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Investigation of Antibacterial Activities of Indian Costus Root Extracts against Human Pathogenic Bacteria

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ABSTRACT

Indian costus is traditionally famous due to its potential therapeutic activities. The study aimed to examine the antibacterial activities of Indian costus root samples obtained from different countries. The roots were extracted using ethanol and water, and phytochemical screening showed the presence of various compounds. The antibacterial activities of the extracts were tested against clinical bacterial isolates (*Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli and Klebsiella pneumonia*), using different methods. The results demonstrated varying inhibition percentages on different bacterial strains when exposed to the extracts using microtiter plate assay. While the disc diffusion method showed negative results, the agar well diffusion method revealed positive results for hot ethanolic extracts against *Streptococcus pyogenes* and *Klebsiella pneumonia*. Additional testing of the most sensitive bacteria to Soxhlet extract showed inhibitory effects at lower concentrations (6.25mg/ml).

1. Introduction

Indian Costus is scientifically known as *Saussurea lappa*, and is available in India and sub-alpine regions [1]. It belongs to the Compositae family, with a 6 cm long root and a distinct odor. It is well known for its significant medical importance for the treatment of skin diseases, cold, rheumatism, typhoid fever, malaria and leprosy. Dehydrocostus lactone and costunolide are the most bioactive compounds responsible for its biological activities [2]. Furthermore, Indian Costus extracts have been found to be effective as antibiotics against a broad range of human multi-resistant microorganisms [3].

In Saudi Arabia a study was conducted on costus acetic acid extract in which, terpenoids account for 79 % of phytochemical screening, and the extract exhibited potent antibacterial effects against various bacteria [4].

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The ethanolic extract of *Saussurea lappa* showed an inhibition zone of 18 mm at 200 mg/ml on *S. pneumonia* and 13 mm *on E. coli*, while the aqueous extract produced inhibition zones of 16 mm and 15 mm on *S. pneumonia,* and 10 mm on *E. coli* at 200 mg/ml, respectively [5]. Increased antibacterial activities with higher extract concentrations, particularly against Gram-positive bacteria. *S. aureus* showed the highest sensitivity, while *Salmonella typhi* was the most resistant to the ethanolic extract [3]. In comparison between costus and cidir aqueous extracts, the extracts from *Saussurea lappa* were most effective against *E. coli*, with a 26 mm inhibition zone, they also showed good results against *Bacillus subtilis* and *S. aureus*, with 24 mm zones of inhibition. Methanolic and ethanolic extracts of the roots were tested on various bacteria, showing greater susceptibility of gram-positive compared to gram-negative bacteria [6]. Alaagib and Ayoub [7] found that the extracts were rich in important phytochemicals, such as alkaloids, coumarins, flavonoids, sterols, saponins and tannins, and exhibited greater susceptibility towards gram-positive than gram-negative bacteria, the ethanolic extract was bacteriostatic on MRSA at 2000 µg/ml and bacteriocidal at 6000 µg/ml [7]. Similarly, it exhibited bacteriostatic and bacteriocidal effects on other bacteria at different concentration levels [8].

2. Methodology

2.1 Indian Costus Root Samples

Three Indian costus roots samples were collected from local herbal markets and resembled from Libya, Egypt and Turkey.

2.2 Preparing Root Powder

The roots were washed, dried, crushed into small particles using a mortar, ground to a fine powder with an electric grinder and then stored in airtight containers for the next experiment.

2.3 Preparing Root Extracts

2.3.1 Aqueous extracts

Following the method of Akoul and Ghreeb [5], 25 g of powdered roots were mixed with 250 ml of distilled water for 24h in the shaker at room temperature, following this, the mixtures were filtered by gauze and centrifuged (MD, France) at 3000 rpm for 15 minutes. Then the supernatant was dried using an oven (Hamilton, UK) at 45°C for 48 h.

2.3.2 Ethanolic extracts

An amount of 25 g from powdered root samples were added to 250 ml of ethanol (96 %). The mixture was homogenized using vortex, then filtered twice by gauze and concentrated by a rotary evaporator (Heidolph, Germany) at 45°C [5].

2.4 Hot Ethanol Extract

Using the Soxhlet apparatus, 25 g of roots powders were placed in an extraction thimble, and the distillation flask was filled with 200 ml of 96 % ethanol, then parts of the apparatus were completely constructed. Heating was started at 45 °C for 6 h. After extracting three samples for three consecutive

days, the ethanol was evaporated by rotatory evaporator, and the dried extracts were kept in a sterile container for the next experiment [9].

2.5 Collection and Preservation of Bacteria

The study included two gram-positive bacteria [*Staphylococcus aureus* (MRSA) and *Streptococcus pyogenes* (*S. pyogenes*)] and two gram-negative bacteria [*Escherichia coli* (*E. coli*) and *Klebsiella pneumonia* (*K. pneumonia*)]. These bacteria were collected and identified by microbiology laboratory at Misurata Medical Center. All bacterial samples were cultured on slant agar at 37℃ for 24h and then stored at 4℃ until they were used.

2.6 Antibiotics Susceptibility Tests

The bacteria were tested for their antibiotics resistance activity using disc diffusion method as published by Bauer *et al*., [11]. Six standard antibiotic discs: vancomycin 5 µg, imipenem 10 µg, cefoxitine 30 µg, penicillin 10 µg, cefotrixone 30 µg and erythromycin 15 µg were applied. The zone of inhibition around each disc was measured in millimeters after being incubated at 37℃ for 24 h, diameter of the discs were deducted. Means and standard deviations were calculated, and results were recorded according to the guidelines of Clinical and Laboratory Standards Institute [10].

2.7 Determining the Antibacterial Activities of Indian Costus
2.7.1 Disc diffusion method

Antibacterial activities of Indian costus extracts against tested bacteria were determined following the method of Bauer *et al*., [11]. Filter paper discs (Diameter: 6 mm) were saturated with 100 µl of each stock solution (100 mg/ml) separately, then the discs were allowed to dry, and the pathogenic bacteria were swapped by inoculating loop on nutrient agar (Liofilchem, Italy) plates, after that the discs were placed on the surface of previously swapped plates and incubated at 37℃ for 24h. The experiment was repeated in triplicate [11].

2.7.2 Well diffusion method

Following the method of Perez *et al*., [12], pathogenic bacteria that were completely inhibited in microtiter plate assay were further cultured on nutrient broth for 24h at 37℃ to get active strains. Meanwhile, the nutrient agar was prepared following instructions of the manufacturer and when its temperature reached 40℃, (1 %) of bacterial broth (1 × 108 cfu/ml) was poured and mixed thoroughly. An amount of 25 ml of the mixture were poured into the Petri dish plates and left to solidify, wells with 7 mm diameter were then made by a sterile cork borer, the base of each well was covered by nutrient agar. By applying a micropipette, 100 µl of stock solution of extracts (100 mg/ml) were carefully introduced to each well separately. Although, wells were labeled with a marker, ciprofloxacin (5 µg) was referred as a positive control, while, nutrient agar with bacteria poured on separate dishes were marked as a negative control. All plates were incubated overnight at 37℃. After that, the diameter of inhibition zones around each well was measured in millimeter, otherwise, absence of zone around well indicates no activity of the examined solution. The experiment was done in triplicate [12].

2.7.3 Microtiter plate assay

The 96-well assay was used to determine the antibacterial activities of each extract against different tested pathogenic bacteria, a loop full of each pathogenic bacteria was inoculated in 30 ml of nutrient broth (oxoid, UK) separately and incubated overnight at 37℃. Microtiter plate assay was performed following Magnusson and Schnürer, [13]. Pipetting 100 µl of inoculated nutrient broth with pathogenic bacteria was added in each well in Microtiter plate and 100 µl of (100 mg/ml) extracts solutions were added to them. Wells filled with 100 µl of inoculated nutrient broth and 100 µl nutrient broth with tested bacteria marked as a positive control group, while 100 µl of extracts stock solutions with 100 µl of nutrient broth were used as negative controls. The plate was kept in at 37℃ (Memmert, Germany) for 24h, and optical density (OD) of bacterial growth in each well was measured at 630 nm using an Eliza reader (Bioteck, USA). The experiment was repeated in duplicate and readings were interpreted as the percentage of inhibition using the formula:

Growth inhibition % = (optical density of initial bacterial growth (nutrient broth inoculated with bacteria) – optical density of inhibited bacterial growth (after addition of extract solutions)/optical density of initial bacterial growth) × 100 [14].

2.7.4 Determining MIC and MBC of extracts against bacteria

Minimum inhibitory concentration (MIC) of extract bacterial growth and minimum bactericidal concentration (MBC) of extract that inhibit 99.9 % of bacteria [15], is used to determine the MIC and MBC of extracts against pathogenic bacteria. Two serial fold dilutions of extracts (100, 50, 25, 12.5 and 6.25 mg/ml) were prepared, then 100 µl of each concentration was added to 100 µl of bacterial broth separately in a microtiter plate, extract with nutrient broth (without pathogenic bacteria) considered as a negative control (-C), nutrient broth and nutrient broth with pathogenic bacteria were used as a positive control (+C), the plate was then incubated for 24h at 37℃, the optical density of bacterial growth was measured at 630 nm using Eliza reader, results interpreted following the formula:

Growth Inhibition % = optical density of initial bacterial growth (nutrient broth inoculated with bacteria) – optical density of inhibited bacterial growth (after addition of extract solution)/ optical density of initial bacterial growth × 100.

The MBC was determined by further plating of bacteria with higher inhibition percentages on a nutrient agar plate and then incubated for 24 h at 37°C.

3. Results and Discussion

3.1 Antibiotics Susceptibility Tests of Target Bacteria

MRSA was resistance to Vancomycin, Penicillin, Cefoxitine, Cefotraixone and Erythromycin, with IZDs of 11, 7, 4 and 23 mm, respectively. Similarly, *S. pyogenes* exhibited resistance activity to Penicillin, Cefoxitine, Cefotriaxone and Erythromycin, with IZDs of 20, 19, 9 and 21 mm, respectively. However, *S. pyogenes* showed sensitivity to Vancomycin, with a diameter zone of 17 mm. *E. coli* and *K. pneumonia*, showed resistance to all antibiotics tested. *E. coli* sample produced IZDs of 11, 11 and 13 mm when tested with Vancomycine, Penicillin and Cefoxitin, respectively. Although, no inhibition zone was observed around the Ceftriaxone disc., *K. pneumonia* sample was resistant to all tested

antibiotics, except from Cefoxitin and Erythromycin with inhibition zone of 19 and 11 mm, respectively. All tested bacteria were susceptible to Imipenem (Table 1).

There were no significant differences ($p > 0.05$) between mean IZDs of hot A, B and C extracts and Cefotriaxone, against *S. pyogenes.* However, significant differences ($p < 0.05$) were found between the extracts and antibiotics: Penicillin, Erythromycin, Vancomycine, Cefoxitine and Imipenem. Also significant difference ($p < 0.05$) were recorded in comparing mean IZDs formed by hot A, B and C with antibiotics: Penicillin, Erythromycin, Cefoxitine and Imepenem against *K. pneumonia.*

In contrast to the current study, Deabes *et al*., [3] suggested that costus extracts which was prepared using an ultrasonic process formed inhibition zones larger than those formed by the positive control (Cefotriaxone 1 mg/ml) against gram-positive bacteria. However, the study also found that Cefotrixone had a greater effect on gram-negative bacteria compared to costus extracts. The efficacy of antibiotics relies on the fact that they are entirely synthetic and manufactured under precise and critical conditions, which make them highly effective.

Table 1
Antibiotics sensitivity tests results

Antibiotic	VA	P	FOX	CRO	E	IMP
Bacteria	Inhibition zone diameter (IZDs)					
MRSA	11 ± 1.41 (R)	7 ± 1.41 (R)	11 ± 1.41 (R)	4 ± 0.00 (R)	23 ± 1.41 (S)	34 ± 1.41 (S)
S. pyogenes	17 ± 1.4 (S)	20 ± 2.8 (R)	19 ± 1.41 (R)	9 ± 1.4 (R)	21 ± 1.4 (R)	36 ± 1.4 (S)
E. coli	11 ± 1.41 (R)	11 ± 1.41 (R)	13 ± 1.41 (R)	0.00 ± 0.00 (R)	22 ± 0.00 (I)	39 ± 1.41 (S)
K. pneumonia	0.00 ± 0.00 (R)	0.00 ± 0.00 (R)	19.0 ± 1.41 (R)	0.00 ± 0.00 (R)	11 ± 1.4 (R)	24.0 ± 1.41 (S)

Note: Mean ± Standard deviation (SD), S=sensitive, R=resistant, I=intermediate, 0=no zone of inhibition, VA=Vancomycin, P=Penicillin, FOX=Cefoxitine, CRO=Cefotraixone, E=Erythromycin, IMP=Imipenem

3.2 Antibacterial Activities of Indian Costus

3.2.1 Microtiter plate assay

The growth inhibition percentages of target bacteria by extract solutions after 24h of incubation ranged from 0 to 100 %. It was observed that ethanol extract A had no effect on *S. aureus*, *E. coli* or *K. pneumonia*. Interestingly, the most potent inhibitory effect was observed when all aqueous extracts were used against all tested bacteria. Remarkably, total inhibition percentages of *S. pyogenes* were detected from hot extracts solutions of A, B and C. Ethanol extract C and *K. pneumonia* with A, B and C hot extracts solutions.

The percentage of inhibition of *S. aureus* was 24 % by ethanol extract C solution, while it was 13.4, 37.7 and 50.2 % by hot A, B and C solutions respectively. On the other hand, *E. coli* was sensitive to ethanol extract B, C and hot A, B and C solutions with inhibition percentage of 12.5, 27.24, 58.8, 44.4 and 65.9 %, respectively. It is noteworthy that the growth inhibition percentages for *E. coli* were higher than those for *S. aureus* when using the same extract solutions. *K. pneumonia* was inhibited by 25.5 and 81.5 % with ethanol B and ethanol C extracts. While *S. pyogenes* inhibition percentages were (67.55 and 73.3 %) with ethanol B and ethanol C solutions respectively (Table 2).

In a study by Idriss *et al*., [4], *S. aureus* was more susceptible to acetic acid extract of costus than *E. coli*, disagreeable with the outcome of the present study. Moreover, the results of the current study disagrees with the findings of Ahmed and Coskun, [16] which indicated that the growth of *E. coli* and *K. pneumonia* was not affected by the aqueous and methanolic extracts of Indian costus at a concentration of 200 mg/ml. Although, the findings of a study conducted by Mihnas *et al*., [17],

revealed that the activity of aqueous extract on *S. aureus* and *E. coli* was comparatively lower than that of methanolic and ethanolic extracts, these results are contrast with those of the present study. It is important to emphasize that all the studies mentioned above relied on the use of disc or well diffusion methods in determining the antibacterial effect of Indian costus.

Alternatively, the extract may be active against some bacterial strains but not against others as in findings mentioned by Parekh and Chanda, [18]. The origin of the plant, extraction method, solvent concentration, polarity and type of active constituents extracted, all influence the plants effectiveness [19].

Table 2
Inhibition percentages of target bacteria by Indian costus extracts using microtiter plate assay

Extract solution	Bacteria (Percentage of inhibition)			
	S. aureus	*S. pyogenes*	*E. coli*	*K. pneumonia*
Aq. A	100.00	100.00	100.00	100.00
Aq. B	100.00	100.00	100.00	100.00
Aq. C	100.00	100.00	100.00	100.00
Eth. A	00.00	67.55	00.00	00.00
Eth. B	00.00	73.30	12.50	25.50
Eth. C	24.00	100.00	27.24	81.50
Hot A	13.40	100.00	58.80	100.00
Hot B	37.70	100.00	44.40	100.00
Hot C	50.20	100.00	65.90	100.00

3.2.2 Disc diffusion method

The results of the current study were negative for all tested extracts. The disc diffusion method used to test antibacterial activity relied on the extracts ability to diffuse from the disc to the agar, which did not occur in this case [20]. These results were consistent with a previous screening study of *Saussurea lappa* and eleven other plant extracts, which also produced no zones of inhibition using the disc diffusion method [18].

3.2.3 Agar well diffusion method

Hot (Soxhlet) extracts of A, B and C were tested against two bacteria, and all three extracts showed inhibition of visible growth. The IZDs against both bacteria ranged between 4 to 9 mm. specifically, the hot C extract solution had an effect on *S. pyogenes*, with an IZD of 9 mm, while the same extract solution against *K. pneumonia* had an IZD of 5 mm. The IZD of hot B against *S. pyogenes* was 5 mm, and against *K. pneumonia* was 4 mm, while the IZD of hot A against *S. pyogenes* was 4 mm, and against *K. pneumonia* was also 4 mm.

The tested bacteria were inhibited by positive control (Ciprofloxacin), resulting in a visible growth IZD of 13 mm against *S. pyogenes* and 18 mm against *K. pneumonia*. These zones were wider than the ones formed by the tested extracts. There was no significant difference between the effects of hot A and B against *S. pyogenes* ($p > 0.05$). However, when hot A and B were compared with hot C, there was a significant difference ($p < 0.05$) in their effects against *S. pyogenes*. A significant difference ($p < 0.05$) was recorded between extracts and Ciprofloxacin. Against *K. pneumonia*, there was no significant difference ($p > 0.05$) recorded between A, B and C hot extracts, but there were significant differences ($p < 0.05$) recorded between the antibacterial activities of the extracts and Ciprofloxacin.

Table 3
Inhibition zones diameter by hot ethanolic extracts solution (100 mg/ml)

Bacteria	*S. pyogenes*	*K. pneumonia*
Solution		
Hot A	4 ± 0.0	4 ± 0
Hot B	5 ± 1.4	4 ± 0
Hot C	9 ± 1.4	5 ± 1.4
CIP	13 ± 1	18 ± 2
LSD	3.4	12.9

Note: Mean ± standard deviation (SD), CIP= Ciprofloxacin, LSD= least significant difference

The results of the present study are in agreement with Deabes *et al*., [3], which showed that the antibacterial effect of 50 mg/ml ethanolic extract was positive and dose-dependent (10, 20 and 50 µl). The study also found that gram-positive bacteria were more sensitive to the extracts than gram-negative ones. A study of Alaagib and Ayoub's, [7], which examined the antibacterial activity of methanolic and water Indian costus extracts prepared by the Soxhelt method, showed that gram-positive bacteria were more sensitive to the extracts than gram-negative bacteria using well diffusion method. The current study found that using a hot ethanolic extract at a concentration of 100 mg/ml showed a similar antibacterial effect to the study conducted by Abdallah *et al*., [6], with concentration of 500 mg/ml of ethanolic extract prepared by maceration method, which showed an inhibitory zone diameter of 6.5 mm against *S. pneumonia* (gram-positive), while *K. pneumonia* (gram-negative) was inhibited by a zone of 6 mm. Another study by Akoul and Ghreeb, [5] used concentrations of 50, 100, 150 and 200 mg/ml of Indian costus ethanolic extracts. The study showed that a concentration of 100 mg/ml had an antibacterial effect against *S. pneumonia* with inhibitory zone diameters of 17 mm, the ethanolic extract with same concentration inhibited *E. coli* with an inhibitory zone diameter of 12 mm.

The aqueous extracts of solutions A, B and C used in this study did not show any activity against the tested bacteria in the well diffusion method. This lack of activity may be attributed to the concentration of active constituents. It is possible that the aqueous extract stocks would need to be more concentrated than 100 mg/ml to diffuse through the medium. These findings are in agreement with Hasson *et al*., [8], who also found that the aqueous extract of Indian costus did not inhibit tested bacteria. On the other hand, ethanolic extracts showed antibacterial activity that was dose-dependent. Similar results were obtained by Malu *et al*., [21], who observed that the water extract of ginger did not inhibit the tested bacteria. These findings were consistent with the results of this study.

On the other hand, plant extracts were exposed to deterioration by light, humidity and temperature during common extraction procedures and storage, which can affect their potency, this was also discussed by El-Mahmood and Ameh, [22].

3.2.4 Determining MIC and MBC using broth dilution methods

The inhibition percentages of the bacteria that were tested were determined using serial dilutions of Hot C extract. The results showed that *S. pyogenes* was totally inhibited with an initial concentration of (100 mg/ml). The second concentration of (50 mg/ml) inhibited the growth of (94.70 %) of the inoculated bacteria. The inhibition percentages were (59.60, 26.35 and 4.99 %) using concentrations of (25, 12.5 and 6.25 mg/ml), respectively. The efficacy of C extract solution in inhibiting *K. pneumonia* was total inhibition with a concentration of (100 mg/ml). The inhibition

percentages were found to be (98.66, 89.31, 41.22 and 12 %) with the concentrations of 50, 25, 12.5 and 6.25 mg/ml, respectively (Table 4). The results indicated that at (100 mg/ml) the hot C extract solution recorded as MBC for both *K. pneumonia* and *S. pyogenes*. The lowest dilution tested (6.25 mg/ml) was found to be the MIC for both bacteria.

Table 4
Inhibition percentage of *S. pyogenes* and *K. pneumonia* using serial dilutions of hot C extract

Dilutions	6.25 mg/ml	12.50 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Tested bacteria			Inhibition (%)		
S. pyogenes	4.99	26.35	59.60	94.70	100
K. pneumonia	12	41.22	89.31	98.66	100

According to Idriss *et al*., [4] when the ratio MBC/MIC is less than 4, this indicates that the extract has bactericidal effect, if the ratio is more than or equal to 4, that means the extract is bacteriostatic. The present study showed that the tested extract is bacteriostatic, in contrast to the acetic acid extract in Idriss's study was bactericidal against other tested bacteria *S. aureus, B. cereus, E. coli, S. enterica and P. aeruginosa*. In this current study a concentration of 100 mg/ml was as MBC for *K. pneumonia*, while costus extracts had a minimum concentration of 150 mg/ml, and a MBC of 200 mg/ml against *K. pneumonia* isolated from the respiratory tract in a study conducted by Othman [23]. Results from Hasson *et al*., [8] found that the MIC and MBC of *Saussurea lappa* ethanolic extract against *K. pneumonia* were as 6 mg/ml, which agreed with MIC of current study extract (6.25 mg/ml) against the same bacteria. According to the recent study, the MIC of hot ethanolic C extract was found to be 6.25 mg/ml against *S. pyogenes,* which is lower than the MIC (50 mg/ml) discovered for gram-positive *S. fecalis* when using the methanolic extract of costus. Additionally, the present study extract C were effective against *K. pneumonia* in comparison to the study conducted by Ahmed and Coskun, [16] which found complete resistance by *K. pneumonia* against the tested methanolic extract at a concentration of 200 mg/ml. As shown in the above results, the extract MBC against *S. pyogenes* was lower than the MBC of red ginger ethanolic extract which was 200 mg/ml by Assegaf *et al*., [24] against the same bacteria.

4. Conclusions

The extracts of root samples from Libya, Egypt and Turkey inhibited the growth of tested pathogenic bacteria. Microtiter plate assay proved its accuracy in evaluation of the antibacterial activity of pathogenic bacteria compared to other methods. The extraction of the active compounds from the active extracts is suggested, including purification and evaluating them against multi antibiotic resistant pathogenic bacteria.

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