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Extraction and Purification of Siderophores Produced by Uropathogenic *E. coli* Local Iraqi Virulent Strains

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Abstract

Background Siderophores are crucial for the proliferation of many disease-causing bacteria and also have significant functions in the transportation of non-iron metals, defense against oxidative stress, antibiotic effectiveness, interactions between different species, and the ability to cause disease.

Objective For extraction and purification of siderophores from uropathogenic *Escherichia coli (E. coli)* standard strain.

Methods Isolation and identification of *E. coli* was carried out only for urinary tract infection (UTI). Enhancement of the production of siderophores was done by investigating the elements that impact their production. Siderophores were detected by using Chrome azurol S (CAS) assay quantitatively in M9 minimal medium salts 5x. Extraction of siderophores using the Jadhav and Desai (1992) technique. Purification of siderophores by Sephadex LH-20 column and high-performance liquid chromatography (HPLC).

Results A quantitative calculation revealed that 44.6% of siderophores were extracted and purified from uropathogenic *E. coli*.

Conclusion Uropathogenic *E. coli* rely heavily on iron uptake or siderophores in the development of UTI in individuals. CAS assay is the most reliable technique for detecting siderophores.

Keywords Siderophore, Sephadex LH-20, HPLC, UPEC, UTI

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List of abbreviations: CAS = Chrome Azurol S, *E. coli* = *Escherichia coli*, HPLC = High performance liquid chromatography, UPEC = Uropathogenic *Escherichia coli*, UTIs = Urinary tract infections

Introduction

Early Urinary tract infections (UTIs) are the most commonly observed diseases in clinical practice worldwide [(1)]. Due to the anatomical configuration of the female lower urinary system and its proximity to the reproductive organs, women have a higher vulnerability to UTIs compared to males. Due to the shorter length of the female urethra compared to the male, germs have limited ability to spread [(2)]. The main risk factors for the occurrence of UTIs encompass elderly, female, experiencing prolonged hospital stays, having debilitating underlying diseases, and undergoing numerous urine catheterizations [(3)]. UTIs are primarily caused by Gram-negative bacteria from the Enterobacteriaceae family. Among these, uropathogenic *Escherichia coli* (UPEC) is responsible for 80% of UTIs cases worldwide [(4)].

Escherichia coli (*E. coli*) is a Gram-negative bacterium, it is capable of surviving with or without oxygen, and it does not produce spore.

Additionally, it has the ability to produce an enzyme called catalase, but it does not produce an enzyme called oxidase. It is classified under the Enterobacteriaceae family. The test results indicate a negative reaction for Vogues-Proskauer, urease, H_2S production, phenylalanine deaminase, and gelatin liquefaction. However, it shows a positive reaction for indole and methyl red. In addition, it generates acid and gas when metabolizing glucose, mannitol, maltose, lactose, and sucrose. It is categorized as a gamma-proteobacterium belonging to the Enterobacteriaceae family [5]. For growth, DNA synthesis, electron transport, and metablossoming, bacteria require iron. However, because iron in the human body primarily exists as transferrin, bacteria secrete substances known as "siderophores," which chelate iron and subsequently bind to a specific surface receptor on the bacteria. The colonization process in UTIs caused by UPEC requires the use of iron obtained via this approach.

In medicine, siderophores are employed to treat illnesses including cancer and malaria by delivering antibiotics to resistant bacteria (the Trojan horse method) [6]. Siderophores are quite varied structurally and have a molecular weight of 200–2000 Da [7].

Accordingly, the aim of this study was extraction of siderophores and purification by high performance liquid chromatography (HPLC) of UPEC isolated from UTIs Iraqi patients.

Methods

Bacterial strains

Fourteen bacterial standard strains that were previously isolated and identified by Vitek 2 system as UPEC from UTI Iraqi patients. Its biological ability to produce siderophore was revealed and after making molecular detection of siderophores' genes using conventional polymerase chain reaction (PCR). The strain containing the three siderophores' genes was chosen. Bacteria were cultured in M9 minimal medium salts 5x (Hi Media, India) with trace elements.

Optimization of siderophores production

The production of siderophores was maximized by researching the factors that affect it. Depending on the bacteria, culture media, cultural circumstances, iron concentrations, carbon-nitrogen sources, and presence of metals, siderophores can vary greatly in concentration within the medium [8].

Quantitative method

The bacterial culture was standardized to a 0.5 McFarland turbidity, equivalent to a concentration of 1x10^8 colony forming unit (CFU)/ml. The siderophores' quantitative estimation was conducted by collecting the supernatant from bacterial cultures that were cultivated in M9 broth [9]. Half ml of the liquid portion of each bacterial culture was combined with 0.5 ml of a chemical reagent called Chrome Azurol S (CAS). After a duration of 20 min, the measurement of the amount of light absorbed by the mixture was taken at a wavelength of 630 nm. The concentration of siderophores in the culture supernatant was determined and expressed as a percentage of siderophores unit (psu), which was calculated according to the following formula:

psu = (Ar-As) *100/Ar

= (0.334-0.185) *100/0.334

= 44.6%

Absorbance of reference (Ar) = CAS solution +uninoculated broth

Absorbance of sample (As) = CAS solution + cell-free supernatant of sample.

Extraction of siderophores

The bacterial suspension is subjected to centrifugation at a speed of 8000 rpm for a duration of 20 min. The liquid portion is made acidic to a pH of 2. Then, an equal amount of ethyl acetate was added to extract the siderophore. The mixture is vigorously shaken in a water bath at a temperature of 50°C to

remove the ethyl acetate layer. Finally, the resulting extract is spread out on open petri dishes and dried in an oven set at 50°C.

Determination of the mass of the crude extract without any moisture content

In order to ascertain the chemical composition of siderophore molecules, a bacterial supernatant is utilized for the experiment; this involved adding 1 ml of a 2% aqueous solution of FeCl3 to 1 ml of the sample. The outcome is affirmative, as determined by the absorption of wine color at 490 nm in the ultra violet (UV) spectrophotometer [10].

Purification of siderophores

1) Sephadex LH-20 column

1. The gel was prepared according to the instructions of Pharmacia Fine Chemicals, where it was suspended in methanol.
2. The air was degassed using a vacuum pump.
3. The gel was filled into a glass column to give dimensions of (2 x 20) cm.
4. Equilibration was done using methanol: deionized water (3:1) volume ratio.

The method of work:

1. The sample on the surface of a Sephadex LH-20 gel, which had previously been equilibrated on the column with methanol: deionized water at a flow speed of (30) ml/hr.
2. The recovery buffer was passed over the column and the fractions were collected at a rate of 5 ml for each recovered fraction.
3. The absorbance of each recovered fraction was measured using CAS reagent. Fractions pooled, analyzed and lyophilized by reverse phase HPLC [11].

Detection of siderophores

The universal CAS assay was used to determine whether or not the bacterial strains were capable of generating siderophores. Before beginning the experiment, all glassware was cleaned in deionized water after being rinsed with 3 mol/l hydrochloric acid (HCl) to eliminate iron [12].

2) HPLC

The quantification of individual phenolic compounds was conducted using reverse phase HPLC analysis. This analysis employed a Sykamn HPLC chromatographic system equipped with a UV detector, Chemstation software, and a Zorbax Eclipse Plus-C18-OSD 25 cm, 4.6 mm column. The temperature of the column was 30°C. The gradient elution procedure was conducted using eluent A (methanol) and eluent B (1% formic acid in water, v/v). The elution was performed in the following manner: from 0 to 4 min, 40% B; from 4 to 10 min, 50% B. The flow rate used was 0.7 ml/min. The samples were injected with a volume of 100 µl, and the standard was also injected with a volume of 100 µL. This process was carried out automatically using an autosampler. The measurements were taken at a wavelength of 280 nm [13].

Results

Quantitative method

The concentration of siderophores in the supernatant was quantified as psu and observed that *E. coli* strain produced 44.6% of siderophores in quantitative estimation.

Purification of siderophores

1) Sephadex LH-20 gel

Partial purification of the siderophores was achieved when the fractions tested positive for sideophores content were pooled, concentrated, and further purified by the Sephadex LH20 column. Altogether, the initial fractions were colorless. Fractions (11-21) were pale yellow the color intensity increased gradually that mean these fractions contained Siderophores as in the figure (1).

Detection of siderophores by CAS

After collecting the fractions, CAS reagent is added, and a spectrophotometer was used to determine the optical density at 630 nm (OD 630). The color changing from blue to wine was observed, which indicates the presence of siderophores (Figure 2).

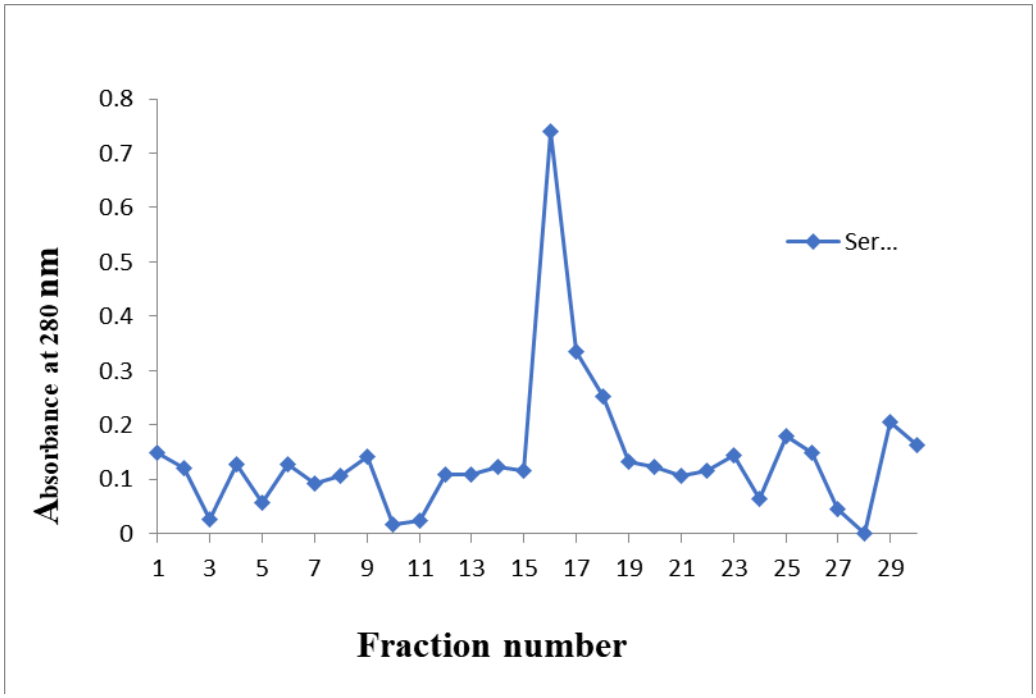


Figure 1. Analysis of siderophores purification in *E. coli* at a wavelength of 630 nm

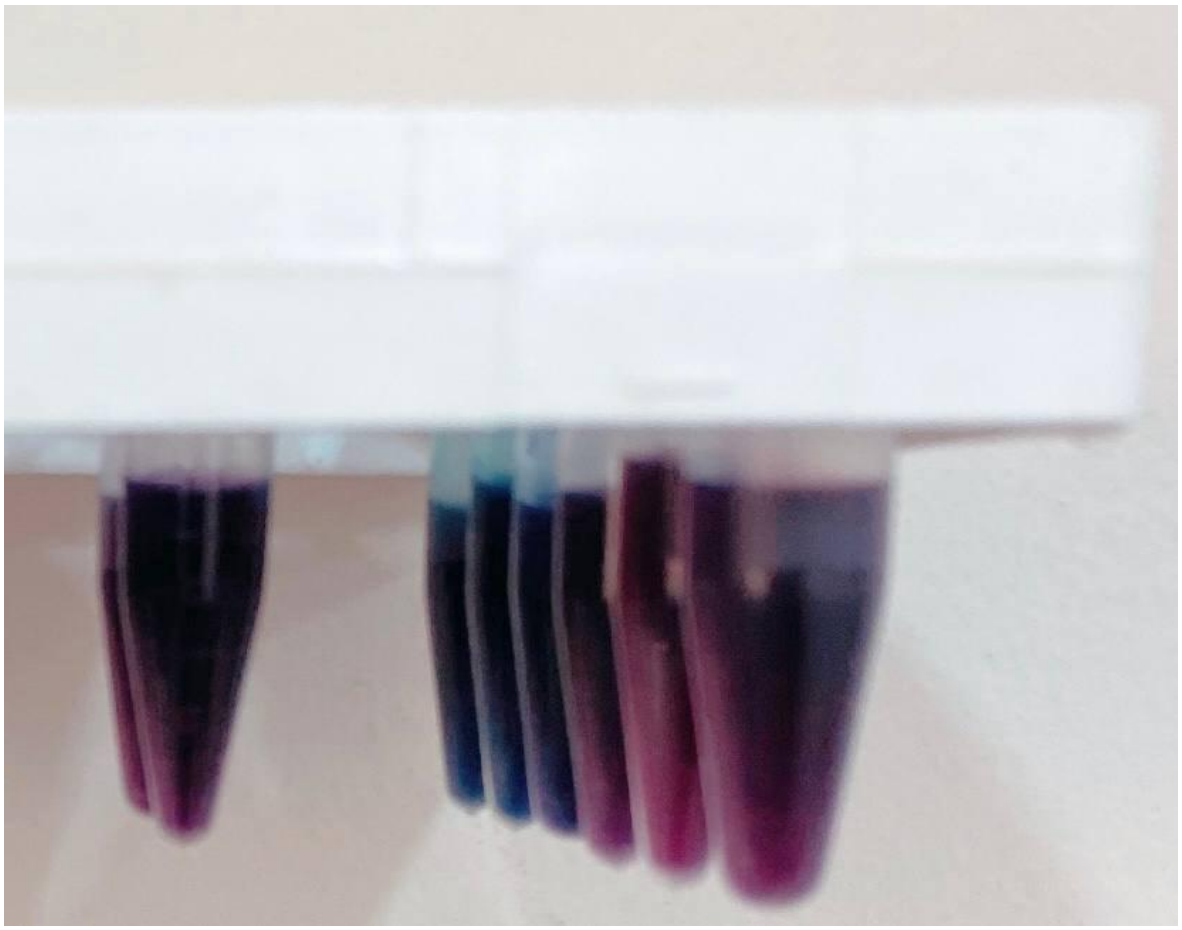
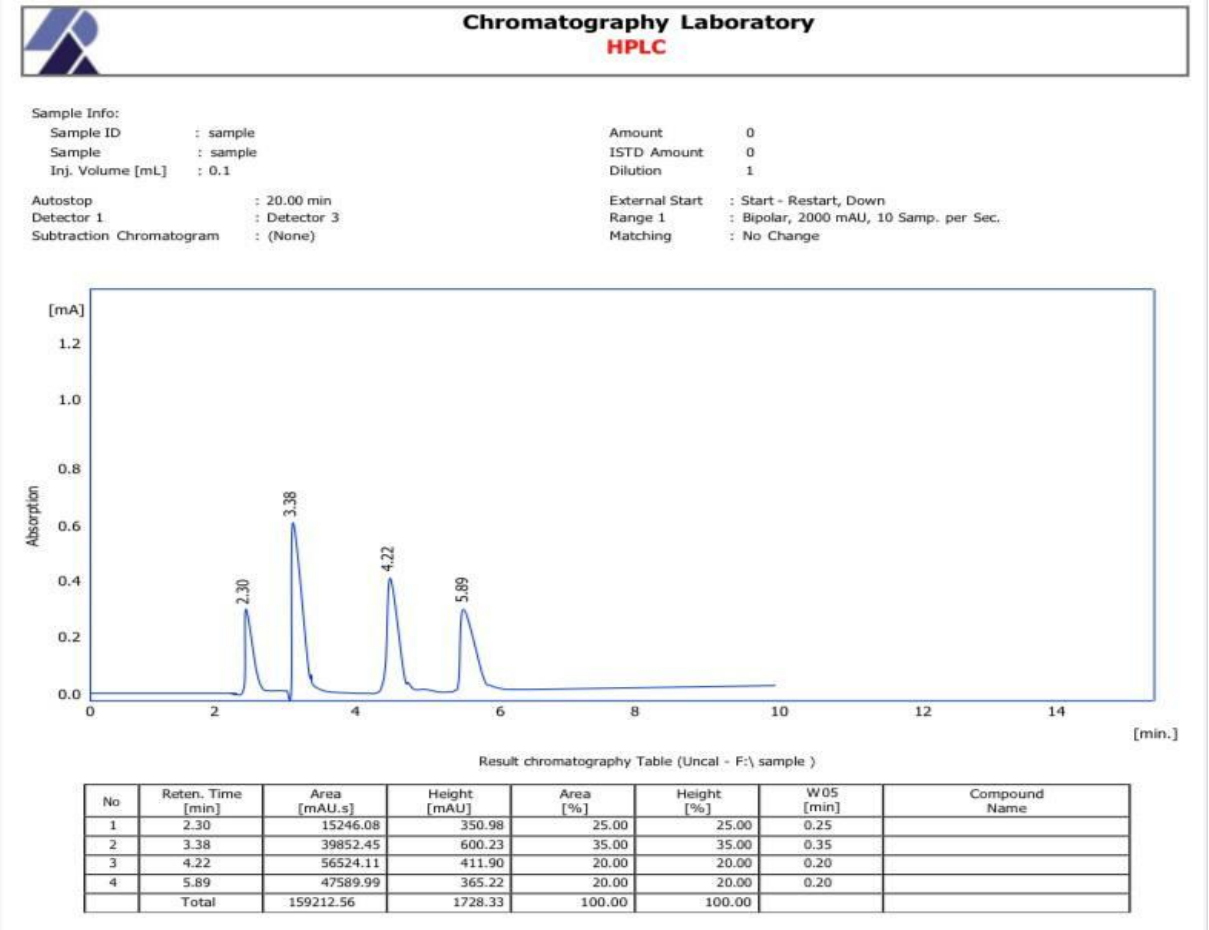


Figure 2. CAS reagent-based detection of siderophores

2) HPLC

The siderophores extracted and purified from the *E. coli* culture were analyzed using HPLC. The retention durations observed were comparable to those of the standard. A prominent peak was detected at a wavelength of 280 mm over a retention duration of 2.30 min, close to the peak observed in standard having a retention time of 3.38, 4.22, and 5.89 min, respectively, confirming the purity of siderophores' samples. The purified siderophores was thus tested for its antimicrobial efficacy (Figure 3).



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.30	15246.08	350.98	25.00	25.00	0.25	
2	3.38	39852.45	600.23	35.00	35.00	0.35	
3	4.22	56524.11	411.90	20.00	20.00	0.20	
4	5.89	47589.99	365.22	20.00	20.00	0.20	
	Total	159212.56	1728.33	100.00	100.00		

Figure 3. HPLC of siderophores purified from uropathogenic *Escherichia coli*

Discussion

In the study conducted by Bnyan et al. (2010), it was shown that 76.6% of UPEC were observed to have the ability to synthesis siderophores, whereas just 5% of *E. coli* fecal isolates had this capability. This study demonstrates a higher occurrence of siderophore synthesis in *E. coli* strains obtained from UTI patients compared to strains obtained from fecal samples. Moreover, the capacity to generate siderophores can serve a distinctive feature for classifying these strains as UPEC [14]. There is no evidence of any iron contamination throughout this period. Prior research by Goel et al. (1998) suggested that elevated iron levels can impede the synthesis of siderophores. Furthermore, the findings indicate that iron-binding proteins, which are synthesized in an iron-deficient environment, have the ability to bind to iron that is already bound to siderophores [15].

Iron-binding proteins are present in the membranes of cultures that have been grown under conditions where there is a restricted amount of iron available. Siderophores have the ability to attach to iron and facilitate its transportation to bacterial cells by means of outer membrane receptors. Iron is a vital element necessary for the proliferation of bacteria. The visibility of siderophores occurs 20 hr after their formation. Introduce the medium and let the bacterial cells to leave. The concentration of siderophores in the culture supernatant reaches its maximum level, indicating that siderophore generation happens at the same time as growth. Hence, after bacterial cells have settled, it is imperative to eliminate these compounds by employing a bacterial filtrate. Exercising caution is crucial when evaporating the ethyl acetate layer to complete dryness using a water bath set at 50°C, since such temperatures can lead to the denaturation of amino acids that are conjugated with phenolates in siderophores [11].

The crude extract was estimated to have a weight of 137 mg/l, which is lower compared to the findings of Hussien et al. (2013) [16], who reported a weight of 235 mg/l for the pyoverdin extract from *Pseudomonas aeruginosa (P. aeruginosa*). The variance in weight can be ascribed to differences in the extraction media, experimental conditions, and the microorganism employed for manufacturing. In a separate investigation, the extract had a weight of 200 mg/l, and siderophores were also obtained from *P. aeruginosa*. The chemical makeup of the isolated molecules indicates a phenolic structure, as demonstrated by the color of the extract when 2% aqueous $FeCl_3$ indicator was introduced. The indicator was absorbed at a wavelength of 490 nm in the UV spectrophotometer.

Pal and Gokarn (2010) [17], found that there is no significant difference in the synthesis of siderophores between commensal and clinical bacterial isolates. They suggested that the formation of siderophores may be necessary for virulence, but it does not directly determine it.

In conclusion, *E. coli* in UTI produces siderophores in a high percentage due to the fact that it contains the three siderophore genes, which means that it will be more virulent than bacteria that do not contain the genes.

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Author contribution

All authors contributed directly to the creation of this paper and approved the final version that was submitted.

Conflict of interest

The authors declare no conflict of interest.

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