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Detection of Antimicrobial Activity of *Aspergillus terreus* Against Clinical Isolates of *Serratia marcescens*

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Abstract: Out of a total of fifty samples, thirty-five isolates were identified as *Serratia marcescens*. These diverse clinical samples were collected over a three-month period, from October 2023 to December 2023, from several hospitals in Baghdad, including Fatima Al-Zahraa Hospital, Al-Sader Hospital, Ibn Al-Balady Hospital, and Al-Imam Ali Hospital. The clinical samples primarily included urine from patients with urinary tract infections (UTIs). All isolates were cultured on nutrient agar, MacConkey agar, and blood agar, and their identities were confirmed through biochemical testing and the Vitek 2 compact system. Based on phenotypic virulence factors, the *S. marcescens* isolates showed varying positive patterns: 32 out of 35 (91.42%) for protease production, 35 out of 35 (100%) for motility, 27 out of 35 (77.14%) for hemolysin production, and 22 out of 35 (62.85%) for Prodigiosin pigment production. The susceptibility of the S. marcescens isolates to two carbapenem antibiotics (Imipenem and Meropenem) was evaluated using the disk diffusion method. The sensitivity tests revealed high resistance to both IPM and MEM, with resistance rates of 34.28% (12 isolates) and 42.85% (15 isolates), respectively. A bioactive compound extracted from *Aspergillus terreus*, isolated from soil, demonstrated significant activity against *S. marcescens* at varying concentrations. Many of these fungal metabolites exhibited potent anti-disease efficacy, and secondary metabolites were found to stimulate host defenses through various signal transduction mechanisms.

Keywords: Multidrug resistance, Carbapenem, Virulence factors.

Introduction

Serratia is a Gram-negative, rod-shaped bacterium measuring 0.9–2 µm in length and 0.5–0.8 µm in diameter. It is facultatively anaerobic, motile, and non-spore-forming. The bacterium is catalase-positive but oxidase-negative and produces a red pigment called Prodigiosin, which gives rise to red or pink colonies on nutrient agar media. This pigment is associated with the bacterial cell membrane (Mahdi et al., 2013). *S. marcescens* produces several virulence factors, including enzymes such as phospholipases, lipases, nucleases, chitinases, and proteases. Additionally, it has the ability to form biofilms on both abiotic and biotic surfaces. This biofilm formation facilitates bacterial colonization and persistence on medical devices like prostheses and catheters, contributing to increased antibiotic resistance (Abbas & Hegazy, 2020). Studies have shown that infections caused by certain strains of *Serratia marcescens* are challenging to treat due to their high resistance to various antibiotics, including aminoglycosides, β-lactams, and fluoroquinolones (Stock et al., 2003).

This highlights the need for new treatment strategies. Microorganisms produce a diverse range of industrially significant metabolites during their growth phases, such as dyes, enzymes, pigments, and antibacterial agents.

During the stationary phase of microbial growth, organisms like fungi, actinomycetes, and bacteria synthesize chemical compounds known as antimicrobial agents through metabolic processes (Alkhulaifi et al., 2019; Albaayit, 2021). The rich chemistry of natural products primarily stems from secondary metabolites, a class of compounds with significant therapeutic potential (Albaayit et al., 2021; Albaayit, 2020). Some of the most potent secondary metabolites developed into therapeutic drugs have originated from filamentous fungi (Ahmed et al., 2019). Thus, this study aimed to isolate and identify *Serratia marcescens* from UTI patients, evaluate their antibiotic resistance profiles, and characterize their phenotypic virulence factors. Additionally, the study investigated the antimicrobial potential of an extract derived from *Aspergillus terreus*, isolated from soil, against *S. marcescens*. The research further sought to assess the capability of the extract to produce antimicrobial agents and perform chemical identification of the active purified compound.

Method

Sample Collection

Over a three-month period, from October 2023 to December 2023, fifty diverse clinical samples were collected from various hospitals in Baghdad, including Fatima Al-Zahraa Hospital, Al-Sader Hospital, Ibn Al-Balady Hospital, and Al-Imam Ali Hospital. The clinical samples primarily consisted of urine.

Isolation and Identification of *Serratia marcescens*

All clinical samples were directly cultured on blood agar plates and incubated at 37ºC for 24 hours. Colonies appearing opaque, grayish, or white on blood agar were subsequently recultured on MacConkey agar plates for further identification and incubated at 37ºC for another 24 hours. To obtain pure colonies, the selected colonies were subculture on fresh MacConkey agar plates (Mekhael & Yousif, 2009). Final identification and confirmation were carried out using the VITEK® 2 Compact system, which provided accurate species-level identification based on automated biochemical profiling

Antibiotic Sensitivity Test

The antibiotic sensitivity of the isolates towards Imipenem and Meropenem was assessed using the disc diffusion method (Kirby-Bauer method). Antibiotic discs were carefully placed on the surface of the medium, which had been inoculated with the bacterial isolates, using sterilized forceps. The plates were then incubated at 37°C for 24 hours. After incubation, the zones of inhibition surrounding the discs were measured in millimeters (mm). The results were compared with the Clinical Laboratory Standards Institute (CLSI, 2023) guidelines to classify the isolates as susceptible (S), resistant (R), or intermediate (I).

Fungal Culture and Isolation of *Aspergillus terreus*

The fungi were isolated from a soil sample using the serial dilution agar plate method under sterile conditions. For serial dilution, 9 mL of sterilized distilled water was added to each of five test tubes. The first test tube, labeled 10^{-1}, was inoculated with 1 g of the soil sample (Bizuye et al., 2013). To isolate fungi, potato dextrose agar (PDA) was used as the growth medium. To inhibit bacterial growth, the PDA plates were supplemented with 0.2 g/L of streptomycin. From each test tube, 0.1 mL of the diluted sample was spread onto the PDA plates, which were then incubated at 30°C for 6–7 days. By the fifth day, morphologically distinct fungal colonies appeared on the plates. These colonies were individually subcultured to obtain pure cultures. The pure fungal colonies were then transferred to PDA slants and stored at 4°C for future use (Sakthiselvan et al., 2015).

Extraction of Bioactive Compounds from *Aspergillus terreus*

On day 16, the natural products were extracted by harvesting the fungal cells, which were then centrifuged at 3000 RPM for 5 minutes. The resulting pellet was dissolved in ethanol at a concentration of 1g/10 mL. The mixture was sonicated at 24,000 Hz to break the cells. After sonication, the mixture was centrifuged again at 3000 RPM for 10 minutes, and the supernatant (leachate) was collected. The ethanol was removed from the supernatant using an evaporator set at a temperature below 40°C to concentrate the extract. The extract was then

dissolved in a small amount of distilled water, and bioactive compounds were precipitated by adding 70% ammonium sulfate. The mixture was cooled at 3000 RPM for 30 minutes to separate the precipitate. The ammonium sulfate was removed from the precipitate by dialysis, and the contents of the dialysis bag were transferred to a discater, a container with low pressure. This process was performed to obtain the protein-containing natural products as dry matter, as described by Siddiquee et al. (2012).

Results and Discussion

Identification of *S. marcescens* Isolates

Fifty urine specimens from UTI patients were cultured on MacConkey agar and blood agar media. Suspected late lactose- and non-lactose-fermenting isolates, as well as hemolytic and non-hemolytic isolates of *Serratia marcescens*, were further identified using catalase and oxidase tests. The isolates were then confirmed using the VITEK2 GN ID Card system. Out of the collected Serratia species, 35 isolates were identified as *S. marcescens.*

Cultural Characteristics

For the primary identification of *Serratia marcescens* obtained from urine samples, isolates were identified and characterized based on their morphology, cultural characteristics, and biochemical tests. On MacConkey agar, *S. marcescens* appeared as pale-colored colonies due to their non-lactose-fermenting nature (Slonczewski and Foster, 2009). As shown in (Figure 1), it is crucial not to confuse the red-pigmented Serratia colonies on MacConkey agar with lactose fermentation, as the pigment, prodigiosin, is a non-soluble compound (Mahlen, 2011). Microscopically, *S. marcescens* was observed as Gram-negative rods. Biochemically, all Serratia isolates were catalase-positive, as shown in (Figure 2), and oxidase-negative (Mohammed et al., 2018).

Figure 1. *S. marcescens* colonies on MacConkey agar

A **B**

Figure 2. *S. marcescens* catalase test positive on slide (A) and macConkey agar (B)

Protease Test

As shown in Figure 3, 32 (91.42%) of *S. marcescens* isolates in this study were positive for protease enzyme production, while 3 isolates were negative. Proteases are enzymes that hydrolyze peptide bonds in proteins, breaking them down into amino acid monomers (Rawlings et al., 2011). *S. marcescens* produces metalloproteases, which exhibit toxic effects on host cells. These enzymes play a critical role in breaking down proteins in the surrounding medium, providing a nutrient source for the bacteria. Proteases are considered key

virulence factors, aiding pathogenic microbes in penetrating host tissues and utilizing proteins as a nutritional source, as highlighted in research by Al-Shmgani et al. (2024).

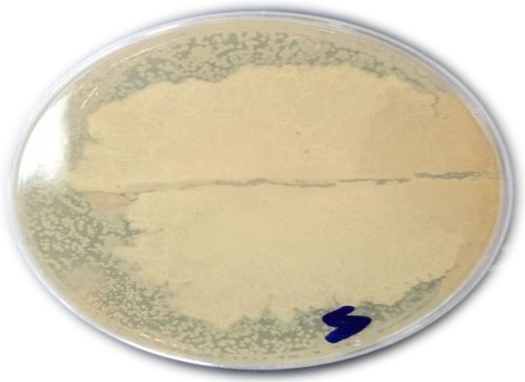

Figure 3. Positive protease test of *S. marcescens* on milk agar

Motility Test

All 35 (100%) *S. marcescens* isolates were found to be motile when tested using semi-solid motility medium. When grown in liquid medium (Figure 4), *S. marcescens* cells exhibited swimming behavior and displayed distinct morphological characteristics. Motility is considered one of the most significant virulence factors of *S. marcescens*. This bacterium employs various mechanisms for movement, including swimming facilitated by its flagellum (Mahmoud, 2015).

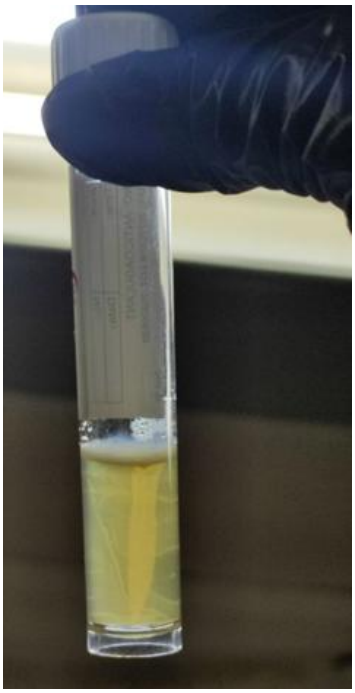

Figure 4. Positive swimming motility test of *S. marcescens*

Hemolysin Production

Out of 35 *S. marcescens* isolates cultured on blood agar, 27 (77.14%) exhibited beta hemolysis, while 8 isolates showed gamma hemolysis, as illustrated in (Figure 5). To confirm the diagnosis of *S. marcescens*, the isolates were grown on blood agar, which serves as an enrichment medium and helps distinguish between hemolytic and non-hemolytic isolates. On blood agar, *S. marcescens* colonies appeared as large, round, red, opaque, moist colonies with hemolytic activity (Ibraheem et al., 2023).

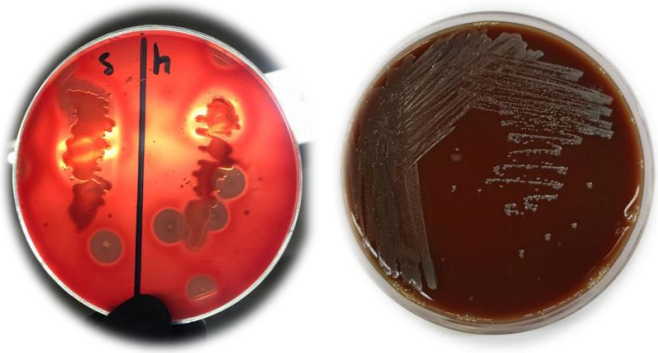

Figure 5. *S. marcescens* colonies on blood agar

Prodigiosin Production

Serratia marcescens has the ability to produce prodigiosin, a secondary metabolite commonly associated with its isolates (Mahmoud, 2015). As shown in Table 1, 22 (62.85%) of the *S. marcescens* isolates in this study were identified as prodigiosin producers when cultured on nutrient agar, as depicted in (Figure 6). The remaining 13 isolates were non-pigment producers. Pigment-producing isolates demonstrated the ability to produce prodigiosin at both 28°C and 37°C. One of the most distinctive features of Serratia colonies is their red pigmentation, attributed to prodigiosin. This pigment is produced by several Serratia species, including *S. marcescens*, *S. rubidaea*, and *S. plymuthica*, while some other Serratia species do not produce prodigiosin (Bdaiwi et al., 2019).

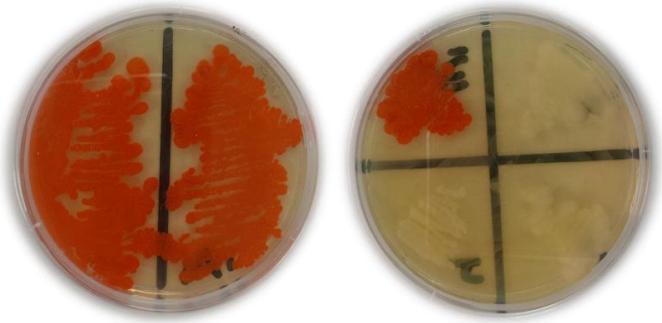

Figure 6. Pigmented and non-pigmented *S. marcescens* isolates on nutrient agar medium

Table 1. The pigmented and non-pigmented *S. Marcescens*

Description	Isolates number
Pigment produce isolates	22
Non pigment produce isolates	13
Total Isolates	35

Antibiotics Susceptibility Test

The susceptibility of *S. marcescens* isolates to two antibiotics from the carbapenem class (imipenem and meropenem) was determined using the disk diffusion method. The results were interpreted according to the recommendations of CLSI (2023). The antibiotic resistance patterns of the studied isolates are presented in Table 2 and Figure 7. The sensitivity test results revealed that S. marcescens isolates exhibited high resistance to both meropenem (MEM) and imipenem (IPM).

Table 2. Determination of diameter zone of antibiotics test of *S. marcescens* isolates

No.	R(%)	I(%)	S(%)
IPM	12 (34.28%)	6 (17.14%)	17 (48.57%)
MEM	15 (42.85%)	4 (11.42%)	19 (54.28%)
CLSI	<19 mm	20-22 mm	>23

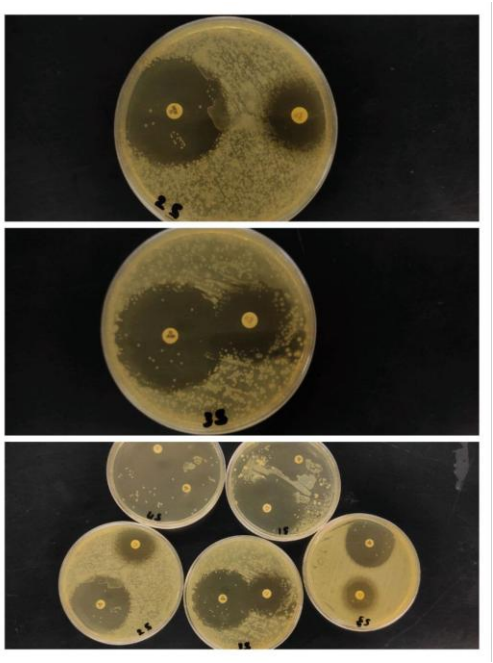

Figure 7. Antibiotic test of *S. marcescens*

One of the most remarkable scientific achievements in history is the discovery and subsequent development of antibiotics. Penicillin remains one of the most effective and safe antibiotics. Since its discovery, thousands of antibiotics have been isolated from soil microbes; however, only about 50 are still in use due to the toxicity of many others to humans (Aminov et al., 2010). The misuse and overexploitation of antibiotics have led to the development of multidrug resistance in harmful microorganisms. Consequently, there is an increasing demand for novel antimicrobial compounds effective against drug-resistant pathogens (Albaayit et al., 2021).

The emergence, selection, and spread of bacterial resistance to multiple antibiotics highlight the urgent need for innovative methods to prevent and treat bacterial infections (Albaayit et al., 2022). Notably, numerous fungal species have demonstrated efficacy against bacterial, viral, and fungal infections resistant to current treatments, making them a valuable source of natural antimicrobial compounds. Antibacterial and antifungal activities can be used to identify potential antimicrobial molecules extracted from various stages of fungal growth (Al-ani & Albaayit, 2018).

Antimicrobial of Bioactive Compound Extraction from Fungi

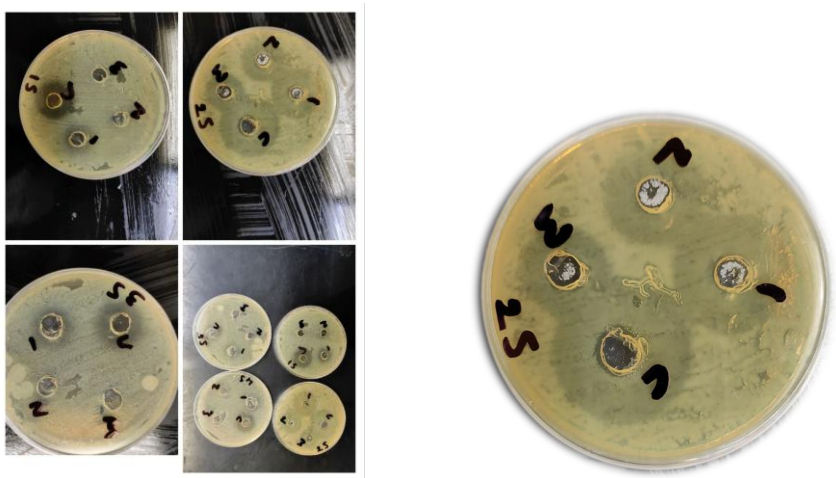

Figure 8. Bioactive extract product on *S. marcescens*

Soil is a crucial source of microorganisms that produce bioactive substances, many of which have demonstrated anti-disease efficacy. Secondary metabolites produced by these microorganisms stimulate host defenses through

various signal transduction mechanisms. The bioactive compound showed significant activity against *S. marcescens*, with inhibition zone diameters ranging from 8 to 33 mm at different concentrations, as illustrated in (Figure 8).

According to the findings of the study, chemical analysis of fungi from the Aspergillus species, obtained from soil, has significantly advanced in recent years, leading to the discovery of new molecular structures with a broad range of pharmacological properties, making them valuable sources of antimicrobial agents (Elkhayat et al., 2016). In contrast, this study highlights the enormous potential of filamentous fungi to produce a wide array of bioactive compounds, which have applications in antibiotics, anticancer treatments, and antifungal drugs (Hawar et al., 2023).

A novel metabolite, asperfumin, produced by the endophytic fungus *Aspergillus fumigatus* CY018, has been shown to inhibit *Candida albicans*, as reported in similar studies by other researchers (Cardoso et al., 2020). Members of the Aspergillus genus are well-known producers of secondary metabolites, such as polyketides (e.g., aflatoxins), non-ribosomal peptides (e.g., ferricrocin), and indole terpenes. These metabolites are considered important resources for new drug exploration (Al-Samarraie et al., 2021).

Four fungal strains—*Aspergillus flavus* (NCIM No. 524), *Aspergillus fumigatus* (NCIM No. 902), *Penicillium marneffei*, and *Trichophyton mentagrophytes* have all been reported to exhibit resistance to the antifungal properties of isochrontriazoles and thiadiazole in similar studies by other researchers. Isoquinoline derivatives from isochromen dione have been discovered to possess significant biological characteristics (Saeed & Zaman, 2006). These isoquinoline derivatives are known to exhibit a range of biological properties, including narcotic, anti-angiogenic, antiallergic, anti-inflammatory, antifungal, antimalarial, antibacterial, antiviral, and anticancer effects (Notte & Leighton, 2008).

This study's results align with previous research showing that various metabolites of isocoumarins possess bioactivities such as cytotoxicity, antimicrobial properties, algicidal effects, protease inhibition, acetylcholinesterase activity, antimalarial properties, immunostimulation, and plant growth regulation, among others (Guo et al., 2020). Additionally, similar studies suggest that dihydroisocoumarins, primarily derived from natural sources such as microbes, bacteria, and fungi (including endophytic, soil, and marine fungi), have significant biological potential (Orfali et al., 2020).

Conclusion

Serratia marcescens is an important cause of nosocomial infections in hospitals, with the highest isolation rate observed from urine samples. The incidence of infection is higher among females compared to males. The isolates demonstrated resistance to carbapenem class antibiotics, with the highest levels of resistance observed against imipenem (IPM) and meropenem (MEM). Additionally, *S. marcescens* produces various phenotypic virulence factors, including hemolysin, protease, and prodigiosin, each exhibiting different patterns. The bioactive compound extract of *Aspergillus terreus* extract, demonstrated high-level activity against *S. marcescens* isolates.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPSTEM Journal belongs to the authors.

Conflict of Interest

* The authors declare that they have no conflicts of interest

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