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Original article

Antimicrobial activity of siderophores on multidrug resistance bacteria and *Candida albicans* isolated from urinary tract infection Iraqi patients

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ABSTRACT

Background: Urinary tract infections refer to the presence of harmful microorganisms in either the urethra or bladder or the ureter and pelvis of the kidney. Bacteria that live in environments with low iron concentrations create siderophores. **Objectives:** This research aims to determine antimicrobial activity of siderophores on multidrug resistant bacteria, *Candida albicans (C. albicans*) and anticancer activity of siderophore on cancer cell lines. **Methods:** This study was conducted using a cross-sectional design for 100 urine samples obtained from Iraqi patients suspected of having UTI. Who visited the Baghdad teaching hospital. 45 of the samples were multidrug resistant bacteria cultured on blood agar, and 20 of the samples were *Candida albicans* cultured on Sabouraud dextrose agar and incubated at 37 °C for 24 hours. The diagnosis was documented using the Vitek 2 system. Siderophores were extracted from a UPEC standard strain by using ethylacetate, purified by Sephadex LH-20 gel and identification by HPLC. Siderophore was detected was done using Chrome azurol S in M9 minimal salts medium. **Results:** The siderophores demonstrated significant antibacterial effects against multidrug resistant bacteria on Gram positive and negative but its effect is higher in Gram positive than Gram negative. Furthermore, siderophores don't demonstrate any effect on *C. albicans*. The cytotoxic effects of these siderophores were tested on breast cancer cell lines to assess their potential as anticancer agents. **Conclusion:** Siderophores extracted and partially purified from UPEC have both anticancer activity against breast cancer and antimicrobial activity against challenging infections like those caused by multidrug resistant bacteria.

Introduction

The most prevalent illnesses seen in clinical practice globally are urinary tract infections [1]. The anatomical configuration of the female lower urinary system, in conjunction with its near closeness to the reproductive organs, is the primary factor contributing to the higher susceptibility of women to urinary tract infections compared to males. The female urethra's short length reduces the proximity at which bacteria can enter the body [2]. Between 70 -95 percent of urinary tract infections globally are associated with uropathogenic E. coli. This bacterium can develop resistance to the most recently discovered antibacterial therapy [3]. Fungal urinary tract infection usually means urinary tract infection (UTI) caused by Candida species with

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special attention to Candida albicans as the major fungal infectious agent [4].The finding of *Candida* species in urine is usual finding and is called candiduria. There is an increase in the frequency of UTI caused by *Candida* especially in critically ill patients [5]. Siderophores are small iron-binding molecules with a molecular weight of less than 14 kDa. They are produced in significant amounts when there is a shortage of iron. The three primary categories of siderophores are hydroxamate, catecholate, and carboxylate [6]. Siderophores used as antimicrobial agents: The rise of drug resistant bacteria and the resulting worsening of nosocomial infections are among the world's most pressing health challenges [7]. Because of this, the World Health Organization (WHO) released a list of the bacteria that are most important and in need of new medicines in 2017. The list started with microorganisms that belong to the group of Gram-negative pathogens, including *Pseudomonas aeruginosa, Acinetobacter baumanni, and Enterobacteriaceae*. Pathogens such as *Streptococcus pneumoniae* and *Staphylococcus aureus* rank second. Consequently, siderophores are useful tools for inhibiting drug resistant pathogenic bacteria, either on their own or in combination with antibiotics [8]. Siderophores used as anticancer agents: Iron is an essential mineral for all cells, however cancerous cells require significantly more of it because of how quickly they divide [9]. Thus, it has been demonstrated that lowering the quantity of iron in the cellular environment of siderophores inhibits the growth and proliferation of cancer [10]. Bacteria use specialized receptors in their outer membrane to take in ferric bound siderophores. Enterobactin, salmochelin, yersiniabactin, and aerobactin are four forms of siderophores that some strains of *Escherichia coli (E. coli)* can produce to aid bacteria in iron limited environments like the urinary system [11]. The aim of this research: Firstly, obtained of siderophores extracted and purified from uropathogenic *E. coli*. As well as detection of siderophores using Chrome azurol S assay to determine the antimicrobial activity (antibacterial and antifungal) of siderophores against multidrug resistance bacteria and *Candida albicans* isolated from urinary tract infection by using agar diffusion method after determination of MIC and cytotoxical effects of siderophores on breast cancer cell lines (MCF-7, AMJ13) and compared to normal human fibroblast (NHF)

Materials and methods

Samples collection

A total of 100 midstream urine samples were obtained from individuals suspected of having a urinary tract infection. Who visited the Baghdad teaching hospital and educational laboratories in the medical city. between the period from September 25th, 2023, to December 28th, 2023.Used a sterile container, collected midstream urine (20-30 ml), transported to the lab as soon as possible, recorded the collection time, date, patient name, and any relevant notes and inspected the sample upon lab arrival.

Isolation and identification of UTI isolates

The culture that yielded bacterial growth ($\geq 10^5$ colonies) was considered positive, and it was microbiological evidence of a UTI. Bacteria were isolated by culturing on blood agar and MacConkey agar, determined their macroscopic characteristics. Microscopic examination was also done after Gram stain under light microscope 100x. For fungi, urine samples were subjected to culture on Sabouraud dextrose agar plates, which were supplemented with 0.5 mg of chloramphenicol per 1000 ml. The all plates were then placed in an incubator set at a temperature of 37 °C and observed for any signs of growth after 24 hours. The culture plates were inspected to assess the visual characteristics, dimensions, pigmentation, and structure of the colonies. The Vitek-2 compact system was used to validate the bacterial and fungal identification in accordance with the manufacturer's guidelines (Biomerieux, France).

Extraction of siderophore

As per the Jadhav and Desai (1992) procedure, the bacterial suspension is subjected to centrifugation at a speed of 8000 rounds per minute for a duration of 20 minutes. The liquid portion is made acidic to a pH of 2. Then, an equal amount of ethyl acetate is 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 involves adding 1 ml of a 2% aqueous solution of $FeCl_3$ to 1 ml of the sample. The outcome is

affirmative, as determined by the absorption of wine color at 490 nm in the UV spectrophotometer [6].

Detection of siderophores using Chrome azurol S (CAS) assay

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].

Purification of siderophores by Sephadex LH-20 gel

1. The gel was prepared according to the instructions of (Pharmacia Fine Chemicals, Sweden) where it was suspended in methanol (CHEM-LAB, Belgium).

2. Degassing the air using a vacuum pump.

3. Fill the gel 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 (length 20 cm and diameter 2 cm) with methanol: deionized water at a flow speed of (30) ml/hour.

2. Pass the recovery buffer over the column and collect the fractions 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.

Identification of siderophores by high performance liquid chromatography (HPLC)

The quantification of individual phenolic compounds was conducted using reverse phase HPLC (Shimadzu, Japan) analysis. This analysis employed a Sykamn HPLC chromatographic system equipped with a UV detector, Chemstation software, and a Zorbax Eclipse Plus-C18-OSD 25cm, 4.6mm 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 minutes, 40% B; from 4 to 10 minutes, 50% B. The flow rate used was 0.7 mL/min. The samples were injected with a volume of 100 ul, and the standard was also injected with a volume of 100 ul. This process was carried out automatically using an autosampler. The measurements were taken at a wavelength of 280 nm [13].

Antimicrobial activity of siderophores

The agar well diffusion method

To assess the antibacterial efficacy of siderophore, the agar diffusion assay is widely used. The basis for this experiment is that the diameter of a growth inhibition zone surrounding the sample is measured, and the microbial inoculum's volume is applied to the entire agar surface in order to inoculate the surface of the plate. Next, a sterile cork borer is used to make an aseptic circular hole (5 mm) in the agar, and an appropriate volume (100µg) of siderophore extract at the selected concentration is added to the well. The agar plate is then placed in an incubator set at a temperature of 37 °C for 24 hours to allow the antimicrobial agents [14] to diffuse into the agar medium and suppress the tested microbial strain's growth. The zone of inhibition [15] was calculated by quantifying the diameter of the inhibitory zone.

Cytotoxic activity of siderophores against cancer cell lines

Culture of cell conditions

The cell lines were cultured in Minimum Essential Medium (MEM) (US Biological, USA) containing 10% (v/v) fetal bovine serum (FBS) (Capricorn Scientific, Germany), 100 IU penicillin, and 100 µg streptomycin in a humidified atmosphere at 37°C. Cells that were growing exponentially were used in the experiments [16].

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT procedure

The cell lines were placed on a 96-well microplate (NEST Biotech, China) at a density of 10000 cells and kept at a temperature of 37 °C for 72 hours until a complete layer of cells was formed. The cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Elabscience, China). The cells were exposed to a range of concentrations (1000, 500, 250, 125, 62.5, and 31.75 ug). 28 µl of MTT dye solution (2 mg/ml) was applied to each well 72 hours after the infection. The incubation continued for three hours. After adding 100 µl of DMSO to each well, the wells were incubated for 15 minutes. Using a microplate reader, the optical density was determined at 492 nm. The cytotoxicity percentage was determined using the following equation:

$$\text{Cytotoxicity\%} = \frac{(\text{OD Control} - \text{OD Sample})}{\text{OD Control}} \times 100$$

The OD sample refers to the optical density of the wells that have been treated, whereas the OD control represents the average optical density of the wells that have not been treated [17].

Analytical statistics

The application Statistical Analysis System (SAS) (2018) was utilized to find out how various factors affected the research parameters. In order to compare the means of the research substantially, the least significant difference (LSD) test (Analysis of Variation, ANOVA) and Tukey's were utilized [18].

Results

Urinary tract infection isolates

Microorganisms obtained from isolation were *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Bacillus cereus, Pseudomonas aeruginosa, Proteus mirabilis, Streptococcus pneumoniae, Enterococcus faecalis, Staphylococcus haemolyticus* and fungi is *Candida albicans* only

Extraction of siderophores

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.

Detection of siderophores by Chrome azurol S (CAS)

CAS reagent was added, and a spectrophotometer was used to determine the optical density at 280 nm. The color changing from blue to wine was observed, which indicates the presence of siderophores.

Purification of siderophore by Sephadex LH-20 gel

By Using Sephadex LH-20 gel for purification of siderophore results shows in figure (4-8) that fraction 16 shows a significant increase in absorbance at 280 nm compared to previous fractions, indicating the presence of a considerable amount of siderophore. The fraction 17 has the highest absorbance, suggesting it contains the highest concentration of siderophore among all fractions. Fraction 18**,** the absorbance starts to decline from the peak at fraction 17, but it is still relatively high, indicating a significant amount of siderophore is still present. Fractions 16 to 18 are critical as they encompass the peak of siderophores concentration as in the **figure (1).**

Identification of siderophores by high performance liquid chromatography (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 2.30min, close to the peak observed in standard having 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 as in the **figure (2).**

Antimicrobial activity of siderophores

When applying siderophores to test its effectiveness on multidrug resistant bacteria by using the agar well diffusion method, found that Gram-positive bacteria (*Staphylococcus aureus, Staphylococcus haemolyticus, Streptococcus pneumoniae, Enterococcus faecalis, and Bacillus cereus)* were more inhibitory than Gram-negative bacteria(*Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Proteus mirabilis*) and the inhibition zone was measured in millimeters, but *Candida albicans*, no inhibition was shown as in the **table (1).**

Cytotoxic activity of siderophores against cancer cell lines

Cytotoxic effects of siderophore on MCF-7 untreated cells appear a dense, compact structure with a purple color while treated cells appear lighter in color and appear less dense, indicating that the Siderophore has affected their viability or proliferation, but on AMJ-13 untreated cells shows dense cluster of cells with some areas where the cells are more spread out while treated cells shows fewer cells and they appear to be more spread out across the field of view. This suggests that siderophore may affect the proliferation or viability of the AMJ13, leading to a reduction in their number compared to the untreated sample. Finally, NHF untreated cells, shows the cells distributed more sparsely between them, while the treated, shows the cells more densely packed and closely spaced together. This comparison suggests that the siderophore may affect on cell proliferation or density as shown in **figure (3).**

The results shows in AMJ-13 cell line the siderophore compound at a concentration of 1000 µg/ml killed 61.5% of the cells , 500 µg/ml killed

49.6% of cells , 250 µg/ml killed 42% of cells ,125 µg/ml killed 23.4 % of cells ,62.5 µg/ml killed 15.7% of cells ,31.2 µg/ml killed 5.7% of cells as in **figure (4),** There are significant differences when comparing the concentrations with each other and when comparing the concentrations with the control, except for the last concentration of 31.2, which is not significant with the control, and the killing rate was 5.7

The lethal dose for half the number of cells of AMJ-13 was 155.1 µg/ml as in **figure (5).**

In MCF-7 cell line shows the siderophore compound at a concentration of 1000 µg/ml killed 67.1% of the cells , 500 µg/ml killed41.2% of cells , 250 µg/ml killed 27.6% of cells ,125 µg/ml killed 18.7 % of cells ,62.5 µg/ml killed 14.6% of cells ,31.2 µg/ml killed 10.2% of cells as in **figure (6)**, There are significant differences when comparing the concentrations with the control and when comparing the concentrations with each other except for the concentration of 250 µg/ml with 125 µg/ml, 125 µg/ml with 62.5 µg/ml, 125 µg/ml with 31.25 µg/ml and 62.5 µg/ml with 31.2 µg/ml , which is not significant

The lethal dose for half the number of cells of MCF-7 was 261.0 µg/ml as in **figure (7).**

In NHF cell line shows the siderophore compound at a concentration of 1000 µg/ml killed 5.2% of the cells, 500 µg/ml killed 3.5% of cells, 250 µg/ml killed 2.8% of cells ,125 µg/ml killed 2.5% of cells ,62.5 µg/ml killed 1.8% of cells ,31.2 µg/ml killed 1.3% of cells as in **figure (8).** There aren't significant differences when comparing the concentrations with each other and when comparing the concentrations with the control.

The lethal dose for half the number of cells of NHF was 137.1 µg/ml as in **figure (9).**

Table 1. Antimicrobial activity of siderophore.

Bacteria and fungi	**Diameter of inhibition zone (mm)**
Staphylococcus haemolyticus	20
Streptococcus pneumoniae	18
Staphylococcus aureus	15
Enterococcus faecalis	15
Bacillus cereus	15
Escherichia coli	13
Klebsiella pneumoniae	12
Acinetobacter baumannii	12
Pseudomonas aeruginosa	11
Proteus mirabilis	10
Candida albicans	0.00
LSD (*P*-value)	6.941 ** (0.0001)
** ($P \leq 0.01$).	

Figure 1. Purification of siderophores by Sephadex LH-20 gel.

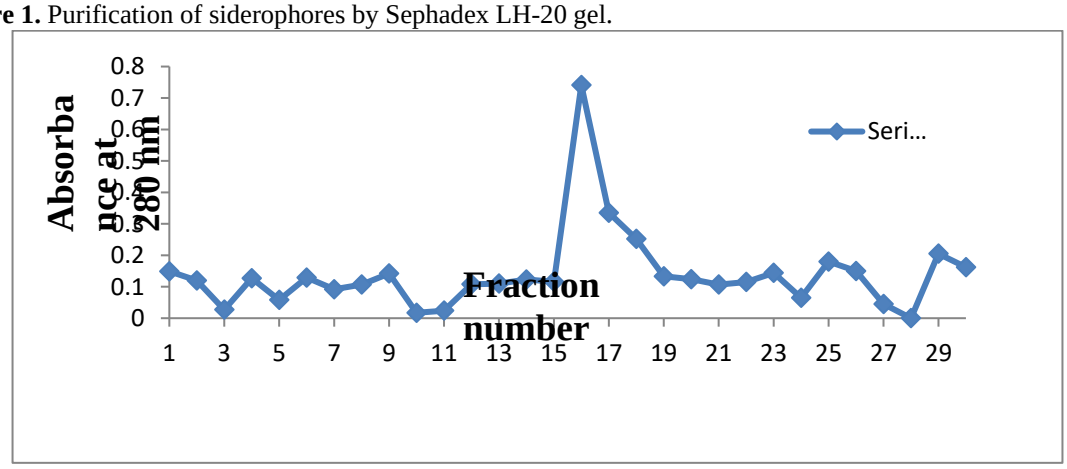
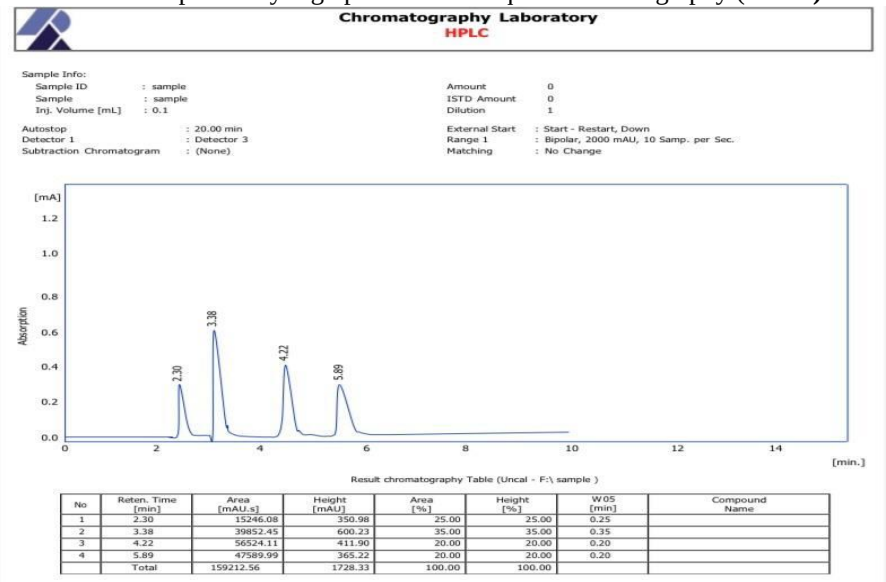
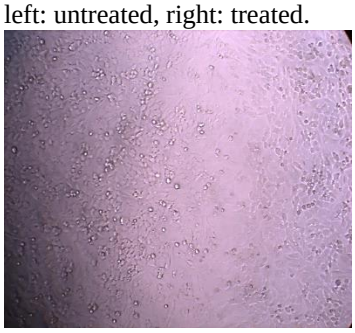


Figure 2. Purification of siderophores by high performance liquid chromatography (HPLC**).**

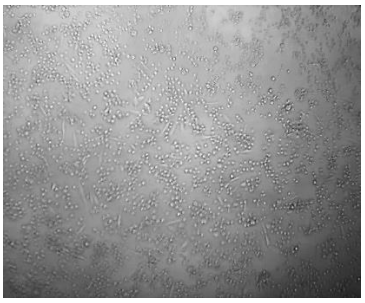


No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.30	15246.08	350.98	25.00	25.00	0.25	
2	3.38	39852.45	600.23	35.00	35.00	0.35	
3	4.22	56524.11	411.90	20.00	20.00	0.20	
4	5.89	47589.99	365.22	20.00	20.00	0.20	
	Total	159212.56	1728.33	100.00	100.00		

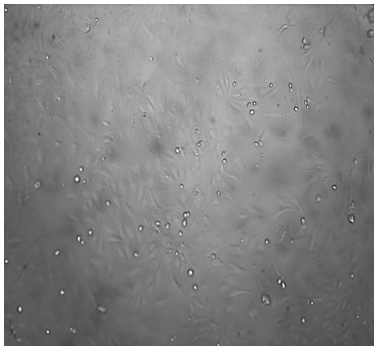
Figure 3. Morphological change of siderophore on (MCF-7, AMJ-13, NHF) under inverted microscope (10X), left: untreated, right: treated.



MCF-7 (un-treated)



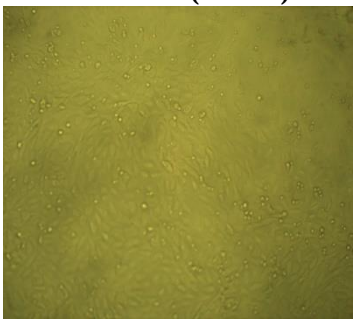
MCF-7(treated)



AMJ-13 (un-treated)

AMJ-13 (treated)

NHF (un-treated)



NHF (treated)

Figure 4. Cytotoxicity and optical density of siderophore on AMJ-13 cell line.

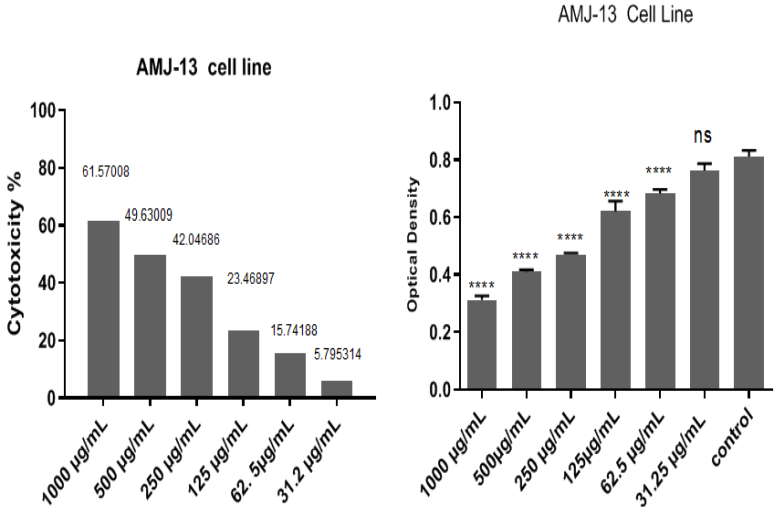


Figure 5. Half maximal inhibitory concentration IC50 effect of siderophore on AMJ-13 cell line.

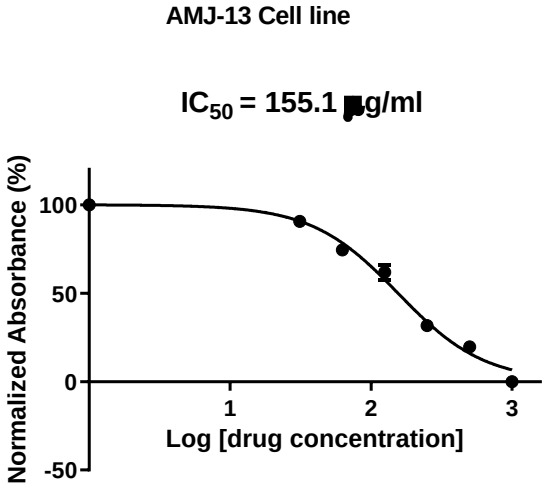


Figure 6. Cytotoxicity and optical density of siderophore on MCF-7 cell line.

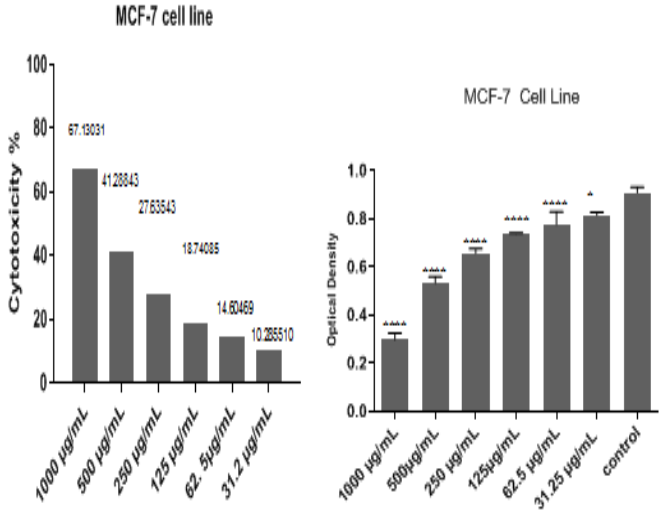


Figure 7. Half maximal inhibitory concentration IC50 effect of siderophore on MCF-7 cell line.

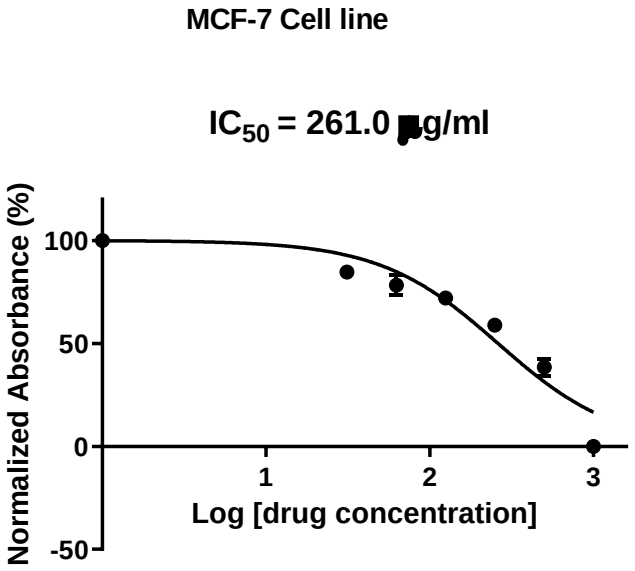


Figure 8. Cytotoxicity and optical density of siderophore on NHF cell line.

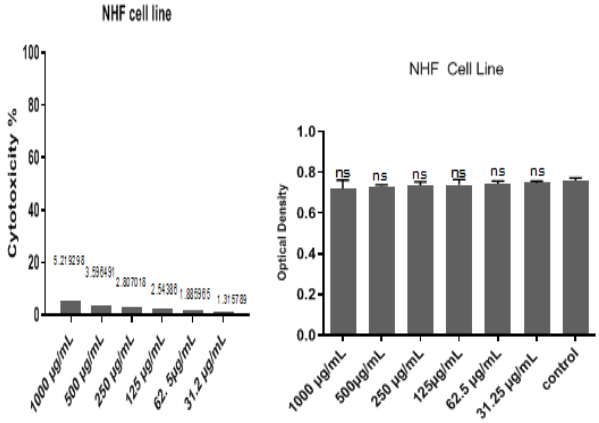
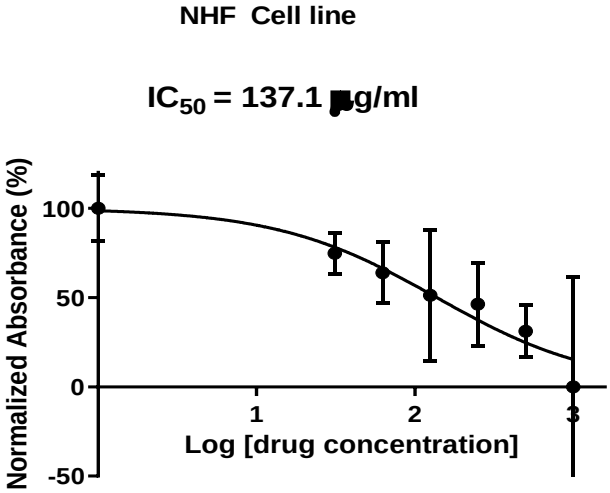


Figure 9. Half maximal inhibitory concentration IC50 effect of siderophore on NHF cell line.



Discussion

Siderophores are molecules produced by bacteria to sequester iron from the environment, which is vital for their growth and survival. The differential inhibitory effect of siderophores from *Escherichia coli* (a Gram-negative bacterium) on Gram-positive bacteria compared to other Gram-negative bacteria can be attributed to several factors: Iron availability and competition: Gram-positive bacteria often have a different set of iron acquisition systems compared to Gram-negative bacteria. Siderophores from *E. coli* might be more effective in outcompeting the iron acquisition systems of Gram-positive bacteria, thereby depriving them of essential iron more effectively [19]. Cell wall structure: Gram-positive bacteria have a thick peptidoglycan layer and lack an outer membrane, which might make them more susceptible to the iron-chelating effects of siderophores. In contrast, Gram-negative bacteria have an outer membrane that can act as a barrier, potentially making them less affected by siderophore-mediated iron deprivation [20]. Specificity of siderophore receptors: Gram-negative bacteria like *E. coli* have specific receptors on their outer membrane to recognize and transport their own siderophores. Other Gram-negative bacteria might share similar mechanisms or receptors, allowing them to resist the siderophore-mediated competition more effectively. Gram-positive bacteria, lacking these specific receptors, might not efficiently compete for iron against *E. coli* siderophores [21]. Bacterial Interference Mechanisms: *E. coli* might produce not only siderophores but also other antibacterial compounds or mechanisms that specifically target Gram-positive bacteria. These could work synergistically with siderophores to inhibit Gram-positive bacteria more effectively. Environmental and ecological Factors: In natural environments, *E. coli* might coexist with Gram-positive bacteria in niches where iron is limited. The evolution of their siderophores might have been driven by the need to outcompete Gram-positive bacteria specifically, leading to the observed differential inhibition [22].

Siderophores from *E. coli* do not inhibit *Candida albicans* due to several reasons: Iron acquisition mechanisms: Candida albicans has its own specialized systems for acquiring iron, including siderophore-independent mechanisms. It can obtain iron from heme, ferritin, transferrin, and other host iron-binding proteins through reductive iron uptake and heme uptake pathways. These mechanisms can by pass the need for siderophore-mediated iron acquisition [23]. Siderophore specificity: Siderophores are often highly specific to the organisms that produce them. The receptors and transport systems for siderophores in *E. coli* are not compatible with those in *Candida albicans*. Therefore, the siderophores produced *by E. coli* are not recognized or utilized by *C. albicans*. Ecological niches and competition: *E. coli* and *C. albicans* often occupy different ecological niches and may not directly compete for iron in the same way. *Escherichia* coli typically resides in the gut, where it has evolved specific mechanisms to outcompete other gut bacteria for iron. In contrast, *C. albicans* can reside in different body sites and has adapted to different iron acquisition strategies suited to those environments. Iron regulation and response: *Candida albicans* has a robust regulatory system for iron homeostasis. This system includes transcription factors that regulate the expression of genes involved in iron uptake and storage. These regulatory mechanisms can mitigate the effects of limited iron availability caused by *E. coli* siderophores [24].

Siderophores from *E. coli* may inhibit breast cancer cell lines primarily due to their ability to chelate iron, an essential nutrient for cellular growth and proliferation. Here are the key mechanisms involved: Iron deprivation: Siderophores are molecules that bind and sequester iron from the environment. Cancer cells, including breast cancer cells, have a high demand for iron to support their rapid growth and metabolic activities. By chelating iron, siderophores reduce the availability of this critical nutrient, thereby inhibiting cancer cell proliferation and inducing apoptosis (programmed cell death). Selective Toxicity: Some siderophores may have direct cytotoxic effects on cancer cells, possibly due to their unique structural properties or their interaction with specific cellular targets. Interference with iron-dependent enzymes: Many enzymes involved in DNA synthesis and repair, mitochondrial function, and cellular respiration require iron as a cofactor. Siderophore-mediated iron chelation can inhibit these enzymes, disrupting essential cellular processes and leading to cancer cell death. [25]. Induction of iron regulatory mechanisms: The presence of siderophores can trigger cellular responses that attempt to compensate for iron deprivation, such as upregulation of iron transporters and ferritin. These compensatory

mechanisms can further stress cancer cells and contribute to their inhibition [26].

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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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