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Effect of *Piper cubeba* fruits extract on Hemolysin production of *E. coli* isolated from urinary tract infection

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Abstract: Thirty five *E .coli* isolates were collected from patients suffering from long term urinary tract catheters and urinary tract infections for both genera with age ranging between (11-60) years. The isolates were identified according to cultural, microbiological, biochemical testes. Effect of *Piper cubeba fruits* on Hemolysin production was tested and the result showed that *P. cubeba* fruits extract have effect on hemolysin production, It was found 10 isolates from 24 producer isolates lost their ability to produce hemolysin after incubated bacteria with Minimum inhabiting concentration(MIC) of Piper cubeba fruits extract phenotypically. While screening Hly gen for 20 isolates showed 18 isolates have Hly gen and 7 of them lost Hly gen after treating with the extract. Hemolysin protein concentration was measured by Bradford method, isolates grown without the extract were have protein concentration higher than isolates that grown with the extract(3.5mg/ml and 1mglml) respectively. Statistical analysis of these result show significant differences between them and the "P value" was 0.003.
Key words : *Escherichia coli* , Hemolysin ,*Piper cubeba.*

Introduction

Virulent bacteria are able to produce molecules that dynamically inhibit the immune response of the host, so enhancing bacterial persistence and tissue damage. The genes encoding virulence factors of UPEC are localized on chromosomal gene clusters “pathogenicity islands”[1,2].Bacterial virulence factors play a vital role in determining whether an organism will invade the urinary tract and the level of infection acquired. Uropathogenic *E.coli* (UPEC) and pathogenic strains of this microorganism are infect the urinary tract by expressing specific virulence factors that allow adherence and colonization of the lower urinary tract[3,4]. *E. coli* hemolysin is a main virulence factor for some pathogenic strains of the bacterium involved in human extraintestinal diseases like urinary tract infections, meningitis, peritonitis and septicemia[5]. Hemolysin are pro-forming proteins, they caused destruction of red blood cells, with subsequent release of hemoglobin that can occur by any one of the numerous substances such as a bacterial hemolysin that appears to aid the invasive power of bacteria[6]. *E. coli* produce many types of hemolysin, that most common type of which alpha-hemolysin. It non-specifically adheres to the cell membrane[7].α-haemolysin (HlyA) is a lipoprotein and most important secreted virulence factor of uropathogenic *E. coli* which associated with upper UTIs such as pyelonephritis[8]. Hemolysin has a number of effects on the host, largely due to the formation of unregulated pores for ion transmission across the membranes of a variety of cell types[9,10]. HlyA requires post translational acylation of lysines at residues 564 and 690 for activation and oligomerizes to form pores in eukaryotic cell

membranes in a calcium-dependent manner[11] and causes calcium oscillations in epithelial cells via a mechanism that requires pore formation`[12]. HlyA and CNF1 are frequently co- expressed in clinical uroepathogenic E .coli cell isolates, and strains that express both toxins are more commonly isolated from patients with hemorrhagic UTIs[13,14]

Recently, attention to medicinal plant studies related to virulence factors inhibition as a target activity is increasing , several bioassays to assess virulence factors have develop for several microorganisms, mainly bacteria and yeast. This is an important source of compounds to investigate new anti-virulence factors mechanisms of microbes. Many medicinal plant metabolites have antimicrobial activity[15,16]. Practitioners of traditional medicine think that the components of plants are unique as they contain both active ingredients and "inactive" components that are play a role in enhancing the wellbeing of their patients[17]. Many virulence factors can be neutralized via plant compounds. A broad field of studies on this subject is further on,science advances in phytochemistry and molecular microbiology providing new features that will end in virulence factors based new therapystrategies[18]. *Piper cubeba*(cubeba) or tailed pepper is a plant in genus *Piper* . *Piper cubeba* usually known as cubeb, tailed pepper (because of the stalks attached), Java pepper (in Java) and kemukus (in Indonesia)[19]. Cubeba is a perpetual plant, with climbing stem, round branches , ash colored and rooting at the joints and it leaves are from 4 to 6 and a half inches long by one and a half to two inches broad, ovate-oblong, acuminate and very soft Flowers arranged in spikes at the end of the branches . *cubeba*is one of the popular medicinal plants[20]. It is used to treat genitourinary disease Kidney and Bladder calculi[21] gonorrhea dysentery, syphilis, abdominal pain and asthma[22] also it use as gastroprotactive[23]. The effect of *Piper cubeba* extract on Hemolysin production in uropathogenic isolates have not been compared before so in the present study, we have evaluated the effect of *P. cubeba*extracts on Hemolysin of *Escherichia coli* that isolated from urinary tract infection patients .

Material and methodsCollection and drying of *Piper cubeba fruits* :

Piper .cubeba fruits were collected from the local market of Babylon province (Iraq) then it was washed thoroughly three times with sterile distilled water. The materials were air dried under hot air oven at 55°C for 3 h and powdered. The powdered samples were hermetically conserved in separate clean container until the time of the extraction.

Extract preparation

Hot water were used to prepare the extract of *P. cubeba*. An amount of 30g. of pulverized fruit in 100ml of hot water (100 c◦) and adjust to magnetic stirrer for 5h. Preparation was filtered through a sterilized whatman No.1 filter paper[24-26] Filtered extracts were air dried at 40˚C for 48 h, then stored in labeled sterile bottles in a deep freeze at -18˚C until further use[27].

Phytochemical analysis of *P .cubeba* Fruits extract: Hot aqueous extract were tested chemically to identify its chemical compounds according to[28]

Bacterial strain

In this study 35 *E.coli* isolates were collected from patient suffering from long term urinary tract catheters and urinary tract infection for both genera with age ranging between (11-60) years in Babylon Province, Iraq during a period from September 2015 to February 2016.isolates were identified according to morphology, microscopic examination and biochemical tests.Bacterial cultures were maintained on nutrient broth as a basal medium, supplemented with 15% glycerol, and kept at 4˚C until used[29].

Escherichia .coli was grown with (25 mg / ml) MIC concentration of *P . cubeba* hot aqueous extract at 37 c for 24h in tests of detection hemolysin production and protein concentration (Antibacterial activity and MIC concentration of hot aqueous extract of *P. cubeba* was published in other research).

Hemolysin production:

Plate hemolysis test will be done by using 5% blood agar to detect hemolysin production. The bacteria will be inoculated on to blood agar and incubated for 24 h at 37^0C. Hemolysin production will be detected by the presence of a zone of complete lysis of erythrocytes around the colony and clearing of the medium[30]

Extraction of hemolysin produced by *E.coli*.

It was done according to [31] with some modified. Bacterial cultures were grown in 50 mL of brain hart infusion broth at 37°C overnight. Culture supernatants were harvested by centrifugation for 15 min at 9000 rpm and then filtered through a 0.45-µm filter.

Precipitation of Hemolysin

Hemolysin precipitate from crude extraction by ammonium sulphate with saturated ratio 75%. this method was done according to[31].

Measurement of Hemolysin concentration:

Bradford method[32] was used to measure Hemolysin concentration.

Bacterial genomic DNA extraction:

Favor PerpGeno DNA Mini Kit was used to extract genomic DNA from *E coli* isolates following the manufacture's protocol. inoculum of *E coli* were prepared at density up to 10. Bacterial pellets were harvested via centrifugation at 400rpm for 1 min After that, the supernatant was discarded and the bacterial cells pellets were used in genomic DNA extraction and the extraction was done according to company instruction. The eluted genomic DNA was stored at -20 until use

Polymerase chain reaction (PCR):

Assay was performed by using specific primer for detection hemolysin toxin gene (hlyA).The primer pairs used in this study were listed in table (1)

Table 1 : Primer Pairs Sequences and Amplicon Size of hemolysin toxin gene (hylA) of *E.coli*

Group	Virulence factor	Primers	5'-sequence-3'		Amplicon size (bp)	Reference
Pore-forming	Hemolysin	*hlyA*	F	'5-AACAAGGATAAGCACTGTTCTGGCT-3'	1,177	33
			R	'5-ACCATATAAGCGGTCATTCCCGTCA-3'		

Result

In this study 35 *E. coli* isolates were collected from patient suffering from long term urinary tract catheters and urinary tract infection whose did not take any drug and for both genera with age ranging between (11-60) years during a period from September 2015 to February 2016 All urine samples were plated on nutrient MacConkey and Blood agar plates and incubated at 37 for18- 24 hours. Identification of pure isolate was done by observing morphological, cultural and biochemical characters according to[34].

In the present study *E. coli* was common among people aged (21- 30) years and the lowest percentage were among people aged (51-60) as show in table (2). This result was similar to the result of [35] it was found that the lowest percentage of *E. coli* was high within the age group 26-36 years . In other study a high percentage of *E. coli* in people with ages (11-21) years[46].

Table 2: Percentage of *E.coli* isolates according to the age groups.

Categories of patient	N O. *E coli* isolates	Percentage
11- 2 0 years	8	30. 7%
2 1-3 0 years	10	45.4%
31 – 4 0 years	9	29%
41 – 5 0years	5	25%
51-60yeares	3	14.2
Total	35	29.1%

Also the results showed a high incidence of UTI in females than males. This difference in occurrence could be due to several clinical factors, including anatomic differences, hormonal effects, and behavioral patterns[37,38].

phytochemical screening of hot aqueous P. *cubeba* fruits showed it was containingglycosides, phenols, Flavonoids and tannin.

To detect effect of hot aqueous extract of *P. cubeba* on hemolysin production *E. coli* isolates incubated with the MIC concentration (25mg /ml) of hot aqueous extract at 37 for 24h **.**

Hemolysin production:

Hemolysin are pro-forming, cytolytic toxin proteins secreted by some *E. coli* isolates, which is as well referred to as alpha-hemolysin[5,6,39].In this study 24 from 35 *E .coli* isolates were able to produce hemolysin on blood agar, while 11 isolates did not producer . This results agreed with the result of[40] It was found that (68.45 %) of isolates were hemolytic. In other study demonstrated that among 220 isolates of *E.coli* from urinary tract infections, 41.36% hemolytic[42],and disagree with[43] they found that among 200 urinary isolates of *E.coli*, 21% hemolysin positive . whereas[43] found that (90%)of *E.coli* isolates produced hemolysin .

To assayed the effect of *P. cubeba* fruits extract on production ofhemolysis .Bacterial cells were incubated with plant extracts for 24 h at 37 °C. After incubation it was grown on blood agar media .The result showed that *P. cubeba fruits* extract possessed effect on hemolysin produced by *E coli* and 10 isolates from 24 isolates lost their ability to produce hemolysin after incubated bacteria with plant extract to become 14 isolates only produced hemolysin. According to study of[44], Among the 87, Chinese herb-susceptible uropathogenic*E. coli* strains, 6 (6.9%) caused clearing of blood agar around areas of bacterial growth.

Piper cubeba fruits extract inhibited the release of the toxin to the bacterial medium so bacteria that lost their ability to hemolysis RBC on blood agar that may be due to the presence of phytochemical constituents especially polyphenolic compound, tannins and flavonoid. There is evidence that a type of coordination between defense mechanisms of host and plant metabolites that can inhibit different virulence factors expressions may aid the host to overcome an infection. This may explain the bacteriostatic, bactericidal activity of medicinal plant extracts that traditionally used in managing urinary complaints[45,46].

Extraction precipitation and measurement protein concentration of hemolysin

Cooling centrifugation method was used to extraction hemolysin produced by *E coli* according to[31]. Ammonium sulphat with saturation ratio (75%) used to precipitate crude hemolysin that had been obtained . Hemolysin protein was measured by Bradford method[32]. In comparison between isolates that grown with and without *P. cubeba* extract in hemolysin concentration, the result show that the isolates grown without the extract have protein concentration higher than isolates that grown with the extract (3.5mg /ml, 1. 3mg/ml) respectively .Statistical analysis of these result show significant differences and P value was 0.003.

Genotypic detection of Hemolysin production

The PCR amplification for genomic DNA used to detection presence of Hemolysin in *E. coli* alone and that grown with the MIC concentration of *P. cubeba* fruits extract. Screening (Hly) gen for 20 *E . coli* isolates was show 18 isolates of *E. coli* without the extractwere able to produce hemolysin. while bacteria were grown with the extract lost hemolysin synthesis and the gen coding to hemolysin, observed that 7 from 18 isolates exposed to the extract lost the gen of hemolysin as show in figure (1). The production of active extracellular α- hemolysin needs the products of the four linked genes hlyC, hlyA, hlyB, and hlyD. α-Hemolysin is formed as an inactive polypeptide and converted in its active form by the addition of a fatty acid group catalyzed by the hlyC protein. The production of α-hemolysin is signal peptide independent and mediated by a specific membrane translocator system encoded by hlyB and hlyD[5, 47,48] reported that eugenol (active component in clove oil) reduced the production of alpha hemolysin of *S. aureus*. The same effects may be happened in presence of *P. cubeba* fruits extract due to the variety of active compounds in it . or the extract of *P. cubeba fruits* interfered with production of one or more of the four genes hlyC, hlyA, hlyB, and hlyD or may interfered with the membrane translocator system encoded by hlyB and hlyD .

The aforementioned result suggests that *P. cubeba fruit extract* can be used to control toxin production in microorganisms, and may be toxin mediated pathology in humans and other toxin .

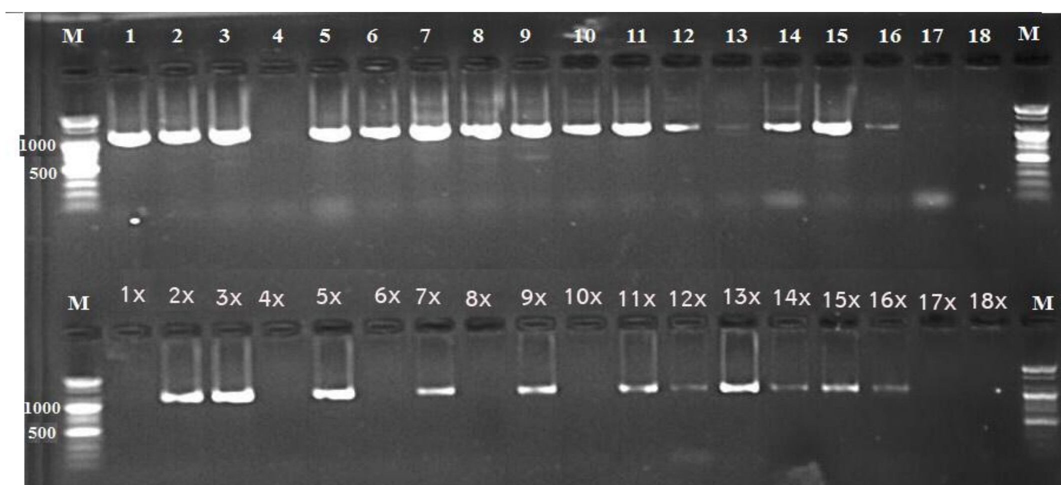

Figure (1) Gel electrophoresis of the amplified products of *Ecoli*(*Hyl*) gene on (2%) agarose. M : Marker 1000 bp , (1-18) represents number of bacteria grown without P. cubeba , (1x-18x)represent gen of bacteria grown with *P. cubeba* extract .

Conclusion

Hot aqueous extract of *Piper cubeba* fruits have activity on Hemolysin production in *E. coli*. *Piper cubeba* may serve as supplementary agents that might enhance standard conventional antibacterial therapy in UTIs. However, Action of *Piper cubeba* on bacterial cells is still not fully known and requires further investigation.

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