colonizing the kidneys [18] .

The effect of *C.roseus* leaves extract on *hpmA* gene expression

Our results exhibit the significant effect of *C. roseus* leaves extracts on *hpmA* gene expression. It has been showed that the expression of *hpmA* gene was decreased at concentration(75%, 50% mg /ml), whereas the expression was stop and the gene band was absent at the concentration 100% mg/ml compared to control fig (3).

The *C. roseus* contains alkaloids such as vinblastine, vincristine, and flavanoids active compounds in the plant that has the ability to penetrate the bacterial cell and interference with DNA[19]. Previous study referred that vincristine binds to DNA molecule through intercalation between DNA bases and H-bond with sugar-phosphate backbone and also bases [20], while others reported that alkaloids can be complex with extracellular and soluble proteins and to complex with bacterial cell walls [21] .

The non-expression of the *hpmA* gene which responsible for the production of hemolysin enzyme may result in the inhibition of nucleic acid synthesis leading to protein synthesis blocking and inactive enzymes secretion [22]. Other reason for the lack or absence of gene expression of the *hpmA* gene in the high concentrations of plant extract may be due to the effect of the effective compounds of the extract on the inhibition of the *hpmB* gene which responsible for activation *hpmA* gene in *Proteus vulgaris* [23]. On the other hand, the *hpmB* is necessary for the extracellular secretion and hemolytic activity of the structural hemolysin *hpmA* and the cleavage of the N-terminal peptide of the *hpmA* need to *hpmB* to activate and transport the hemolytic *hpmA* protein to outside the cell detecting and characterizing *P. vulgaris* hemolysin *hpmA* is necessary to elucidate its importance as a virulence factor [24].

CONCLUSIONS

It was investigated that the ethanolic leaves extract of *Catharanthus roseus* showed potential antibacterial activity against human pathogens (*Proteus vulgaris*). Also it was that the plant extract was able to block the gene expression activity of *hmpA* in *P. vulgaris* at higher extract concentrations. Depth studies of this plant extract will be helpful for elucidation the molecular mechanism of blocking the action of *hpmA* gene.

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