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Biological Activity of *Saussurea Costus* Extract on MDR Enterobacter Spp

Shareef Saleh Mahdi Al-khateeb, Nisreen Ahmed Mahdi,
Sarah Mohammed Hussein al-Thabhawee, AMJED MOHAMMED SAHIB ALMUSAWI
Imam Ja'afar Al-sadiq University (Ijsu), Department of Medical Laboratory Techniques

Abstract: Enterobacter spp. are common pathogens causing a variable spectrum of the infection are a main reason of nosocomial and opportunistic infection in hospitals also can be found in the gastrointestinal tract. The aim of this study is to diagnose Enterobacter spp. that cause bacteremia, infections for both the urinary as well as respiratory tracts, inflammations of joins and endocardium, infections of ophthalmic, intra-abdominal and bones among other extra intestinal infection multiple resistance to a group of antibiotics in patients admitted to the Al-Sadr Teaching Hospital in Al-Najaf.

Where 102 samples were collected, among 102 clinical specimens from different sources were 50 (48.32%) positive bacterial growth as following 12 (23.7%) return to gram-positive and 38 (76.3%) as gram-negative compared with 52 (51.67%) with no growth. This study also showed 6 clinical isolates of Enterobacter return to different species however these species ware isolated from various infections such as UTI, vaginal infection, burn wound infection, knee fluid, throat infection and intestinal infection and this explains the ability of this pathogen to infection various parts of human which may be due to the important virulence factors which help it to opportunist any conditions for infection , Where he tested the drug sensitivity of isolates and used 13 types of antibiotics by the method of tablet spread.

Key points: Nanotechnology, antibacterial activity of Saussurea costus, MDR bacteria, Antibacterial Activity of Plant Extract.

INTRODUCTION

Enterobacter spp. are common pathogens causing a variable spectrum of the infection (Davin-Regli, 2015). are a main reason of nosocomial and opportunistic infection in hospitals also can be found in the gastrointestinal tract (Salimiyan et al., 2020). *Enterobacter* species can cause bacteremia, infections for both the urinary as well as respiratory tracts , inflammations of joins and endocardium, infections of ophthalmic, intra-abdominal and bones among other extra intestinal infection (Mortazavi et al., 2018). They have a characteristic ability to develop Broad-spectrum antimicrobial resistance during therapy (Tepeli and Zorba, 2018). In fact, there are six species belong to the *E. cloacae* complex Which are closely related involved *E. cancerogenus, E. asburiae, E. Nimipressuralis, E. kobei, E. dissolvens* as well as *E.hormaechei* (Silva et al., 2018). The Enterobacter cloacae complex (ECC) is a common nosocomial bacteria that can cause a variety of illnesses among human involve: pneumonia, urinary tract infections, and septicemia(Annavajhala et al., 2019) .The appearance of multidrug resistance (MDR), such as resistance to carbapenems (meropenem, imipenem, and ertapenem) has made an increased interest in these organisms (Elshamy and Aboshanab,2020) Commercial drugs of beta-lactams, which account for 60% of all antimicrobial use worldwide (penicillins, cephalosporins, carbapenems, and monobactams) (Ogunrinuet et al., 2020) They are preferred because of their efficiency, low toxicity, and activities

that ability prolonged or returned through chemical modification (Palacios et al., 2020) The beta-lactamases which are associated with AmpC gene are located or mediated on chromosomes for some genus of Enterobacteriaceae such *Enterobacter* Infections due to bacteria (possess AmpC gene) that Produce beta-lactamases that cleavage of most penicillin's drugs are Usually related to important mortality and morbidity (Ahmad et al ., 2019) Enterobacter spp. Are Resistance for a variety of antibacterial drugs or may become resistant Through therapy making it difficult to choose the best treatment (Li and Webster, 2018). There is a scarcity of molecular data from Iraq on the Evolution of antibiotic resistance in Enterobacter spp., and the information That is accessible is limited to the presence of extended-spectrum beta lactamases in this bacterium. Surveillance allows for the tracking of the Development of drugs resistance among organisms and the implementation Of control measures to limit the spread of resistance; therefore the current the study involved resistance and virulence markers genes among Enterobacter spp. isolated from patients in Najaf City, Iraq .

However, there is currently inadequate research available in the literature regarding the antibacterial property of *S. costus*. More research is needed to evaluate *S. costus* antibacterial activity. The objective of this study is to evaluate the susceptibility of Enterobacter spp. to various root extracts of *S. costus*.

Dolomiaea costus, formerly known as Saussurea costus, commonly known as costus, Indian costus, kuth, or putchuk, is a species of thistle in the genus Dolomiaea native to South Asia and China. Rishi (Hindu) mystics of Kashmir especially ate this plant. Essential oils extracted from the root have been used in traditional medicine and in perfumes since ancient times (Kasana et al., 2020).

The emergence of multidrug-resistant microorganisms has been reported all over the world, which has created a serious global medical crisis (Nawab *et al*. 2011). Even though many new antimicrobial agents have been produced over the past decades, microbial resistance is continuously increasing toward different antimicrobial agents. In many cases, resistance emerges only a few years after the introduction of the medication (Njimoh *et al*., 2015).

The therapeutic value of *S. costus* lies in some active biochemical constituents that produce different physiological effects on the body. These biochemically active substances mainly include flavonoids, alkaloids, sesquiterpenes, phenolic compounds, tannins, carbohydrates, and glycosides (Abdelwahab *et al*., 2019). Many investigations have reported various therapeutic properties of *S. costus* root, such as anti-parasitic, anti-inflammatory, wound healing, immuno- stimulatory, antioxidant, hepatoprotective, choleretic, antiulcerogenic, larvicidal, gastro-protective, cytotoxicity, cardiotonic, and anticancer activities (Al Otibi *et al*., 2020).

Materials and Methods

Sterilization and Culture Media Preparation

All of the media utilized in the current study ware made according to the instructions from the manufacturer, fastened. Was autoclaved at 121°C for 15 minutes to sterilize them. After cooling the medium to 45°C in a Water bath, a blood agar base was completed with 5% blood and poured into sterile Petri dishes (Shareef *et al*., 2019).

Solutions and Reagents

Reagents Preparation All reagents were prepared according to MacFaddin, (2000).

Catalase Reagent

The ability of Microorganisms to produce catalase was tested using Hydrogen peroxide (H_2O_2) at a concentration of 3 % .

Oxidase Reagent

It was prepared by dissolving 100 mg of tetramethyl p-phenyl Diamine-dihydrochloride in 10 ml of sterile distilled water (without light). It was used to identify a pathogen's capacity to produce oxidase.

McFarland Standard (0.5)

It was made depending on Barry, (1976) as follows: First: Solution A: melting 1.175 g. of ($BaCl_2.2H_2O$) in 100 ml Distill Water. Second: Solution B: 1 ml of (H_2SO_4) was added to 100 ml of distilled Water. Then, 0.5 ml from the first solution (A) was transported to 99.5 ml From the second solution (B). The McFarland standard tubes were sealed with Para-film to block Evaporation and saved for up to 6 months in the dark at room temperature. The exactitude of the density of the mad 0.5 McFarland standard was examined by using a spectrophotometer. The absorbance of the wavelength of 625 nm should be between 0.08 and 0.1 (CLSI, 2023) to get optical Density that is equivalent to the turbidity of the bacterial suspension Including 1.5×10^8 CFU/ml.

Identification of *Enterobacter* spp. Isolates

All suspected *Enterobacter* spp. Isolates were isolated from clinical Sources after culturing on Blood agar, and MacConkey agar, as well as according to morphology characters then all suspected isolates streaking and incubating overnight at 37˚C to an obtained single colony.

1-Gram Stain Method

For all putative bacterial isolates, a bacterial smear was prepared From pure and young 24 h culture and stained with gram stain. Under a Light microscope, it was utilized to determine the form and grouping (arrangements) of bacterial cells (Collee *et al*., 1996).

2-MacConkey Agar Growth

The MacConkey agar medium was utilized to isolate gram-negative Bacteria selectively. All bacterial isolates were streaked on MacConkey Agar surface and cultured for one day at 37°C. This medium was also used to observe the ability of *Enterobacter* spp to grow on MacConkey agar Medium (Collee *et al*.,1996).

3-Catalase Production Test

This test was done as reported by (MacFaddin, 2000) as following: A Growth from the young and pure colony was confused with 3% of H_2O_2 in clean slide. The production of bubbles indicates a positive result for the capability of bacteria to form catalase.

4-Oxidase Test

A sterile wooden stick was used to combine 2 or 3 drops of a prepared oxidase reagent with a fresh bacterial colony on filter paper. The Presence of violet or purple color within 10-15 seconds as a marker for the Spread of bacteria to create oxidase was designated as a favorable outcome (MacFaddin, 2000).

5-Indole Production Test

It was prepared as described by MacFaddin, (2000) a follow: A new bacterial strain was injected into peptone water, which was Then incubated at 37°C for 24 hours. The test tube was then filled with 1 or 2.

Kovacs reagent drops. Finally, the formation of a pink ring denotes a Positive outcome for bacteria's capability to convert tryptophan to indole.

9-Motility Test

By stabbing down to the middle of the test tube, a new and pure Bacterial colony was incubated and cultured at 37°C overnight. The ability Of bacteria to motile was judged to be positive when turbidity diffused from The stab line (MacFadden, 2000).

Antibiotic Susceptibility Test

Antibiotic susceptibility was carried out for all isolates of bacteria according to Bauer – Kirby (1966) method

1- The tips of 4-5 isolated colonies of the bacteria to test tube containing 5 ml from sterile normal saline in a cell density equivalent to the turbidity of McFarland tube No. 0.5 which approximately equal to bacterial cells density of $5x10^8$ cells/ml

2-The standardized bacterial suspension was dipped into a sterile cotton swab. The excess fluid was removed by rotating the swab Firmly against the inside of the tube above the fluid level. The swab was then streaked onto the dried surface of a Muller-Hinton plate in 2 different planes to obtain an even distribution of the inoculums

3-The sheet covers were replaced and the inoculated panels were allowed to remain on a flat, level surface without disturbance for 3-5 minutes to allow the excess moisture to be absorbed.

4- With the sterile forceps, the selected discs were placed on the Inoculated plate and pressed gently into the agar. Within 15 min the inoculated plates were incubated at 37 ºC for 18 – 24 hours .

5-Using reflected light and a ruler, the diameters of the complete Inhibitory zone were documented and measured after incubation. The Area with no visible growth was used as the endpoint, which was measured to the nearest millimeter

6- The inhibition zones around the disks were assessed using CLSI, (2023).

Methanolic and Hexane extraction :

Methanolic extract was prepared from 100 g of the dried powdered root of *S. costus* soaked with 250 ml of methanol, it was placed on a stirrer and left for 72 hours. Then the content was homogenized and filtered through Whatman No. 1 filter paper. After that, the filtrate was transferred into a round bottom flask and was concentrated using Lauda Alpha RA 8 with a Heidolph Rotatory evaporator to remove the solvent and obtain a dark-gum like crude methanol extract. The same procedure was followed for the preparation of Hexane extract by dissolving 100 g of dried root powder with 200 ml of hexane The extracts were stored at 4°C for further antimicrobial activity testing.

Sterility proofing of the extracts :

The obtained *S. costus* extracts were sterility proofed by transferring two ml of the extract into 10 ml of Mueller Hinton Broth (MHB) and incubating it for 24 hours at 37°C. After the period of incubation, the absence of bacterial turbidity or clearness of the broth was observed and 100 µl was transferred onto a clear blood agar plate to ensure the sterility of the extracts.

Antibacterial activity of methanol plant extract:

The methanol extract was tested to evaluate their antimicrobial activity against *Enterobacer*. spp using agar well diffusion method (Bauer *et al*., 1966). Standardized suspension of *Enterobacer* (1.5 x 10^8CFU/ml) by McFarland standard (0.5N) then swabbed separately onto sterile Muller-Hinton Agar (MHA) plates using sterile cotton swabs. This approach was like all extracted. Using a sterile cork borer, two holes were made in each of the culture plates. 100 µL of methanol extract was added to one of the holes, and 100 µL of hexane extract was placed to the second hole. The culture plates were then incubated at 37°C for 24 hr. The clear zone of inhibition around the hole was computed in mm. The tests were performed in five replicates.

Results and Discussion

1. Patients and Specimens Collection

The research was conducted in most of the private clinical laboratories in Al-Najaf City, Iraq, from September 2023 to May 2024 . An entirety of 102 clinical specimens got from non-duplicate patients were randomly collected and checked to recognize *Enterobacter* spp. isolates. The data in table (4-1) recorded the number and percentage of bacterial growth using different culture media, among 102 clinical specimens from different sources were 50 (48.32%) positive bacterial growth as following 12 (23.7%) return to gram-positive and 38 (76.3%) as gram-negative compared with 52 (51.67%) with no growth. Table (4-1):

Table (4-1): Distribution of Type of Bacterial growth among 102 specimens return to different sources

Status	Number (%)	Bacterial growth
Gram positive	12	50
Gram negative	38	
No growth	52	
Total	102	

There are many signs as well as guides that ware published in a previous local and global researches have demonstrated that *Enterobacter* isolates are important members of Enterobacteriaceae that capable of causing disease and infection in different parts of the human (Singh et al ., 2018).

However, the frequency of the spread of Gram-negative pathogens among clinical specimens may be due to their ability to trigger disease by lipopolysaccharides (LPS), which function as an endotoxin for hospital-acquired infections, and porin channels that occur in the outermost layer, blocking the entry of harmful substances rather than being immune to a wide range of antibiotics. Moreover, according to some studies, the source of Gram-negative infection may be endogenous, but transmission occurs often in hospitalized patients as well as by exogenous routes such as sink taps and hospital personnel's hands (Arbabi et al., 2016).

4.2. Isolation and Identification of *Enterobacter* spp. Isolates

The existing work was reported that the features of *Enterobacter* spp. isolates were gram-Negative pathogen when using gram stain method, bacilli-shape, organized as a straight rod cells, catalase and oxidase examination is positive and negative respectively, non-pigment produced, motile. Usually, the colonies light to white in color, large, smooth, non-hemolysis were seen on blood agar medium and, usually red to pink color on MacConkey agar at 37 ℃ for overnight (Figure 4-1). The conventional biochemical examinations were represented in Table (4-3). However, based on the outcomes of the features of colony morphology, microscopic vision, some main biochemical tests, and Vitek-2-system, it was recorded that out of total 102 specimens obtained throughout the study period, 6(7.3%) of isolates were distinguished as *Enterobacter* spp. as following *E. cloacae* 4 isolates, *E. aerogens* 1 isolate, and *E. hormaechei* 1 isolate, (Table 4-5).

Table (4-3): Phenotypic features and some biochemical tests of *Enterobacter* spp. isolates

Tests of *Enterobacter* spp.	Feature of the result
Gram stain	Negative
Microscopic shape	straight rod
Catalase	+
Voges proskaures	+
Methyl red	-
Oxidase	-
Indole	-
Growth on macConkey agar	+
Simmons citrate	+
Motile	+
Chromagar orientation medium	Teal blue

The data in the table (4-4) exposed distribution of *Enterobacter* spp. isolates as well as number and percentage of bacterial growth among human sex, out of 6 isolates of *Enterobacter* spp. were obtained during the period study, However, 2(47. 61%) was gained from female while 4 (52.38%) was recovered from the male. Table (4-4) Number and percentage of *Enterobacter* spp. according to sex.

Sex	Number (%) of specimens	Gram-negative (%)	*Enterobacter* spp. (%)
Female	60	21	2
Male	42	17	4
Total	102	38	6

Female infections are more common than male infections especially the ratio of females to males in urinary tract infection which may be due to variations in the anatomy of the reproductive system between males and females (Amin et al., 2009). At the same time, the fact that these bacteria are mostly part of the living flora and various virulence factors that lead to their pathological potential and the difference in outcome with other virulence factors of pathogens can be due to their existence in UTI (Wagner *et al*., 2020).

The current study obtained 6 clinical isolates of *Enterobacter* return to different species as shown in table (4-5), however these species ware isolated from various infections such as UTI, vaginal infection, burn wound infection, knee fluid, throat infection and intestinal infection and this explains the ability of this pathogen to infection various parts of human which may be due to the important virulence factors which help it to opportunist any conditions for infection. Haidar *et al*., (2016) pointed that *Enterobacter* species are hospital-acquired causative agents of nosocomial infections. ESBL-producing isolates are the main concern because of their resistance to different classes of antibiotics as well as their infectious nature. However, this finding is lower than a local study done by Abid, and AL –Amaar, (2015) they obtain 84 clinical isolates of *Enterobacter* from 718 specimen return to different clinic source in Thiqar City/Iraq.

Antibacterial Activity of Plant Extract

After we drilled in the planting medium and added plant extracts to it, the results showed an inhibitory activity for each extract, and the effectiveness of each extract was determined by measuring the area of inhibition zone in millimeters.

The extract weight

The plants types	**Samples that added Methanol to them**
Cordiamyxa	**0.35 g**
Pomegranate peel	**1.44 g**
Citrulluscolosynthis	**0.4g**

The plants types Samples that added Methanol to them Cordiamyxa 0.35g Pomegranate peel 1.44 g *Citrullus colosynthis* 0.4g

The results showed a clear inhibitory activity of the plant extracts against the bacteria used in the study, and it was noted after 24 hours of incubation at 37°C a variation in the effect of the concentrations used for the plant extracts in inhibiting the two types of bacteria separately.

* The effect of pomegranate peel extract ranged between 14-16 mm As for the bamboo plant, it had no effect on *Staphylococcus aureus*, and it had an inhibitory effect on *Pseudomonas aeruginosa* by 17 mm.

* As for the bitter melon extract, it had an inhibitory effect on bacteria by (10- 12 mm) .

Table (2.4) The activing of pom./Cordia./Citrullus. at the *Staphylococcus* and *Pseudomonas*

Plants extract	Staphylococcus(+)	Pseudomonasaeruginosa(-)
Pomegranatespeels **(100 mg/ml)**	**16**	**16**
Pomegranatespeels **(250 mg/ml)**	**16**	**16**
Pomegranatespeels **(500mg/ml)**	**15**	**14**
Cordiamyxa peels **(250 mg/ml)**	**Zero**	**17**
Cordiamyx a peels **500)mg/ml)**	**Zero**	**17**
Citrullus colocy **(250 mg/ml)**	**10**	**10**
Citrullus colocy.**(500 mg/ml)**	**12**	**12**

Plants extract *Staphylococcus* (+) *Pseudomonas aeruginosa* (-) Pomegranates peels (100 mg/ml) 16 16 Pomegranates peels (250 mg/ml) 16 16 Pomegranates peels (500mg/ml) 15 14 Cordiamyxa peels (250 mg/ml) Zero 17 Cordiamyx a peels 500 mg/ml) Zero 17 Citrullus colocy (250 mg/ml) 10 10 Citrullus colocy.(500 mg/ml) 12 12

Discussion

The results in table to show the inhibitory activity of the plant extract against *Enterococcus spp.* was noted the high inhibition action in pomegranate peels at 100, 250 and 500 mg/ml in other hand the extract of citrullus appear medium activity against off the *Enterococcus* but the cordiadoesn't effect at the *Enterococcus* colony. when we are discuss this result may be the resistance of cell wall in *Enterococcus* against cordia extract also the natural products of other extract had high activity at staphylococcus . The acting off extracts against *Pseudomonas* in the table 2 appears high inhibition zone in cordia which isn't affected at *Staphylococcus* this leads to the *Staphylococcus* differ from pseudomonas in the cell wall. At the second level of inhibition the peel of pomegranate with 16 mm nature this resulting be active compound of pomegranate peel.

Conclusions:

The percentage of *Enterobacterv spp.* that cause difference infections in this study is high, and its percentage in males is more than females. The proper management of these infections is required in order to help reduce the morbidity and mortality associated with isolated bacteria.

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