

Antimicrobial Activity of Costus Plant Extract Against Methicillin-Resistant *Staphylococcus aureus* (MRSA, I₃)

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Abstract: In the present investigation, a trial was carried out to find biological active agents isolated from *Costus speciosus* to control the resistance that pathogenic microorganisms build against antibiotics. The rhizomes of two costus varieties viz. Qust Hindi and Qust Bahri were carried out. *Costus speciosus* were dried, grinded and extracted with methanol and water. The extracts were tested for their antimicrobial activities against MRSA, I₃, *E.coli*, I₅ and *Candida albicans* isolated from King Fahd Hospital, Al Madinah Al Munawarah, KSA. The aqueous extract of Qust Hindi was more effective compared with methanolic extract and aqueous extract of Qust Bahri against MRSA, I₃ & *E.coli*, I₅. *Candida albicans* was resistant to all plant extracts. Some parameters controlling the activity of this aqueous extract had been studied against pathogenic microorganisms under study. The highest antimicrobial activity obtained at the following conditions: 121 °C; pH 5; shaking at light conditions; 200 rpm (48 h); lactose (1%); non of nitrogen sources; cysteine; non of metallic ions; ascorbic acid (100ppm). Treatment of coleoptiles sections with crude *Costus speciosus* extract exhibited 20% of straight growth of *Hordeum vulgare* coleoptiles. Among all the tested solvent systems, ethyl acetate was found the best solvent for extraction of the antimicrobial substance. Fractions 3, 4, 5, 6, 7, 8 & 9 obtained from column chromatography exhibited the highest antimicrobial activity against MRSA, I₃. TLC plates showed only two colored fractions, fraction I showed pale yellow under normal lab light, and pale fluorescent turquoise color under long wave length, and fraction II gave dark yellow under both normal and long wave length. Fraction I and fraction II gave antimicrobial activity of 25.5 and 16.25mm, respectively against MRSA, I₃.

Keywords: *Costus speciosus*, Qust Hindi, Qust Bahri, MRSA, *E.coli*, *Candida albicans*

1. Introduction

Phytochemicals with biological activity have had great utility as pharmaceuticals. Through the 19th century and into the first half of the 20th century, the primary strategy was to discover of plant compounds and determining the active ingredients of plants (Duke, 1991; Pachlatko, 1998).

In fact, individual plant species may contain over one thousand chemical substances and only a minor fraction of the estimated total of 250,000 to 300,000 plant species has been studied for biomedical application (Tringali, 2001).

The need for new and useful compounds is ever-growing. Drug resistance in bacteria, the appearance of new life-threatening viruses, the recurrent problems of diseases in persons with organ transplants, and the tremendous increase in the incidence of fungal infections in the world's population all underscore our inadequacy to cope with these medical problems (Kayaser and Quax, 2007).

Multiple drug resistance has developed due to indiscriminate use of existing antimicrobial drugs in the treatment of infectious diseases (Service, 1995). In addition to this, antibiotics are sometimes associated with adverse effects. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of the infectious diseases from other plant resources (Cordell, 2000).

Moreover, much attention has been paid to extracts biologically active compounds from plant species (Essawi and Srour, 2000). The use of herbal preparations made from

medicinal plants is widespread in developing countries (Okpuzor and Oloyede, 2009). The healing powers of traditional herbal medicines have been realized and about 65% of the world populations have access to local medicinal plant knowledge system (Tag *et al.*, 2007).

However, Jigna *et al.* (2005) support the folkloric usage of the medicinal plants and suggest that, some of the plant extracts possess compounds with antimicrobial properties that can be further explored for antimicrobial activity.

Hazrat Anas Bin Malik narrates that Rasool Allah (saw) stated " Out of those things which are being used by you for treatment, the cupping and Qust Bahri are the best treatment." (Bukhari)¹. Narrated Um Qais: I went to Allah's Apostle along with a son of mine whose palate and tonsils I had pressed with my finger as a treatment for a (throat and tonsil) disease. The Prophet said, "Why do you pain your children by pressing their throats! Use Ud Al-Hindi (certain Indian incense) for it cures seven diseases, one of which is pleurisy. It is used as a snuff for treating throat and tonsil disease and it is inserted into one side of the mouth of one suffering from pleurisy." (Bukhari)²

Kust, Qust or costus root is the dried rhizome of *Costus speciosus* (Hansel *et al.*, 1994). The plant of Qust, which is known as costus is 2-2.7 meter in length. It is generally found in the marshy and wet places along the rivers at the heights up to 5000 fts. These plants are found extensively in north west and north east sub Himalayan regions along the course of rivers. Plant roots are being used for medicinal

purpose (Mandal, 2007; Anonymous, 2007; Gupta *et al.*, 2008; Khan, 2009).

About 150 species of *Costus speciosus* (Koen.) Sm. (family: Costaceae) have been reported from the tropical regions of the world (Deb, 1983). The rhizomes are useful in curing ailments like burning sensation, constipation, fever, inflammation, worm infection and diseases like leprosy, asthma, bronchitis and anaemia (Mandal, 2007).

Traditionally, Costus rhizomes are indicated in the treatment of cough, fever, skin diseases, snake bite, anemia and inflammation. The juice of fresh tips of young branches is instilled in case of otitis (Dutta and Dutta, 1998; Nehete *et al.*, 2010).

Moreover, *Costus speciosus* was indicated in the case of abortion, anasarca, anemia, arthrosis, asthma, bite, bleeding, blister, bronchosis, burn, cancer, catarrh, childbirth, cholera, cold, constipation, cough, cramp, dermatosis, dysentery, dyspepsia, fever, gastrostis, gravel, headache, hematuria, hiccup, inflammation leprosy, lumbago, malaria, ophthalmia, osteosis, pain, phthisis, pneumonia, rabies, rheumatism, scabies, smallpox, snakebite, stomatosis, swelling, syphilis, thirst, tuberculosis, water retention and worm (Duke *et al.*, 2002). Recently, Revathy *et al.* (2014) reported that, *Costus speciosus* enhances insulin secretion by the islets of langerhans, enhances peripheral glucose utilization and increases serum protein levels. Wide numbers of phytochemical constituents have been isolated from the plant and should be studied more extensively for its therapeutic effects (Srivastava *et al.*, 2011 and 2013). The successive extracts of *Costus speciosus* rhizome have revealed the presence of alkaloids, flavanoids, cardiac glycosides, saponins, sterols and tannins (Buwa and Staden, 2006; Saraf, 2010).

The main constituent is: diosgenin which is the major constituent isolated from *Costus speciosus* (Dasgupta and Pandey, 1970; Yanyong and Yongqing, 1981; Sulakshana *et al.*, 2014). The maximum quantity of diosgenin reported in the stem is 0.65%, in the leaves 0.37% and in the flowers 1.21% (Anonymous, 2007). Other constituents isolated are Tigogenin, dioscin, gracillin β -sitosterol glucoside (Gupta *et al.*, 2008). In addition, Hansel *et al.* (1994) elucidated that, the most active compounds present in *Costus speciosus* rhizome are Steroid saponins (1 to 4%): chief components dioscin and gracillin, aglycones diosgenin, tigogenin; Steroids: sterols, including beta-sitosterol, beta-sitosterol glucoside and Curcuminoids (3 %): including curcumin.

Diosgnin is extensively used as a raw material for the synthesis of important drugs such as corticosteroids and oral contraceptives. The rhizomes of *Costus speciosus* (Koen) Sm are well known for their diosgnin content and also for several saponine (Singh and Thakur, 1982). In addition, Khare (2007) reported that, all parts of the costus plant yield steroidal saponin, diogenin (quantity varies from 0.32-4.0%).

It has been reported that, *Costus speciosus* has many antimicrobial agents against different bacteria such as *Escherichia coli*, *Salmonella enterica* and *Staphylococcus*

aureus (Francis *et al.*, 2002; Chen *et al.*, 2008; Duraipandiyar *et al.*, 2012). Ariharan *et al.* (2012) evaluated *Costus speciosus* for antibacterial activity against pathogenic strains of Gram positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*) and Gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*) bacteria.

Moreover, Malabadi (2005) explained that the hexane, methanol and aqueous extracts of leaf and rhizomes of *C. speciosus* were used for its antibacterial activities against pathogens isolated from infected burn patients (*Shigella*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas*, *Bacillus subtilis* and *Salmonella*). No antibacterial activity was recorded with water extracts. The disc-diffusion method showed significant zone of lysis against all the pathogens studied. The in vitro antibacterial activity was performed against a few pathogens viz. *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Saraf, 2010).

Al-Kattan (2009) indicated that, *Costus speciosus* of India (lute), has an antimicrobial activity against microorganisms that infect the respiratory tract, including fungi viz. *A.niger*, *A.fumigatus* and yeast viz. *Candida albicans*, so in accordance it can be used especially in the treatment of respiratory diseases of microbial origin.

Recently, Qiu *et al.* (2011) elucidate the effects of subinhibitory concentrations of costus oil on virulence factor production in *Staphylococcus aureus*. The data suggested that, costus oil may deserve further investigation for its potential therapeutic value in treating *Staph. aureus* infections.

2. Materials & Methods

2.1. Media Used

- Nutreint Agar Medium (NAM) (HiMedia, India). It was prepared as manufacturer's direction.
- Czapek Dox Agar (g/l). (Czapek's, 1902 and Dox, 1910). It was used as basal medium in physiological studies.

2.2. Types of *Costus speciosus* rhizome used

Two types of costus plant rhizomes were obtained from druggist in Al-Madina Al-Munawarah, KSA; one is known as Qust Hindi and the other is known as Qust Bahari.

2.3. Test organisms used:

MRSA, I3; *E.coli*, I5 and *Candida albicans* were previously isolated by Abo-Shadi *et al.* (2010) and Sidkey *et al.* (2011) from cases with post cesarean wound infections, Department of Maternity and Children, King Fahd Hospital, Al Madinah Al Munawarah, KSA.

2.4. Extraction of Plant Material

Aqueous extraction and methanolic extraction of plant material was carried out according to the method described by Nair *et al.* (2005) with some modifications.

2.4.1. Aqueous extraction

The costus rhizomes were washed thoroughly under running tap water and then oven-dried at a temperature of 40- 57°C for 5 hours. After that, the rhizomes were crushed and blended into powder using an electric blender; only 50g was added to 150 ml of distilled water (1:3) and soaked for 24 hour. Then, slurry is filtered, the filtrate was centrifuged (approximately 5000-8000 rpm) for 5-10 min for clarification. The supernatant was collected and divided into two parts, part was left at 4°C, and the other part was autoclaved at 121°C and 15 lbs pressure and then stored at 4°C. The final concentration of the aqueous extract was (1g/3ml, w/v) (Nair *et al.*, 2005).

2.4.2. Methanolic extraction

Air-dried powdered plant material (50gm) was soaked in 150 ml of 95% methanol (1:3) and kept for 24 hour. Plant extract was filtered through muslin cloth and centrifuged at 5000-8000 rpm for 5-10 min. The supernatant was collected and the solvent was evaporated. It was stored at 4°C in dark airtight bottle for further studies (Nair *et al.*, 2005).

2.5. Antimicrobial activity of *Costus speciosus* rhizome extract against MRSA, I₃

A loopfull of 24 hour of MRSA, I₃ was inoculated in 5 ml sterile saline solution and was adjusted to 28X10⁷ (cfu/ml), 10 µl was seeded in conical flask contains 100 ml nutrient agar medium at 45°C. The inoculated medium was poured into sterile Petri dishes. The assay was performed using diffusion method according to Kavenagh (1972).

2.6. Determination of the MIC of *Costus speciosus* rhizome extract against MRSA, I₃

The Minimum Inhibitory Concentration (MIC) was determined according to Drummond and Waigh (2000). Different concentrations of sterile *Costus speciosus* rhizome extract in sterile distilled water (100/0, 50/50, 25/75, 12.5/87.5, 6.5/95.5 and 0/100) were tested against MRSA, I₃. The mean diameter of inhibition zone measured in mm.

2.7. Parameters controlling the growth of MRSA, I₃ in relation to aqueous *Costus speciosus* (Hindi) rhizome extract

The following parameters were carried out to maximize the antimicrobial effect of costus extract against MRSA, I₃. After determining each parameter, the best result was applied in the subsequent parameters. Triplicates were used for each particular parameter. The antimicrobial activity was assayed. The mean diameter of inhibition zone as well as standard deviation was calculated.

2.7.1. Incubation Temperature

In this respect, aqueous *Costus speciosus* rhizome extract was incubated at different temperatures (0, 30, 40, 70 & 100 °C) at different incubation periods (2, 6, 12 and 24 hr.), also autoclaving of extract at 121°C was carried out. This experiment was constructed to study thermal stability of *Costus speciosus* rhizome extract.

2.7.2. Initial pH's

Aquous *Costus speciosus* rhizome extract was incubated at different pH's (1, 3, 5, 7, 9 and 13) for several time intervals (1/2, 2, 6, 12 and 24 h). This experiment was constructed to study pH stability of *Costus speciosus* rhizome extract.

2.7.3. Dark and light

In this experiment, sterile aqueous *Costus speciosus* rhizome extract was exposed to dark, lab light and direct sun light for time intervals 24 and 48 hr.

2.7.4. Static and shaking

Sterile aqueous *Costus speciosus* rhizome extract was subjected for shaking at 0.0, 100 and 200 rpm for 24 and 84 hr.

2.7.5. Different carbon sources

This trial was aimed to determine the antimicrobial activity of aqueous *Costus speciosus* rhizome extract in combination with different carbon sources. Carbon sources were added at 1% to crude extract. Different carbon sources were used viz. D-glucose, D-fructose, D-maltose, D-lactose, soluble starch and carboxy methyl cellulose. All carbon sources were sterilized with diethyl ether and left over night until diethyl ether was evaporated completely, then they were added to sterile aqueous costus extract (0.01g/1ml).

2.7.6. Different nitrogen sources

For such purpose the following nitrogenous compounds were supplemented to aqueous *Costus speciosus* rhizome extract such as sodium nitrate, sodium nitrite, ammonium nitrate, ammonium chloride, ammonium phosphate, ammonium sulphate and peptone. These nitrogen sources were added in an equimolecular amount to Na NO₃ that present in Dox's agar medium, and sterilized by diethyl ether as previously mentioned.

2.7.7. Different Amino Acids

Twenty two different amino acids were used viz. (L-Lysine-HCl, L-Arginine-HCl, DL-Histidine-HCl, L-Ornithine, L-Aspartic Acid, DL-Glutamic Acid, L-Serine, L-Threonine, DL-Asparagine, DL-Glutamine, L-Tyrosine, L-Glycine, DL-Alanine, L-Valine, Leucine, L-Proline, L-Phenylalanine, L-Tryptophan, Methionine, Cysteine and L-Cystine). The amino acids were added in an equimolecular amount to that present in Dox's agar medium and sterilized by diethyl ether and added to sterile *Costus speciosus* rhizome extract. The antimicrobial activity was evaluated as usual.

2.7.8. Different Metallic Ions

Different metallic ions were used in this respect viz. MnSO₄, MgSO₄, ZnSO₄, NiSO₄ and CuSO₄ with different concentrations: 10, 50 and 100 ppm.

2.7.9. Different Vitamins and Growth Promoters

In this respect, different types of vitamins and growth promoters were used including thiamin, biotin, ascorbic acid, amino benzoic acid, adenosine, riboflavin, and inositol with three concentrations: 20, 50 and 100 ppm. The vitamin sources were separately sterilized by means of diethyl ether. The sterilized vitamin sources were added to sterilized aqueous *Costus speciosus* rhizome extract under aseptic conditions.

2.7.10. Effect of crude costus plant extract on straight growth of *Hordeum vulgare* coleoptiles

Hordeum vulgare grains were soaked at 28°C for about 3 hrs. The grains were then left to germinate at 28°C for 72 hr. Sections of 5mm in length were cut from coleoptiles of uniform length by sterile cutter. The coleoptile sections were placed into a dish containing filter papers wetted with crude *Costus speciosus* rhizome extract to be tested for its biological activity with regard to growth. The dishes were then incubated for 16 hr at 28°C, after which measurements of the length of the sections were carried out. The coleoptiles sections were measured. The mean length of 10 coleoptile sections for each Petri dish was calculated.

2.8. Extraction, Separation and Purification of the antimicrobial substance from *Costus speciosus* rhizome

2.8.1. Solvent for extraction of the antimicrobial substance from *Costus speciosus* rhizome

Different trials of organic solvents systems were used for extraction of the antimicrobial substance from *Costus speciosus* rhizome powder viz.:

- 1- Chloroform 2-Ethyl Acetate
- 3- n-Butanol 4- Diethyl ether
- 5- Chloroform + ethyl acetate (1: 1, v/v)
- 6- Chloroform and n-butanol (1: 1, v/v)
- 7- Ethyl acetate and n-butanol. (1: 1, v/v)

Five ml of each solvent was added to only 0.1 gm of *Costus speciosus* rhizome powder in a glass tube with cover, vortexed for 3min, and repeated for three subsequent days.

The top layer was transferred to small glass vial and about 10 µl was spotted on TLC plate. The organic phase was collected, evaporated under reduced pressure until viscous syrup was obtained. The residual syrup was dissolved in the least amount of solvent and filtered through Whatman No.1 filter paper. The antimicrobial activity of the separated agent was evaluated.

About 500gm of *Costus speciosus* rhizome powder was soaked in 1.5 liter of the best solvent with intervals shaking for three days. The solvent was evaporated under reduced pressure by using a rotary evaporator. The evaporation was continued until viscous brown syrup was obtained. The syrup was tested for its antibacterial activity against MRSA, I₃.

2.8.2. Column chromatography

A glass column of 2.5 × 50 cm diameter was used for such purpose. The column was packed with silica gel (Prolabo). A glass rod was often used to stir the slurry. Once the slurry get homogenous, it was poured cautiously into the empty column and the column was left overnight until the silica gel was completely settled. One ml of crude viscous brown syrup extract was added onto top of the silica. The elution gradients of different solvent mixtures (10 ml each) were added. Thirty fractions were collected (each of 5 ml). Antimicrobial activity was performed for each separate fraction.

2.8.3. Thin layer chromatography (TLC)

Separation of the antimicrobial agents into its individual components has been carried out. Thin layer chromatography

(TLC) plates were used (aluminium sheets silica gel 60 F₂₅₄ pre-coated 20x20 cm with a layer thickness 0.2 mm), E. Merck, Germany.

By using a solvent system composed of (55% ethyl acetate: 45% methanol: 10% butanol: 2% chloroform) which was found to be the best solvent system. Thin-layer chromatography was carried out by spotting 100 µl of active fractions obtained from column chromatography on silica gel plate 10x20cm by means of winCATS Planar Chromatography Manager "sample application CAMAG Linomat 5". Syringe size of 100 µ, spray gas is an inert gas, dosage speed 60nl/s. The 100 µl was spotted in the form of a line with a band length of 40.0 mm.

2.8.4. Detection of separated zones on TLC plates

After development of the plates in the best solvent, the plates were dried at room temperature. Separated zones were visualized in white normal light and UV at 254 nm and by UV at 365 nm (Smith and Seakins, 1976 and Balbaa *et al.*, 1981). The antimicrobial activity against MRSA, I₃ was tested by scraping off the separated zones and studying their activities.

3. Results & Discussion

Historically, *Staphylococcus aureus* has been recognized as an important cause of disease worldwide. This has become a major pathogen associated with both hospital- and community-acquired infections (Panlilio *et al.*, 1992). Moreover, *S. aureus* is uniquely equipped with virulence factors and defense mechanisms that could cause rapidly progressive fatal infections (Weigelt, 2007).

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were resistant to all β-lactam antibiotics. Moreover, MRSA were identified soon after methicillin was introduced into clinical practice (Panlilio *et al.*, 1992). MRSA has become a concern in hospitals worldwide because it can cause bacteremia, pneumonia, surgical site infections, and other nosocomial infections (Simor *et al.*, 2001; Stefani and Varaldo, 2003; Cuevas *et al.*, 2004; and Kuehnert *et al.*, 2005).

Searching for new efficient and cost effective ways for the control of infectious diseases is necessary, as a result of increasing antibiotic resistance of microorganism to conventional drugs, (NCCLS, 2000; Kone, *et al.*, 2004; Hidayathulla *et al.*, 2011). In general, most of the medicinal plants are regarded as "Chemical Goldmines" (Thomas, 1997).

Historically, most of the medicinal preparations were derived from plants. There are a huge number of drugs that are developed from plants, and found to be very active against some diseases (Fabricant and Farnsworth, 2001). The use of complementary and alternative medicines has increased. This has led to enhancing the market for herbal products worldwide (Bodeker and Kronenberg, 2002).

Costus speciosus is the only species in the genus *Costus* that is medicinally important. Moreover, because of the diosgenin content, the genus *Costus* has become very valuable

(Thomas, 1997). Many different pharmacological activities are attributed to *Costus speciosus* like hepatoprotective, antioxidant, antidiabetic, hypolipidemic, and antimicrobial activity (Srivastava *et al.*, 2011). *Costus speciosus*, serves as an important source of many therapeutically efficient chemicals, like diosgenin, steroidal saponins like prosapogenin, α & β of dioscin, furostanol saponins like costusoside I & J, octasanoic acid, cycloartenol and various other constituents (Srivastava *et al.*, 2011).

The current study was concentrated on the antimicrobial activity of *Costus speciosus* rhizomes extract against resistant pathogenic microorganism viz. MRSA, I₃ in comparison with *E.coli*, I₅ and *C. albicans*.

3.1. Antimicrobial activity of aqueous and methanolic extract of *Costus speciosus* rhizome (Two Varieties)

The production of herbal drug preparations (i.e. extracts), using a variety of methods from simple traditional technologies to advanced extraction techniques is the first step in the value addition of medicinal and aromatic plants bioresources (Hanada *et al.*, 2008). In the present investigation, extraction of *Costus speciosus* rhizome with methanol and water was carried out. Two variants of Qust was used viz. Qust Hindi and Qust Bahri. The resistant pathogenic isolates MRSA, I₃ and *E.coli*, I₅ showed susceptibility to aqueous extracts for both Qust Hindi and Qust Bahri. However, *Candida albicans* was resistant to costus plant extracts. MRSA, I₃ was more susceptible than *E.coli*, I₅.

Aqueous extract of Qust Hindi was more effective as antimicrobial agent than Qust Bahri in the case of MRSA, I₃ and *E.coli*, I₅. No bactericidal effect was recorded in case of *E.coli*, I₅, and only bacteriostatic effect. A zone of bacteriostatic inhibition was noticed around a bactericidal inhibition zone with a diameter 41mm for sterile and 34.5mm for non sterile Qust Hindi against MRSA, I₃. Qust Hindi was found to be activated after autoclaving treatment, this indicated that high temperature activate the antimicrobial activity of aqueous Qust Hindi extract (Table 1 and Fig. 1).

In view of the findings of other workers, water is almost shown as the practical solvent that used to extract activity in different ways (Thomson, 1978; Brantner and Grein, 1994). In addition, Tiwari *et al.* (2011) indicated that, the traditional method of treating a bacterial infection was by administering a decoction of the plant or apart there by boiling it in water.

Moreover, Hidayathulla, *et al.* (2011) reported that, the higher degree of solubility of the active principle in the polar solvents such as water and methanol as higher antibacterial activity was recorded in the polar solvent extracts compared to the non polar solvent extracts.

Based on the findings of a number of studies, *Costus speciosus* has many antimicrobial agents against different bacteria such as *Escherichia coli*, *Salmonella enterica* and *Staphylococcus aureus* (Francis *et al.*, 2002; Chen *et al.*, 2008; Thambi and Shafi, 2015)

Table 1: Antimicrobial activity of sterile and non sterile aqueous extract of *Costus speciosus* rhizome (two variants) against test organisms

Test organisms	State	Inhibition Zone (mm)			
		Q.H (Sterile)	Q.H (Non sterile)	Q.B (Sterile)	Q.B (Non sterile)
MRSA, I ₃	Bactericidal	22±0.28	15.5±0.07	16±0.07	15±0.07
	Bacteriostatic	41±0.14	34.5±0.65	36±0.14	35.5±0.21
<i>E.coli</i> , I ₅	Bactericidal	-	-	-	-
	Bacteriostatic	31±0.0	27±0.07	28±0.07	29±0.14
<i>C. albicans</i>	Bactericidal	-	-	-	-
	Bacteriostatic	-	-	-	-

Where the diameter of cork borer = 11mm
 Q.H= Qust Hindi Q.B= Qust Bahri - = No inhibition zone

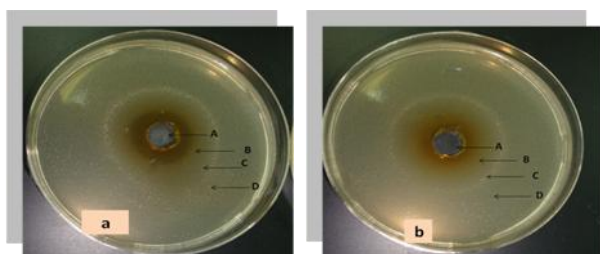


Figure (1): Antimicrobial activity of both (a) sterile and (b) non sterile aqueous extract of Qust Hindi on MRSA, I₃.
 A: Qust Hindi (sterile by autoclaving (a) and non sterile (b))
 B: Bactericidal Zone C: Bacteriostatic Zone D:MRSA, I₃ growth

Moreover, in complete accordance with our results, Saraf (2010) and Swarnkar and Katewa (2009) indicated that, the

aqueous extracts of *Costus speciosus* only appear to have antibacterial activity against *Staphylococcus aureus*.

Table 2: Antimicrobial activity of methanolic extract of *Costus speciosus* rhizome (two variants) against test organisms

Test organisms	State	Inhibition Zone (mm)	
		Methanolic ex of Q.H	Methanolic ex of Q.B
MRSA, I ₃	Bcateriocidal	14.5±0.07	-
	Bacteriostatic	35.5±0.07	24.5±0.07
<i>E. coli</i> , I ₅	Bactericidal	-	-
	Bacteriostatic	30.5±0.21	27±0.07
<i>C. albicans</i>	Bactericidal	-	-
	Bacteriostatic	-	-

Where the diameter of cork borer = 11mm
 Q.H= Qust Hindi Q.B= Qust Bahri - = No inhibition zone

Data represented in table (2) showed that, a bactericidal and bacteriostatic effects were exhibited in case of methanolic extract of Qust Hindi against MRSA, I₃ (14.5mm and 35.5mm, respectively). Moreover, in terms of methanolic extract of Qust Hindi against *E.coli*, I₅ only bacteriostatic effect was obtained. In addition, no inhibition zone detected in the case of methanolic extract of Qust Bahri with *E.coli*, I₅. When *Candida albicans* was studied, no inhibition zone was detected with both variants. In fact, the weakened effect of methanolic extract compared with aqueous extract of Qust may be explained by the presence of other constituents extracted with methanol exerting antagonistic effects or negating the positive effects of the bioactive agents, or it may be the solvent used is not the appropriate one. **Cowan (1999)** reported that, most commonly used solvents were ethanol and methanol, both used as initial extractants in approximately 35% of the studies. However, ethanol and methanol may not have the greatest sensitivity in producing antimicrobial chemicals on an initial screening.

Vijayalakshmi (2008) revealed that methanolic extract of *costus speciosus* rhizomes possess antioxidant and antimicrobial activity.

3.2. Determination of MIC of the antimicrobial agent of aqueous *Costus speciosus* rhizome (Qust Hindi) extract against the growth of MRSA, I₃

Results illustrated in Table (3) showed the minimum inhibitory concentration (MIC) of aqueous *Costus speciosus* rhizome extract (Qust Hindi) against the growth of MRSA, I₃. Data declared that, inhibition zone increased by elevating the concentration of the extract. The minimum inhibitory concentration was (166 mg/ml) against MRSA, I₃.

Table 3: Minimum inhibitory concentration (MIC) of aqueous *Costus speciosus* rhizome (Qust Hindi) extract against MRSA, I₃.

<i>Costus speciosus</i> rhizome extract (mg/ml)	Inhibition zone (mm)
333	22.25±0.28
166	16.5±0.07
83	0
41.5	0
20.8	0
0.0	0

3.3. Parameters controlling the growth of MRSA, I₃ by aqueous *Costus speciosus* rhizome (Qust Hindi) extract

For the purpose of improving the activity of aqueous extract of *Costus speciosus* rhizome as an antimicrobial agent, different parameters were studied.

Results recorded in Table (4) showed that, by increasing the incubation temperature of aqueous extract of Qust Hindi up to 40, 50 and 70°C, the antimicrobial activity against MRSA, I₃ does not affected or it may be fluctuated. But, at 100°C, the antimicrobial activity against MRSA, I₃ increased with time. The same result was obtained with autoclaving. This may be due to the antimicrobial agents obtained from Qust Hindi stimulated by heat.

Table 4: Antimicrobial activity of aq. *Costus speciosus* rhizome (Qust Hindi) extract against MRSA, I₃ in relation to different incubation temperatures and time intervals

Temp.(°C)	Antimicrobial activity (mm)				
	Time intervals (h.)				
	1/3	2	6	12	24
0		35.3±0.09	35.3±0.09	35.3±0.09	35.3±0.09
30		34±0.28	34±0.14	35±0.141	39±0.141
40		35±0.14	35±0.07	35.5±0.21	34.50.07
70		38±0.0	36±0.14	35.0±0.07	36±0.0
100		39.5±0.07	39.5±0.07	41±0.141	41.5±0.21
Autoclaving 121°C	41±0.14				

From the historical point of view the traditional method of treating a bacterial infection was by administering a decoction of the plant or apart there by boiling it in water (**Saraf, 2010**).

On the other hand, the effect of different pH's on the antimicrobial activity of aqueous extract of Qust Hindi against MRSA, I₃ was tested, the results revealed that pH 5 (the original pH of Qust Hindi) gave the least results. The acidic and alkaline pH exhibited activation in the antimicrobial activity of Qust Hindi against MRSA, I₃ (Table 5). No available literature was found concerning this point.

Table 5: Antimicrobial activity of aqueous *Costus speciosus* extract (Qust Hindi) against MRSA, I₃ in relation to different pH's and time intervals

pH	Antimicrobial activity (mm)			
	Time intervals (h)			
	2	6	12	24
1	39.5±0.07	42.5±0.07	42±0.28	40±0.0
3	41.5±0.07	44.5±0.07	45±0.0	44±0.14
5	40±0.0	40±0.0	40±0.0	40±0.0
7	44.5±0.07	45±0.0	45±0.0	44.5±0.07
9	44±0.14	45.5±0.0	45±0.0	45±0.0
13	45.5±0.21	45±0.0	45.5±0.21	44±0.14

Concerning the effect of dark and light on aqueous extract of Qust Hindi against MRSA, I₃, laboratory light and direct sun light showed more activation in the antimicrobial activity comparing with dark (Fig. 2). Direct sun light gave the highest antimicrobial activity, this may be due to heat radiation of sun.

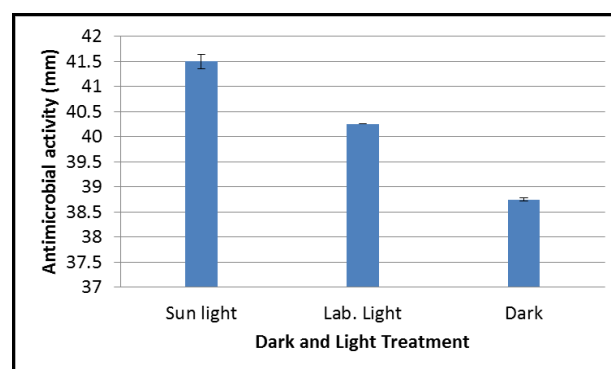


Figure 2: Antimicrobial activity of aqueous *Costus speciosus* extract (Qust Hindi) against MRSA, I₃ in relation to dark and light

Also, increased the shaking up to 200 rpm of aqueous extract of Qust Hindi showed pronounced antimicrobial activity against MRSA, I₃ (43.25mm). This may be due to shaking could facilitate the release of the active substance in the water (Fig. 3).

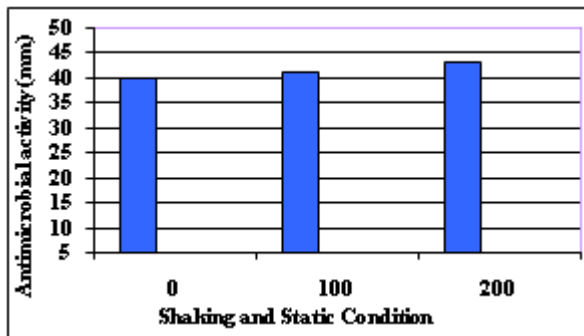


Figure 3: Antimicrobial activity of aqueous *Costus speciosus* extract (Qust Hindi) against MRSA, I₃ in relation to shaking and static

In the present investigation, different carbon sources were used in order to determine the best one which increased the antimicrobial activity of aqueous extract of Qust Hindi against MRSA, I₃. Results represented graphically in fig.(4) showed that, lactose, and carboxy methyl cellulose (CMC) exhibited the highest antimicrobial activity against MRSA, I₃. They may induce synergetic activity with costus extract. Lactose was added to aqueous costus extract in the next step.

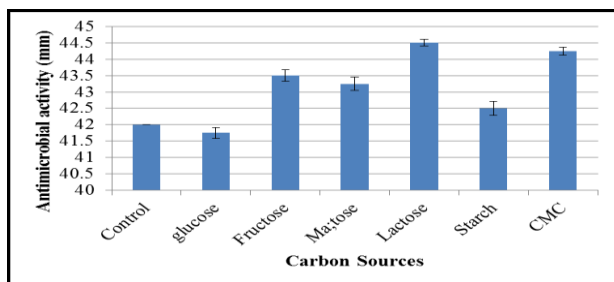


Figure 4: Antimicrobial activity of aqueous *Costus speciosus* extract (Qust Hindi) against MRSA, I₃ in relation to different carbon sources

Data represented in Fig. (5) indicated that, none of the used nitrogen sources (organic or inorganic) induced any further antimicrobial activity against MRSA, I₃ compared with control when added to the aqueous costus plant extract. Therefore, it is suggested not to add any nitrogenous sources in combination with costus extract.

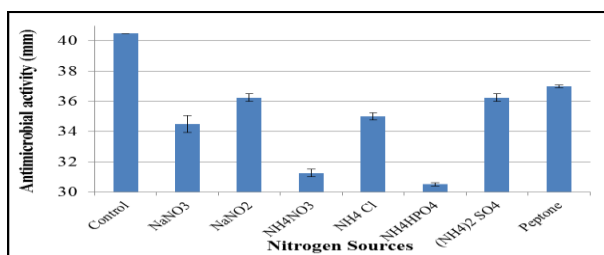


Figure 5: Antimicrobial activity of aqueous *Costus speciosus* extract (Qust Hindi) against MRSA, I₃ in relation to different nitrogen sources

Data in fig.(6) indicated that, cysteine followed by arginine, proline and asparagine in combination with costus plant extract exhibited the highest antimicrobial activity against MRSA, I₃ while, amino acids alone have no additional results compared with control.

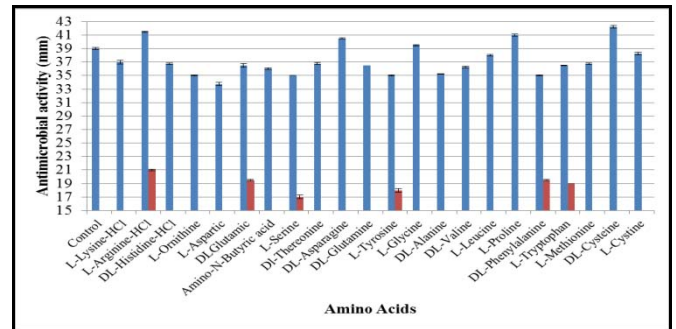


Figure 6: Antimicrobial activity of aqueous *Costus speciosus* extract (Qust Hindi) against MRSA, I₃ in relation to different amino acids

Cysteine is a non-essential amino acid and is regarded as an important structural and functional component of many proteins and enzymes. It is also known as antioxidant agent and its antimicrobial effect has been demonstrated (Riise *et al.*, 1994; Gunduz *et al.*, 2003 and Gurbuz *et al.*, 2005). Also, according to Xiaoqian *et al.* (2011), cysteine has been found to inhibit biofilm formation significantly in *S. aureus*.

By using different metallic ions with various concentrations (10, 50 & 100 ppm) in combination with costus extract resulted in sharply inhibition in the antimicrobial activity against MRSA, I₃ comparing with control. Thus, the addition of any metallic ions must be restricted and avoided.

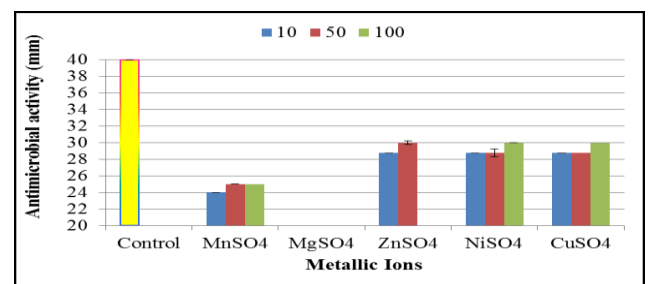
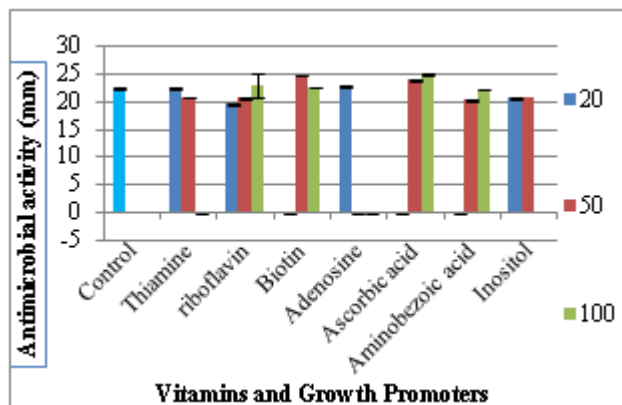


Figure 7: Antimicrobial activity of aqueous *Costus speciosus* extract (Qust Hindi) against MRSA, I₃ in relation to different metallic ions

Data represented graphically in fig. (8) indicated that different vitamins with various concentrations in combination with costus extract had stimulatory effects on the growth of MRSA, I₃. Ascorbic acid at 100 ppm followed by biotin at 50ppm exhibited the highest antimicrobial activity against MRSA, I₃. This finding is in agreement with the fact that ascorbic acid had efficient antimicrobial properties (Shaista *et al.*, 2009). Ascorbic acid has been used to extend the shelf life of various fruits and vegetables (Santerre *et al.*, 1991 and Sapers and Miller, 1995). The results in the present study are supported by the fact that ascorbic acid may enhance or stabilize the flavonoids that are responsible to disrupt the microbial membrane (Tsuchiya *et al.*, 1996).



Where: Control (*Costus* extract + lactose + Cysteine)

Figure 8: Antimicrobial activity of aqueous *Costus speciosus* extract (Qust Hindi) against MRSA, I₃ in relation to different vitamins.

One possible therapeutic approach could be to add ascorbic acid, to appropriate antimicrobial agents, which could work synergistically with the antimicrobial agents against pathogenic resistant microorganisms (Cursino *et al.*, 2005). Amabile-Cuevas and Heinemann (2004) suggested that high doses of ascorbic acid in combination with antimicrobial agents may stimulate the loss of R plasmids and affect the levels of antibiotic resistance in *Staphylococcus*.

3.4. Separation & purification of the active antimicrobial substance from *costus speciosus* rhizome

In the present investigation, the main focus was to provide a more standardized extraction method for the extraction of the antimicrobial agent from *costus* plant rhizome. Among all the tested solvents, ethyl acetate was the best solvent of extraction. The inhibition zone of ethyl acetate extract against MRSA, I₃ was 21 mm (Table 6 and Fig. 9).

Cooper and Kronenberg (2009) reported that methods used in extraction of the plant material can influence the chemical composition of the extracts and potentially the biological activity is not new. The more information on the product that is provided in research, the greater will be the ability to compare among studies and understand differences in results that may emerge. In addition, Thomas (2003) reported that, when screening a material contains an active compound, the problem becomes one of extraction, purification and assessment of the pharmacological activity.

Eloff (1998) ranked of the solvents which are reported in the literatures with the highest frequency for the extraction and their ability to solubilize antimicrobials from plants, and the ease of removal of solvent from the fraction. The author ranked them in the order of methylene dichloride, methanol, ethanol, and water.

Recently, Nehete *et al.* (2010) indicated that, the dried rhizomes of *Costus spicuosus* were grounded and subjected to successive extraction using Soxhlet apparatus. Petroleum ether (60:80), cyclohexane, benzene, ethyl acetate, chloroform, acetone, methanol and water were significantly applied for extractions used in sequence.

In addition, Binny *et al.* (2010) reviewed that, the ethanolic extract of the rhizome of *Costus speciosus* possesses anti-inflammatory and antipyretic properties. Moreover, Verma and Khosa (2009) evaluated the effect of alcoholic extracts of *Costus speciosus* rhizomes. *Costus speciosus* hexane extract and its compounds have an antihyperglycemic action, and are able to ameliorate the diabetic state, representing as an alternate therapy for diabetes (Eliza *et al.*, 2010).

Furthermore, Niño *et al.* (2011) suggested that the *n*-hexane extract from *Costus speciosus* is a great natural antioxidant (antioxidant activity 48%), and this activity could be explained based on its saponin contents, according to the phytochemical screening.

Table 6: Extraction of antimicrobial substance from *costus speciosus* rhizome by different solvent systems

Solvent Used	Antimicrobial activity in terms of mean diameter of inhibition zones (mm)
1- Chloroform	0.0
2- Ethyl Acetate	21.0 ± 0.0
3- n-Butanol	0.0
4-Diethyl ether	0.0
5- Chloroform + ethyl acetate	12.0 ± 0.0
6- Chloroform + n-butanol	0.0
7- Ethylacetate + n – butanol	0.0

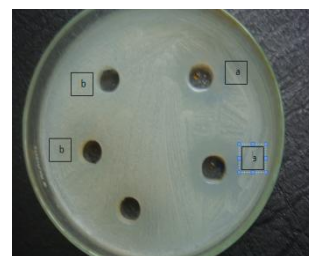


Plate (9): Antimicrobial activity of *Costus speciosus* rhizome powder against MRSA, I₃ (a) extracted with ethyl acetate and (b) ethyl acetate disc as a control

In the present investigation, the antimicrobial substance was purified by column chromatography packed with silica gel and the eluting solvent was composed of ethyl acetate, methanol and butanol in gradient manner. Thirty fractions were collected and each was 5 ml. All fractions were tested against the target organism MRSA, I₃ by using agar diffusion method. Concerning the active fractions eluted from the column chromatography, fractions 3, 4, 5, 6, 7, 8, &9 were found the most active fractions. Fractions 7&8 exhibited the highest antimicrobial activity with 28.6 and 27.25 mm, respectively. On the other hand, fractions 1, 2 and 10-30 didn't exhibit any antimicrobial activity. Moreover, most of the active fractions showed different colors, from dark yellow to deep brown color (Fig 10, 11, 12 & 13).

In view of the findings of other workers, column chromatography packed with silica gel and an eluting solvents composed of various ratios of solvent system was used to fractionation of the crude extract into active fractions (Kumigiri *et al.*, 1996; Tabata *et al.*, 1997; Imnagaki *et al.*, 1998; Momose *et al.*, 2001; El-Henawy, 2006; El-Tantawy, 2008 and Khalifa *et al.*, 2008).

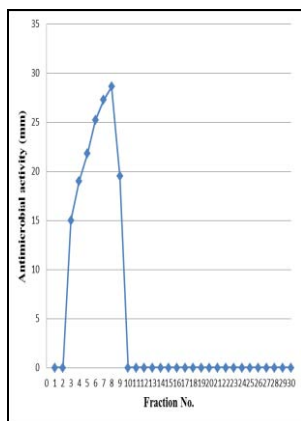


Figure 10: Fractionation pattern of active antimicrobial substances from *Costus speciosus* rhizome and their effect against MRSA, I₃.

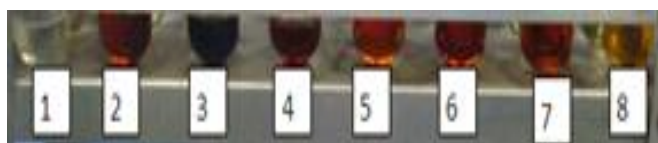
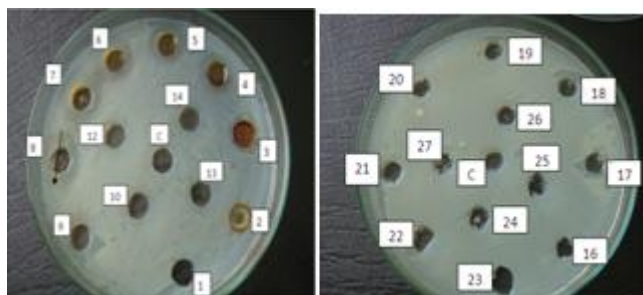


Figure 11: Colored separated active fractions, 3-9



Plates (12&13): Antimicrobial activity of column chromatography separated fractions and their effect against MRSA, I₃, where 3,4,5,6,7,8 &9, are the separated active fractions, and C is control (ethyl acetate).

The active fractions were applied on TLC plates in a trial to separate the active substances, the eluting solvent system was composed of 55% ethyl acetate: 45% methanol : 10% Butanol: 2% Chloroform. In all active fractions, only two areas were obtained; the first gave pale yellow color under normal lab light and pale fluorescent turquoise color under long wave length; the second gave dark yellow color under both normal lab light and long wave length (dominate in fractions 7 & 8). These two colored areas were detected in all active fractions, so it may play a role in the antimicrobial activity. The active fraction I exhibited antimicrobial activity against the test organism under study MRSA, I₃ and gave 25.5mm, while fraction II gave 16.25mm. (Table 7 and Fig. 14).

Table 7: Antimicrobial activity of the active fractions against MRSA, I₃.

Active fractions	Colour		Antimicrobial activity in terms of mean diameter of inhibition zones (mm)
	Normal lab light	Long wave length	
I.	pale yellow	pale fluorescent turquoise	25.5
II.	dark yellow	dark yellow	16.25



Plate (13): Antimicrobial activity of the active fractions against MRSA, I₃.

Although this data may be very important in probing of biochemistry of this plant in the future, there is a need to further carry out spectroscopic studies in order to elucidate the structure of these compounds.

Costus speciosus are known to contain saponins and sapogenins, costusoside I & J, dioscin, dioscin prosapogenin A & B, protodioscin, diosgenin and diosgenin derivatives, gracillin, tigogenin, cadinene, carvacrol, 1,8-cineol, methyl ester paracoumaric acid, cycloartanol, cycloartenol, 31-norcycloartanone, cycloaudenol, lanosterol, daucosterol, beta-sitosterol, stigmasterol, lipids, lauric acid, linoleic acid, myristic acid, oleic acid, palmitic acid, stearic acid, plastoquinone, and vanillic acid (WHO, 2009). Recently, Borkatky *et al.* (2014) reported that, the rhizome of *Costus speciosus* is a source of secondary metabolites like phenolics, flavonoids, tannins, saponins, steroids and glycosides which are reported for various biological effects including antimicrobial and antioxidant activities.

Recently, the oil was found to be active against four Gram positive bacteria namely *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus faecalis*, and *Staphylococcus albus* and Gram negatives namely *E.coli*, *Pseudomonas aeruginosa*, *Klebsiella aerogenes* and *Protieus vulgaris*. The activity may be due to the presence of individual components like α -Humulene and Zerumbone or due to the synergistic effect of the major and minor components (Thambi and Shafi, 2015).

In conclusion, an attempt has been made to evaluate one of the medicinal plants that have attracted considerable global interest in recent years. From the thorough study and investigation of the available literature on *Costus speciosus*, it is clearly found that the extract possess compounds with antimicrobial properties and serves as an important source for treating diseases caused by Methicillin-resistant *Staphylococcus aureus* (MRSA) strains. The rhizome of *C. speciosus* (Qust Hindi) has the potential for use in diet for nutritive and health benefits.

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