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GC–MS Characterization and Biological Evaluation of *Costus speciosus* Rhizomes: Antibacterial Activity and Mechanism of Action against MDR and XDR Bacteria with Anticancer Potential

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Abstract

The rapid emergence of multidrug-resistant (MDR) and extensively drug-resistant (XRD) bacteria represents a major global health challenge, necessitating the exploration of novel antimicrobial agents from natural sources. This study investigated the antibacterial potential *Costus speciosus* rhizome extracts and evaluated the cytotoxic activity of the most active fraction. Rhizomes were extracted using ethanol, methanol, ethyl acetate, and cold water. Antimicrobial susceptibility testing was performed on 29 bacterial isolates from clinical specimens and meat products to determine resistance profiles. Based on resistance patterns, 11 representative MDR/XDR isolates, including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia rubbidea*, and *Hafnia alvei*, were selected for further evaluation using agar well diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. The ethyl acetate extract showed the strongest antibacterial activity and was subsequently fractionated using column chromatography, yielding 43 fractions. Fractions were screened for antibacterial activity, and the most active fraction was further analyzed by gas chromatography–mass spectrometry (GC–MS). Transmission electron microscopy (TEM) was used to investigate the antibacterial mode of action, while cytotoxicity was assessed against HepG2, A-549, and WI-38 cell lines. The ethyl acetate extract showed the highest antibacterial activity, whereas the cold-water extract exhibited weak activity. Among the 43 fractions, nine displayed antibacterial activity, with fraction 4 exhibiting the strongest effect. GC–MS analysis of fraction 4 identified major compounds, including azuleno[4,5-b]furan-2(3H)-one (65.87%), dihydrodehydrocostus lactone (10.54%), and (Z,Z,Z)-1,8,11,14-heptadecatetraene, (9.91%). The MIC and MBC values ranged from 0.062 –1.5 mg/mL and 0.25 – 1.5 mg/mL, respectively. TEM revealed severe cellular damage, including cell wall disruption and cytoplasmic leakage. The active fraction demonstrated potent cytotoxic activity against HepG2 and A-549 cell lines, with IC_{50} values of 5.17 ± 0.49 and 8.27±0.61 µg/mL, respectively, and a CC_{50} value of 23.92±0.86 µg/ml against WI-38 cells, yielding selectivity indices (SI) of 4.63 and 2.89, respectively. In conclusion, the ethyl acetate fraction of *Costus speciosus* represents a promising source of antibacterial and anticancer agents against drug-resistant pathogens.

Keywords

Costus speciosus, MDR/XDR bacteria, bioactive fraction, GC–MS, antibacterial activity, anticancer activity

Introduction

Infectious diseases remain a major health problem, accounting for approximately 20% of deaths in the Americas and up to 50% of deaths in tropical regions. Despite advances in microbiology, sporadic outbreaks caused by drug-resistant bacterial pathogens continue to pose a significant threat **(Zouine, 2024).** The widespread and often inappropriate use of antimicrobial agents for the treatment of infectious diseases has contributed to the emergence and spread of antimicrobial resistance (AMR) among various bacterial species. According to the World Health Organization (WHO), antimicrobial-resistant microorganisms can survive exposure to antimicrobial agents, leading to treatment failure, prolonged infections, increased healthcare burden, and higher mortality rates. The growing prevalence of resistant pathogens has become a major global health concern, particularly among immunocompromised individuals. Notable examples include *Escherichia coli* resistant to cephalosporins and fluoroquinolones, *Klebsiella pneumoniae* resistant to cephalosporins, and methicillin-resistant *Staphylococcus aureus* (MRSA) **(Tanwar et al., 2014).**

Bacteria can develop multidrug resistance through various mechanisms, including antibiotic efflux, enzymatic inactivation of antibiotics, and modification of antibiotic targets. Furthermore, the acquisition and horizontal transfer of antibiotic resistance genes (ARGs), often clustered on mobile genetic elements, facilitate the dissemination of multidrug resistance among bacterial pathogens, posing significant challenges to the effective treatment of infectious diseases **(Syame 2020; Belay, 2024)**.

Multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacterial represent a growing problem worldwide. Their ability to withstand multiple classes of antimicrobial agents is attributed to diverse resistance mechanisms and the acquistion of resistance determinants. Consequently, infections caused by these pathogens are associated with treatment difficulties, prolonged disease duration, and increased clinical burden **(Samy et al., 2022)**.

According to a study published in *The Lancet*, more than 100,000 deaths worldwide were attributed to methicillin-resistant *Staphylococcus aureus* (MRSA) in 2019 alone. Since this strain was initially identified in healthcare settings, it is commonly referred to as hospital-acquired MRSA (HA-MRSA) or healthcare-associated MRSA. The infection can be transimitted through direct contact with contaminated hands, surfaces, or infected wounds **(Alghamdi et al., 2023).**

The limited availability of effective antibiotics, coupled with inadequate preventive measures and the slow development of new antibiotics, underscores the urgent need for alternative antimicrobial therapies **(Frieri et al., 2017).** With the rapid advancement of synthetic chemistry, many conventional herbal remedies have been replaced by synthetic drugs. Nevertheless, medicinal plants continue to attract considerable scientific interest owing to their therapeutic potential and long history of use in traditional medicine. Given their rich diversity of bioactive compounds, medicinal plants represent a promising source for the discovery of novel agents against pathogenic microorganisms **(Mirpour et al., 2015)**.

Recent advances in phytochemical research have demonstrated that plant extracts are rich sources of bioactive constituents, such as volatile oils, alkaloids, flavonoids, terpenoids, and organic acids. These compounds exhibit a broad spectrum of biological activities, including antimicrobial effects against pathogenic microorganisms,

and anticancer activities and have served as valuable lead compounds in the development of many pharmaceutical drugs **(Ahmad et al., 2022).**

Costaceae is a family of medicinal plants that includes *Costus,* the largest genus, comprising over 150 species, including *Costus speciosus*. Crepe ginger is also known as cane-reed, Malay ginger, spiral flag, and wild ginger. *Costus speciosus* is distributed throughout the tropical and subtropical regions of Africa, Asia, and the Americas. *Costus* species are widely distributed across the tropical regions of the world. It has been reported in the sub-Himalayan range, central India, and the Western Ghats. Researchers worldwide have reported the numerous pharmacological properties and biological potential of this plant**.** *Costus specious* has long been used to treat a variety of diseases, including asthma, cough, throat infections, tuberculosis, malaria, leprosy, helminthic infestations, paralysis, headache, rheumatism, and edema. Its compounds and nutrients provide numerous health benefits. It possesses antibacterial, antifungal, and anthelmintic properties, making it effective against many ailments. It also exhibits anti-inflammatory properties and acts as a natural antipyretic agent **(Maji et al., 2020** and **Ravikumar et al., 2021).** *C. speciosus* contains a high concentration of bioactive metabolites of commercial value, such as diosgenin and azulene, as well as considerable therapeutic potential **(Aslam et al., 2023)**.

Although *C. speciosus* has been reported to possess various pharmacological properties, limited information is available regarding its activity against clinically important MDR and XDR bacterial isolates. In addition, the bioactive compounds responsible for its antimicrobial and anticancer effects have not been fully characterized, and the underlying antibacterial mechanism of action remains poorly understood. Therefore, the present study aims to evaluate its biological activities, and to identify its bioactive constituents, In addition, the study investigates cytotoxicity, and potential mechanism of antibacterial action.

Materials and Methods

Plant material

The rhizome powder of *Costus speciosus* was purchased from Ragab Herbal Store, El-Attaba, Cairo, Egypt. The plant material was authenticated and a voucher specimen was deposited by Dr. Mona Marzouk, Prof. of Phytochemistry and Plant Chemosystematics, in the Herbarium of the National Research Centre (GAIRG) under voucher number (M 317).

Bacterial isolates

Twenty-four bacterial isolates of *S. aureus* and *E. coli*, were collected from animal meat and meat byproducts, obtained from Microbiology and Immunology Department, National Research Centre, Dokki, Giza. Five bacterial isolates; *S. aureus* (Sputum), *E. coli* (Urine) *Klebsiella pneumoniae* (Sputum), *Serratia rubbidea* (Sputum), *Hafnia alvei* (Blood)) were obtained from human samples, Biotechnology Laboratory, Faculty of Science (Girls Branch), Al-Azhar University, Cairo. These bacterial isolates were collected from patients during the COVID pandemic. These isolates were identified according to phenotypic and biochemical characteristics by the VITEK 2 system **(Helmy et al., 2023).**

Antimicrobial susceptibility Testing (Kirby–Bauer Disc Diffusion Method)

Antimicrobial susceptibility testing of the bacterial isolates was performed using the Kirby–Bauer disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, Briefly, bacterial colonies were suspended in sterile saline and adjusted to 0.5 McFarland standard turbidity (approximately 1×10^8 CFU/mL). A sterile cotton swab was used to evenly inoculate the bacterial suspension onto the surface of Muller–Hinton agar (MHA) plates. Antibiotic discs were then aseptically placed on the inoculated agar surface using sterile forceps. The plates were incubated at 37°C for 18–24 h. After incubation, the diameters of inhibition zones were measured in millimeters. The bacterial isolates were classified as sensitive, intermediate, or resistant according to CLSI interpretative standards. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as quality control strains **(Gajic et al., 2022)**.

Antibiotic discs used

Antibiotic class	**Antibiotic disc**
β-lactams	Amoxicillin (AML,10 µg) Oxacillin (OX,1 µg)
Macrolides	Erythromycin (E, 15 µg)
Aminoglycosides	Gentamycin (CN, 120 µg) Streptomycin (S, 10 µg)
Glycopeptides	Vancomycin (VA, 30 µg)
Sulfonamides	Trimethoprim and Sulphamethoxazole (SXT, 1.25/23.75 mcg)
Rifamycins	Rifampicin (RD, 5 µg)
Cephalosporins	Cefoxitin (FOX, 30 mcg) Cefuroxime (CXM, 30 µg), Cefotaxime (CTX, 30 µg)
Polymyxins	Colistin sulphate (CS, 10 µg)

Preparation of plant extracts

The dried rhizomes of *C. speciosus* (10 g) were crushed and soaked using 100 mL of water, methanol, ethanol, and ethyl acetate separately for 3 days to a week at room temperature. The extracts were filtered with filter paper of Whatman No. 1, and the filtrates were concentrated using a rotary evaporator at 40°C and stored in the refrigerator in dark colored bottles at 4 °C for later studies **(Mazimba et al., 2015).**

Antibacterial activity of different plant extracts of *C. speciosus*

Agar well diffusion method

Antibacterial activity of different extracts of *C. speciousus* was evaluated using the agar well diffusion technique. The bacterial strains were subcultured onto nutrient agar (Oxoid, UK) and incubated at 37 °C for 24 h. A suspension was prepared in normal saline and adjusted to 0.5 McFarland turbidity (1.5×10^8 CFU/mL), spread onto the Muller-Hinton agar (Oxoid). About 100 µL of the extract were poured into the wells at different concentrations. Standard antibiotics (oxacillin 1 µg/mL for *S. aureus*, gentamycin 120 µg/mL for *E. coli*) were used as a positive control, and distilled water was used as a negative control to determine the sensitivity of the strains. The inoculated plates were incubated at 37 °C for 18-24 h for bacteria **(Syame et al., 2022; Montaser et al., 2026)**. The plates were placed in the refrigerator for 2 hours for extract diffusion. The inhibition zone diameter was measured using a ruler to determine the antibacterial activity.

Minimum inhibitory concentration (MIC)

The MIC value was tested for the microbes sensitive to the algal extract in the agar well diffusion assay. The broth micro-dilution technique was used for the determination of MIC using 96-well microtiter plate. The algal extract was studied at different concentrations from 100 to 0.19 mg/mL. The lowest concentration with no visible turbidity in the test tube was determined as the MIC value **(Ragab et al., 2023).**

Minimum bacterial concentration (MBC)

The MBC and MFC values were determined by sub-culturing from the MIC wells onto Muller–Hinton. The lowest concentration where no visible growth was observed was defined as MBC value **(Khalaf et al., 2022)**.

Column chromatography for the separation and purification of active antimicrobial compounds from *Costus speciosus* extract

A glass column with a diameter of 1×65 cm was used. After the silica was mixed with chloroform as an eluting solvent, the column was filled with activated silica gel. To prevent air bubbles that could cause issues during purification, the mixture was thoroughly stirred with a glass rod. Once the mixture was homogeneous, it was transferred into the empty column and left to settle overnight. To prevent the silica from drying out, a chloroform: methanol mixture (85:15) was continuously added throughout the separation process until it was completed. First, 10 mL of *C. spacious* ethyl acetate extract were added on the top of the silica gel, followed by a mixture of chloroform-methanol. Then, each three milliliters were collected in a tube. Finally, 45 fractions were collected from *C. specious.* All fractions were tested for their activity against MDR pathogenic bacterial isolates using the well agar diffusion method and examined for their purity using TLC assay **(Srivastava et al., 2021).**

Gas chromatography-mass spectrometry (GC-MS)

GC–MS was performed using Agilent Technologies 7890B GC Systems combined with 5977A Mass Selective Detector. A capillary column was used (HP-5MS Capillary; 30.0 m × 0.25 mm ID × 0.25 μm film), and the carrier gas was helium at a pressure of 8.2 psi. The reaction was carried out by adding 300 μL of Bis (trimethylsilyl) trifluoroacetamide (BSA) as a silylation agent to an amount of the sample and heating in a water bath at 80 °C for two hours, and after that, injecting into GC/MS with 1 μl injection. The sample was analyzed with the column held initially for 5 min at 60°C after injection, then the temperature was increased to 300°C with a 20°C/min heating ramp, with a 5.0 min hold. Injection was carried out in split mode (1:5) at 300°C. MS scan range was (m/z): 50–550 atomic mass units (AMU) under electron impact (EI) ionization (70 eV) and solvent delay 8.0 minutes. The constituents were determined by mass fragmentations with the NIST mass spectral search program for the NIST/EPA/NIH mass spectral library Version 2.2 (Jun 2014) (**Ragab et al., 2023).**

Mode of action using transmission electron microscope

The morphological alternations in the bacterial cells (*S. aureus* and *E. coli*) after treatment with ethyl acetate extract of *C. speciosus* were assessed using TEM. Bacterial cells (treated and untreated controls) were harvested by centrifugation at 4000 rpm for 10 min from 24-h-old cultures grown on nutrient broth media and washed twice with phosphate-buffered saline (PBS, pH 7.2). The cell pellets were than fixed in 3% glutaraldehyde in 0.1 M phosphate buffer overnight at 4°C and rinsed in phosphate buffer saline. Post-fixation was carried out using potassium permanganate solution for 5 min at room temperature. The samples were subsequently dehydrated through a gradual series of ethanol ranging from 10% to 90% for 15 min and finally with absolute ethanol for 30 min. After dehydration, samples were infiltrated with epoxy resin and acetone through a graded series until finally in pure resin. Ultrathin sections (approximately 60-90 nm) were cut using an ultramicrotome and collected on copper grids. The sections were double stained with uranyl acetate then lead citrate to enhance contrast. Finally, the grids were examined using a JEOL-JEM 1010 transmission electron microscope (JEOL Ltd, Tokyo, Japan) at 80 kV at the Regional Centre for Mycology and Biotechnology (RCMB), Al-Azhar University **(Amin et al., 2022)**.

Cytotoxicity of the active fraction

Evaluation of cytotoxic effects on normal cell line

Mammalian cell lines: WI-38 cells (human lung fibroblast normal cell line), A549 cells (human lung carcinoma cell line), and HepG2 cells (human liver carcinoma cell line) were obtained from the American Type Culture Collection (**ATCC, Rockville, MD).**

Chemicals Used: Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthia zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and trypan blue dye were purchased from Sigma **(St. Louis, MO, USA)**. Fetal Bovine serum, Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin, and 0.25% Trypsin-EDTA were purchased from Lonza **(Belgium)**.

Cell line propagation:

The cells were grown on DMEM medium (for WI-38 cells) and RPMI-1640 medium (A549 cells and HepG2 cells) supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer, and 50 µg/ml gentamycin. The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and sub-cultured twice a week.

Cytotoxicity evaluation using MTT assay:

The cells were suspended in the medium at a concentration 5×10^4 cells/well in Corning® 96-well tissue culture plates, then incubated for 24 hr. Fresh medium containing different concentrations of the plant extract was added into flat-bottomed microtiter plate (Falcon, JN, USA) (three replicates) using a multichannel pipette. Media or 0.5% DMSO was used in the control wells. Three wells were used for each concentration of the plant material. After incubating for 24 h, the numbers of viable cells were determined by the MTT test. Briefly, the media was removed from the 96-well plate and replaced with 100 µl of fresh culture medium, then 10 µl of the 12 mM MTT stock solution (5 mg of MTT in 1 mL of PBS) was added to each well, including the untreated controls. The 96-well plates were then incubated at 37°C and 5% CO_2 for 4 hours. An 85 µl aliquot of the media was removed from the wells, and 50 µl of DMSO was added to each well and mixed thoroughly with the pipette, and incubated at 37°C for 10 min.

After that, the number of viable cells was ascertained by measuring the optical density at 590 nm using a microplate reader (Sunrise, TECAN, Inc., USA). The percentage of viability was then computed as [(ODt/ODc)]×100%, where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The survival curve of each cell line following treatment with the designated substance was obtained by plotting the relationship between surviving cells and drug concentration. The 50% inhibitory concentration (IC50), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose-response curve for each concentration using GraphPad Prism software (San Diego, CA., USA) **(Mosmann, 1983; Gomha et al., 2015; Huda et al., 2022; and Abdelsalam et al., 2022)**

Microscopic examination of the treated tumor cells

This experiment was performed as previously described in the procedure for antitumor activity. After the end of the treatment at the tested concentration, the plates were inverted to remove the medium, the wells were washed three times with 300 µl of phosphate-buffered saline (pH 7.2), and then the cells were fixed to the plate with 10% formalin for 15 min at room temperature. The fixed cells were then stained with 100 µl of 0.25% crystal violet for 20 min. The stain was removed and the plates were rinsed using deionized water to remove the excess stain, then allowed to dry.

The cellular morphology was observed using an inverted microscope **(CKX41; Olympus, Japan)** equipped with a digital microscopy camera to capture the images representing the morphological changes compared to control cells. The cytopathic effects (morphological alterations) were microscopically observed at 200x.

Results

Antimicrobial Susceptibility Testing (AST)

The antibiotic susceptibility testing (AST) revealed that, out of the 29 tested bacterial isolates, 18 were classified as extensively drug-resistant (XDR), while the remaining 11 isolates exhibited a multidrug-resistant (MDR) profile (Table 1). The XDR isolates showed susceptibility to only two out of eight antibiotic classes tested.

Table 1. Antimicrobial susceptibility pattern of the tested bacterial isolates agaisnt different antibiotics determined by the Kirby-Bauer disk diffusion method according to CLSI guidelines. Results were interpreted as sensitive (S), intermediate (I), or resistant (R) based on inhibition zone diameters.

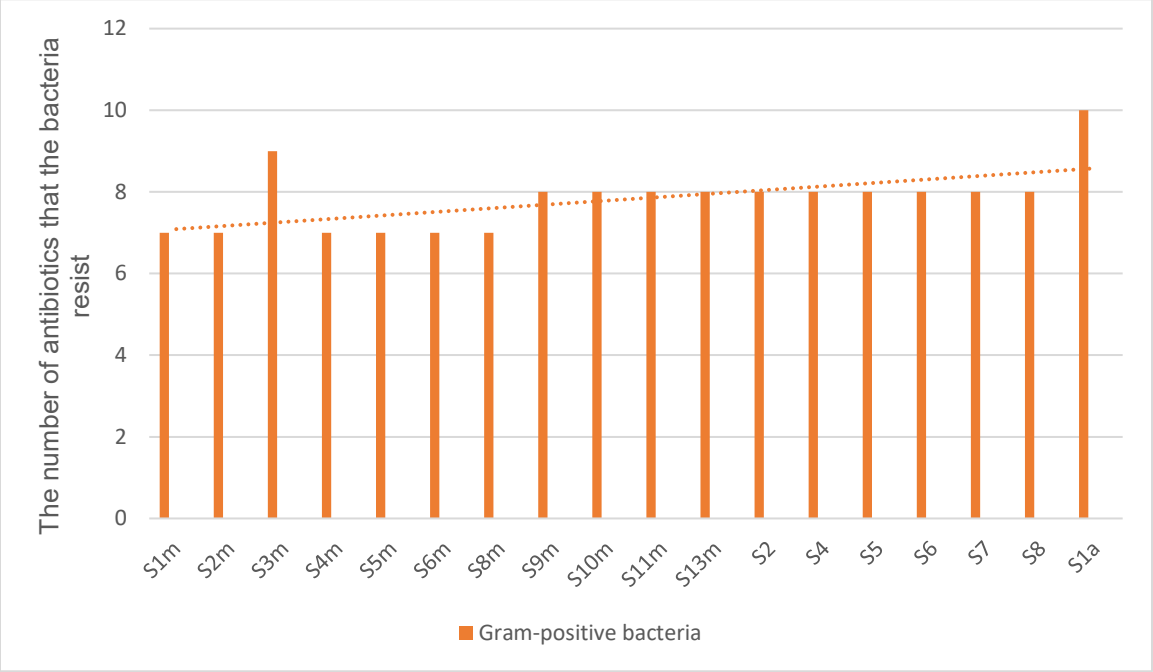
	B-lactams		**Cephalosporins**			**Amino-glycosides**		**Glyco-peptides**	**Sulfo-namides**	**Macro-lides**	**Rifa-mycins**	**Polymyxins**
Antibiotics	**AMX 10** µg	**OX 1** µg	**CTX 30** µg	**CXM 30** µg	**FOX 30** µg	**S 10** µg	**CN 120** µg	**VA 30** µg	**SXT 1.25/ 23.75mcg**	**E 15** µg	**RD 5** µg	**CS 10** µg
Gram-positive bacteria												
S1m*	R	R	R	R	R	S	S	S	R	S	R	-
S2m*	R	R	R	R	R	S	S	S	R	S	R	-
S3m**	R	R	R	R	R	R	S	S	R	R	R	-
S4m*	R	R	R	R	R	S	S	S	R	S	R	-
S5m*	R	R	R	R	R	S	S	S	R	S	R	-
S6m*	R	R	R	R	R	S	S	S	R	S	R	-
S8m*	R	R	R	R	R	S	S	S	R	S	R	-
S9m**	R	R	R	R	R	S	S	S	R	R	R	-
S10m**	R	R	R	R	R	S	S	S	R	R	R	-
S11m**	R	R	R	R	R	S	S	S	R	R	R	-
S13m**	R	R	R	R	R	S	S	S	R	R	R	-
S2**	R	R	R	R	R	S	S	S	R	R	R	-
S4**	R	R	R	R	R	S	S	S	R	R	R	-
S5**	R	R	R	R	R	S	S	S	R	R	R	-
S6**	R	R	R	R	R	S	S	S	R	R	R	-
S7**	R	R	R	R	R	S	S	S	R	R	R	-
S8**	R	R	R	R	R	S	S	S	R	R	R	-
S1a**	R	R	R	R	R	R	R	S	R	R	R	-
Gram-negative bacteria												
E1**	R	-	R	R	R	R	I	-	R	R	R	R
E7**	R	-	R	R	R	S	S	-	R	S	R	R

E8**	R	-	R	R	R	S	S	-	R	R	R	R
E19**	R	-	R	R	R	S	R	-	R	S	R	R
E23**	R	-	R	R	R	R	S	-	R	S	R	R
E26**	R	-	R	R	R	R	S	-	R	S	R	R
E30**	R	-	R	R	R	S	S	-	R	S	R	R
E35**	R	-	R	R	R	S	S	-	R	S	R	R
K**	R	-	R	R	R	R	R	-	R	R	-	R
H**	R	-	R	R	R	R	R	-	R	R	R	R
C**	R	-	R	R	R	R	R	-	R	R	R	R

S = *Staphylococcus aureus*; E = *Escherichia coli*; K = *Klebsiella pneumoniae*; H = *Hafnia alvei*; C = *Serratia rubbidea*

* means multidrug-resistant (MDR) bacteria

** means extensively drug-resistant (XDR) bacteria



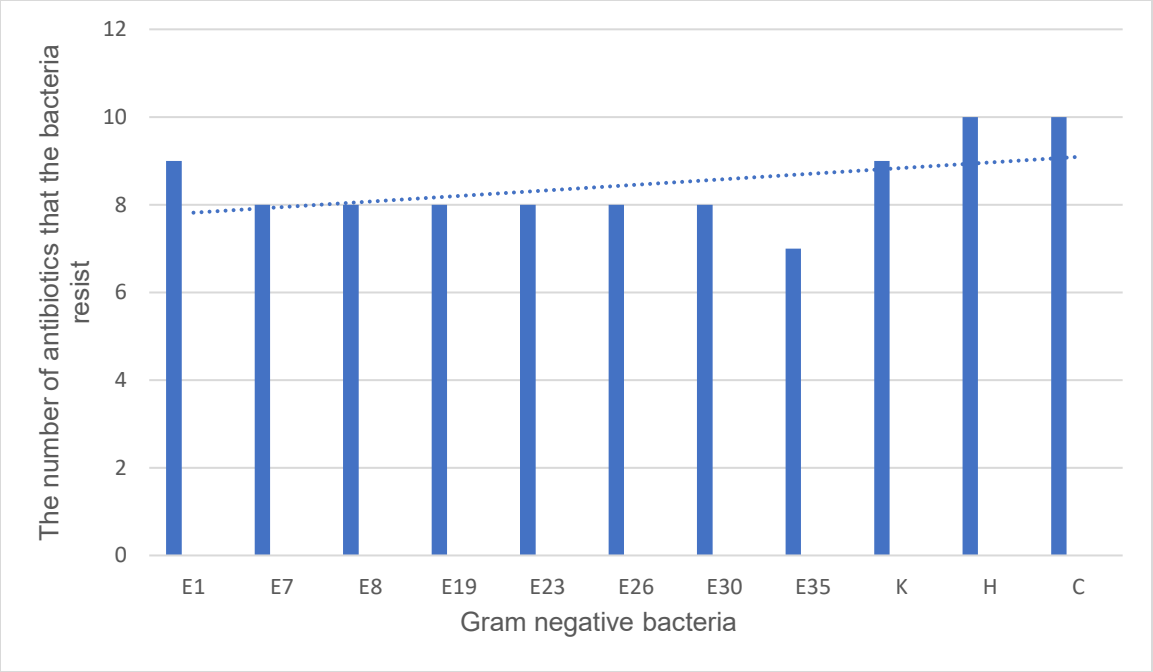


Fig. 1 Antimicrobial susceptibility testing (AST) of the tested bacterial isolates using the Kirby-Bauer disk diffusion method on Muller-Hinton agar. Representative plates showing zones of inhibition around different antibiotic discs after incubation at 37º C for 18-24 h, indicating sensitive, intermediate, and resistant responses according to CLSI guidelines

Antibacterial activity of different extracts of *Costus speciosus* against drug-resistant bacterial isolates

Table 2. Antibacterial activity of different extracts of *C. speciosus* against the tested bacterial isolates expressed as zones of inhibition (mm).

Strain	Cold water extract	Methanol extract	Ethanol extract	Ethyl acetate extract
Gram-positive bacteria				
S1a	22.00 ± 00.00	25.00 ± 00.00	28.00 ± 00.00	31.00 ± 00.57
S9m	20.00 ± 00.00	24.66 ± 0.577	25.00 ± 00.00	30.66 ± 1.154
S11m	16.00 ± 00.00	20.66 ± 1.154	24.66 ± 0.577	25.33 ± 1.527
S13m	18.00 ± 00.00	20.00 ± 00.00	25.00 ± 00.00	27.66 ±1.527
Gram-negative bacteria				
E1	24.00 ± 00.00	25.66 ±1.527	28.00 ± 00.00	32.00 ± 00.00
E19	13.33 ± 1.527	23.00 ± 00.00	27.66 ± 1.527	29.66 ± 0.577
E23	23.00 ± 00.00	21.66 ± 0.577	27.33 ± 1.527	29.33 ± 1.154
E26	24.00 ± 00.00	30.00 ± 00.00	30.00 ± 00.00	30.33 ± 0.577
K	24.00 ± 00.00	27.33 ± 01.527	27.33 ± 01.527	28.00 ± 01.00

H	20.00 ± 00.00	24.66 ± 1.527	28.00 ± 00.00	29.33 ± 0.577
C	22.00 ± 00.00	27.33 ±1.527	27.33 ± 1.527	27.66 ± 1.527

S1a, S9m, S11m, S13m*: S. aureus;* E1, E19, E23, E26*: E. coli*; K: *K. pneumoniae;* H: *H. alvei;* C: *S. rubbidea*. Values are expressed as mean ± SD. Repeated measures ANOVA revealed a significant difference in the inhibition zone diameters among the four extracts ($P < 0.001$). Pairwise comparisons (Paired t-tests with Bonferroni correction) showed that the ethyl acetate extract produced significantly larger inhibition zones than the water ($P < 0.001$), methanol ($P < 0.001$), and ethanol ($P = 0.002$) extracts. The ethyl acetate extract exhibited the highest mean inhibition zone.

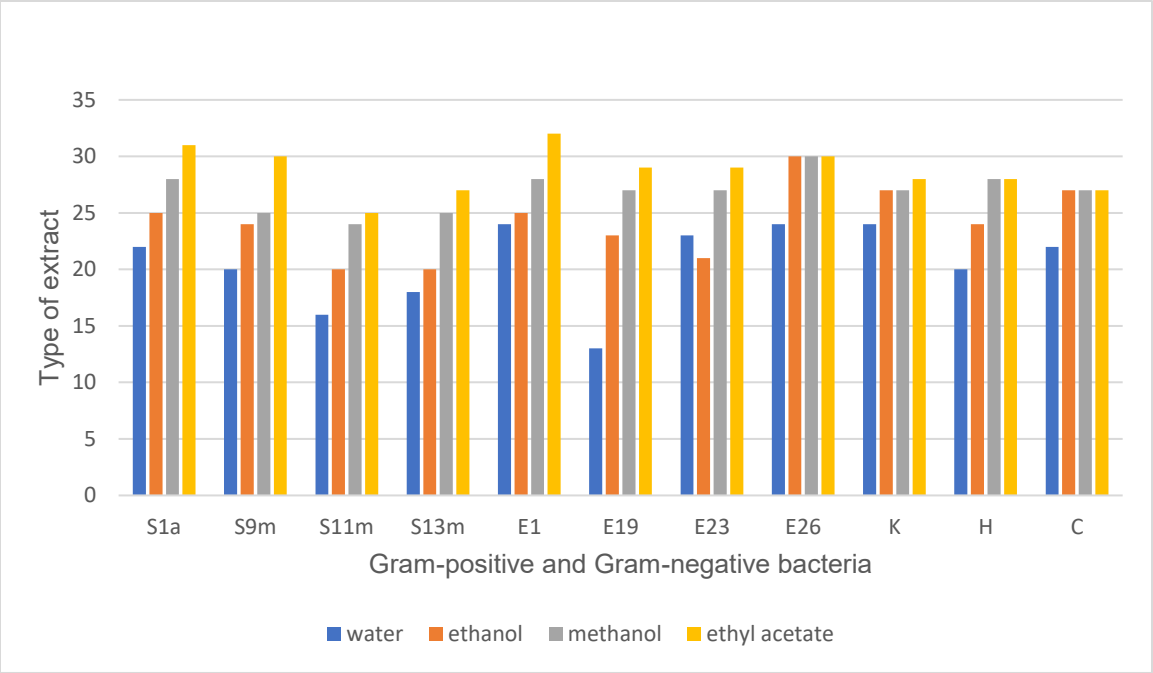


Fig. 2 Antibacterial activity of different plant extracts of *C. speciosus* against drug-resistant bacterial isolates, expressed as zones of inhibition (mm) after incubation at 37°C for 18-24 h. Results are shown as mean ±SD

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethyl acetate extract of *C. speciosus*

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethyl acetate extract against drug-resistant bacterial isolates. Values are expressed in µg/mL

Isolate	**Bacteria**	**MIC** (µg/mL)	**MBC** (µg/mL)
Gram-positive bacteria			
1	*S. aureus* (S1a)	31.25	62.5
2	*S. aureus* (9m)	62.5	62.5

3	*S. aureus* (11m)	62.5	125
4	*S. aureus* (13m)	31.25	62.5
Gram-negative bacteria			
5	*E. coli* (E1)	62.5	125
6	*E. coli* (E19)	31.25	62.5
7	*E. coli* (E23)	31.25	62.5
8	*E. coli* (E26)	62.5	125
9	*K. pneumoniae* (K)	62.5	62.5
10	*H. alvei* (H)	62.5	125
11	*S. rubbidea* (C)	125	250

There was no statistically significant difference in MIC and MBC values between the studied groups of different bacterial species with P values = 0.369 and 0.287, respectively. A statistically significant difference was observed between MIC and MBC values (Wilcoxon signed-rank test, p = 0.007), with MBC values being higher than MIC.

Fractionation of ethyl acetate extract of *C. speciosus* using column chromatography

Data in Figure 3a, 3b, and 3c shows that fraction 4 achieves the maximum antibacterial activity against *K. pneumoniae* (K), *S. rubbidea* (C), *S. aureus* (S13m), *E. coli* (E1), *S. aureus* (S1a), and *Hafnia alvei* (H). However, the fraction 8 has no effect, and this confirms the boundary between the two peaks.

Table 4. Antibacterial activity of different fractions obtained from column chromatography of the ethyl acetate extract of *C. speciosus* against drug-resistant bacterial isolates, expressed as zones of inhibition (mm).

Fraction number	***S. aureus* (S1a)**	***S. aureus* (S13m)**	***E. coli* (E1)**	***K. pneumoniae* (K)**	***H. alvei* (H)**	***S. rubbidea* (C)**
1:2	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00
3	24.00±00.00	26.00±00.00	24.00±01.00	25.00±00.00	24.00±00.00	25.00±01.00
4	26.66±0.577	27.66±00.00	27.00±0.577	28.00±00.00	27.00±00.00	27.00±00.00
5	23.00±01.00	23.00±00.00	23.66±0.577	25.66±0.577	23.66±0.577	24.00±00.00
6	22.33±00.66	21.00±0.577	22.33±00.00	23.00±00.00	22.66±0.577	22.33±0.577

7	20.00±00.00	20.33±0.577	20.33±0.577	20.00±00.00	20.00±00.00	19.33±00.00
8	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00
9	22.00±00.00	22.33±00.00	22.33±0.577	22.00±00.00	22.00±01.00	23.00±00.00
10	22.00±01.00	22.66±00.00	22.66±0.577	22.00±00.00	21.00±00.00	22.00±00.00
11	21.00±00.00	21.33±00.00	21.33±0.577	20.33±00.00	19.33±0.577	21.00±00.00
12	17.33±0.577	15.66±00.00	17.00±00.00	16.00±00.00	18.00±00.00	17.00±00.00
13:43	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00

Values represent inhibition zone diameters (mm) measured after incubation. Data are expressed as mean ± SD of three independent experiments.

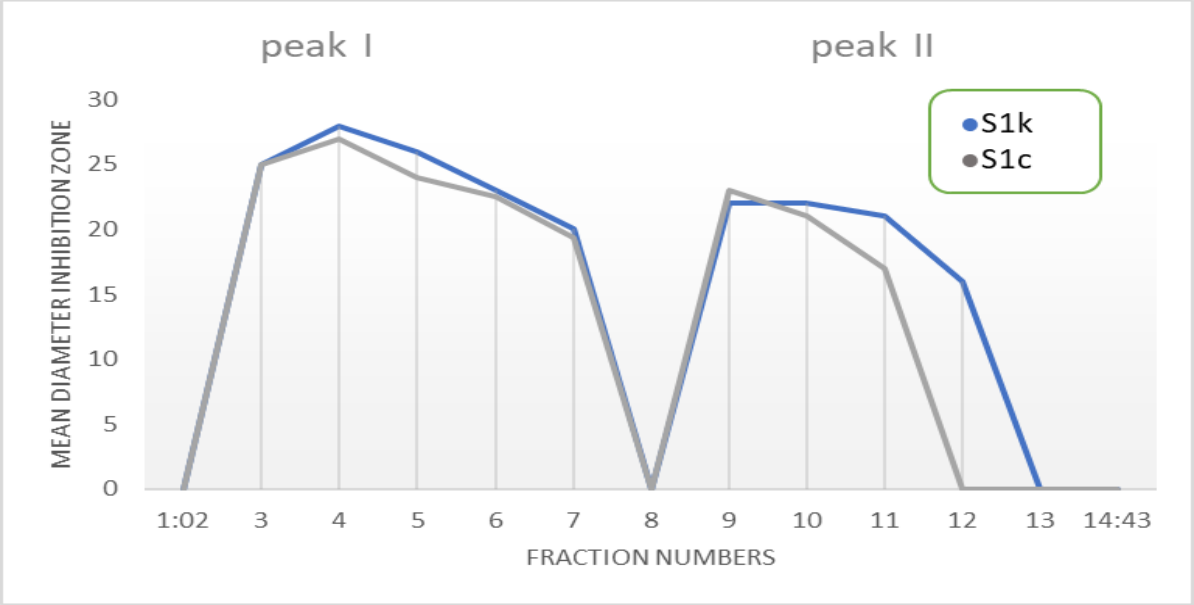


Fig. 3a Antibacterial activity of different fractions obtained from column chromatography of the ethyl acetate extract of *C. speciosus* against *Klebsiella pneumoniae* (K) and *Serratia rubbidea* (C), expressed as zones of inhibition (mm). Results are presented as mean ± SD

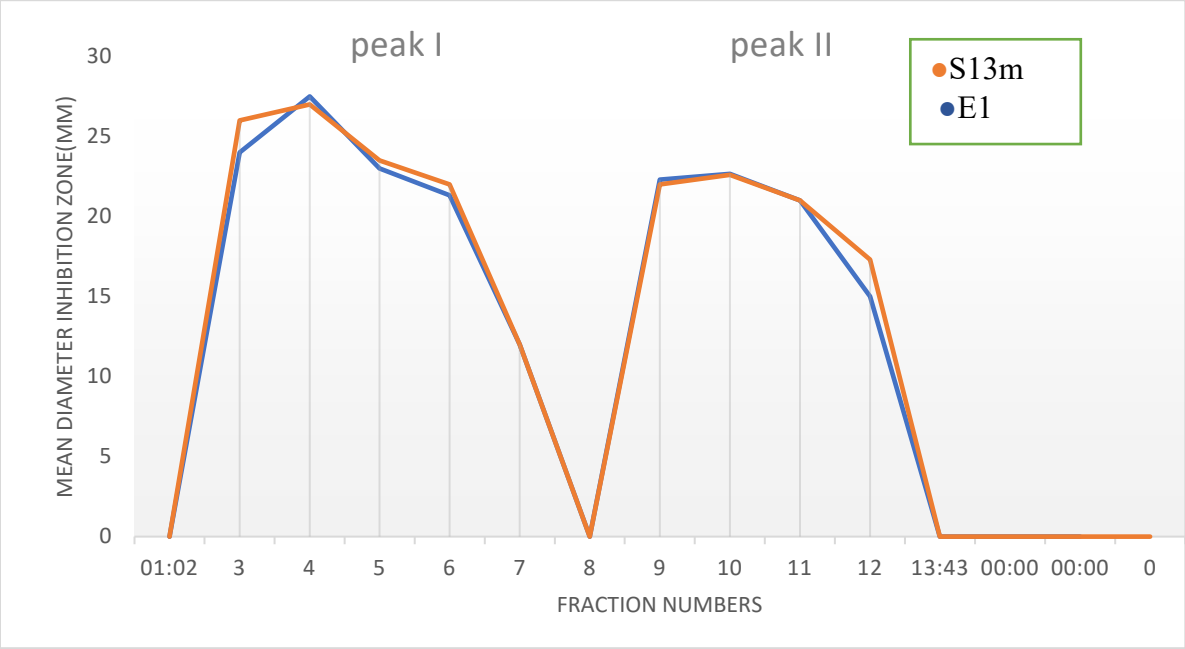


Fig. 3b Antibacterial activity of different fractions obtained from column chromatography of the ethyl acetate extract of *C. speciosus* against *S. aureus* (S13m) and *E. coli* (E1), expressed as zones of inhibition (mm). Results are presented as mean ± SD

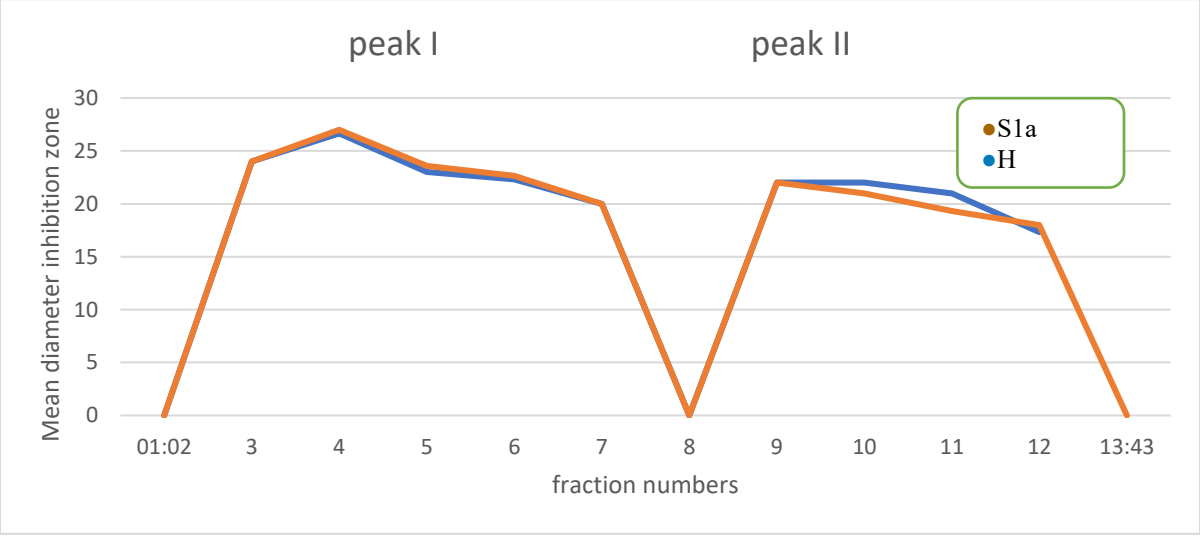


Fig. 3c Antibacterial activity of different fractions obtained from column chromatography of the ethyl acetate extract of *C. speciosus* against *S. aureus* (S1a) and *Hafnia alvei* (H), expressed as zones of inhibition (mm). Results are presented as mean ± SD

Data in Figure 3a, 3b, and 3c shows that fraction 4 achieves maximum efficiency against *K. pneumoniae* (K), *S. rubbidea* (C), *S. aureus* (S13m and S1a), *E. coli*, and *H. alvei*. However, the fraction 8 has no effect, and this confirms the boundary between the two peaks.

Determination of MIC and MBC of fraction (4) against drug-resistant bacterial isolates

Table 5. Minimum inhibitory concentration (MIC) and minimum inhibitory concentration (MBC) of fraction (4) of ethyl acetate extract.

Isolate	**Bacteria**	**MIC (**µg/mL**)**	**MBC (**µg/mL**)**
1	*S. aureus* (S1a)	62.5	125
2	*S. aureus* (9m)	62.5	62.5
3	*S. aureus* (11m)	62.5	125
4	*S. aureus* (13m)	62.5	125
5	*E. coli* (E1)	125	125
6	*E. coli* (E19)	62.5	125
7	*E. coli* (E23)	125	125
8	*E. coli* (E26)	62.5	125
9	*K. pneumoniae* (K)	125	250
10	*H. alvei* (H)	1500	1500
11	*S. rubbidea* (C)	125	250

A statistically significant difference in MIC values was observed among the tested bacterial isolates (Kruskal-Wallis test, $p = 0.074$). However, no statistically significant difference was found in MBC values among the same isolated (Kruskal-Wallis test, $p = 0.129$

GC-MS Analysis of Fraction 4 of Ethyl Acetate Extract of *C. speciosus*

The GC-MS analysis of the selected plant fraction (4) revealed a complex mixture of bioactive phytochemical constituents belonging to different chemical classes, mainly including terpenoids (especially sesuiterpene lactone), fatty acid esters, hydrocarbons, and aromatic derivatives. Among the identified compounds, azulene[4,5-b] furan-2(3H)-one, decahydro-3,6,9-tris(methylene)-, [3aS-(3a. alpha.,6a. alpha.,9a. alpha.,9b. beta] derivative was the predominant constituent based on its relative peak area (65.8734%), indicating that sesquiterpene-related compounds represent the major chemical class in the analyzed fraction. Other notable compounds included dihydrodehydrocostus lactone and (Z,Z,Z)-1,8,11,14-heptadecatetraene with peak area percentages of 10.5377 and 9.9093%, respectively.

Table 6. Phytochemical constituents identified in fraction 4 of the ethyl acetate extract by GC-MS analysis, including retention time (RT), compound name, and peak area percentage.

Peak area	RT	Compound name	Peak area %
1	12.1727	(Z,Z,Z)-1,8,11,14-Heptadecatetraene,	9.9093
2	12.4301	Cyclohexane, 1,5-diethenyl-3-methyl-2-methylene-, (1α,3α,5α)-	1.3404
3	13.0596	2-Methallyl alcohol, TMS derivative	1.9883
4	13.4544	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3aα,6α,7β,7aβ)]-	2.6085
5	14.0151	Dihydrodehydrocostus lactone	10.5377
6	14.347	Azuleno[4,5-b]furan-2(3H)-one, decahydro-3,6,9-tris(methylene)-, [3aS-(3aα,6aα,9aα,9bβ)]-	65.8734
7	20.7384	Isopropyl linoleate	2.7568
8	21.4537	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	2.4164

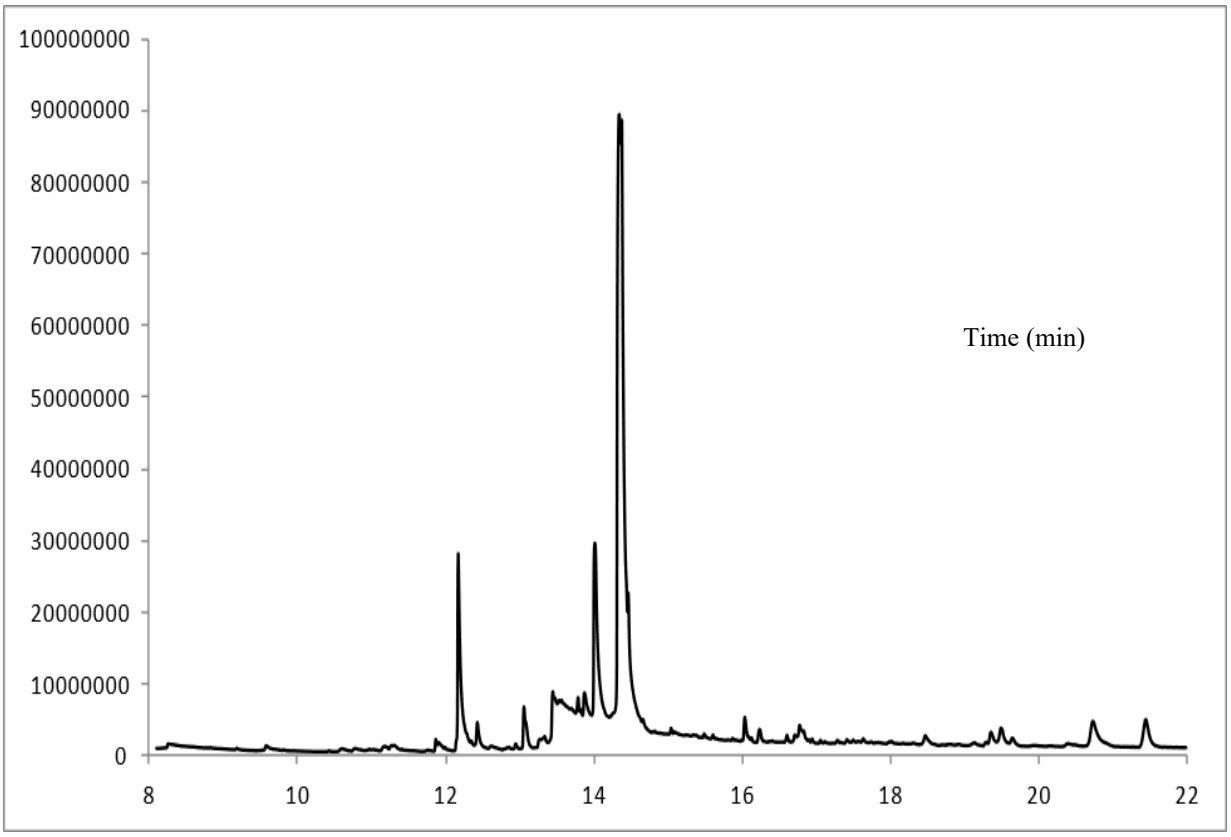


Fig. 4 GC–MS chromatogram illustrating the identified phytochemical compounds in the selected plant fraction, including major constituent such as azuleno[4,5-b]furan-2(3H)-one derivative and other terpenoids, fatty acid esters, and aromatic compounds

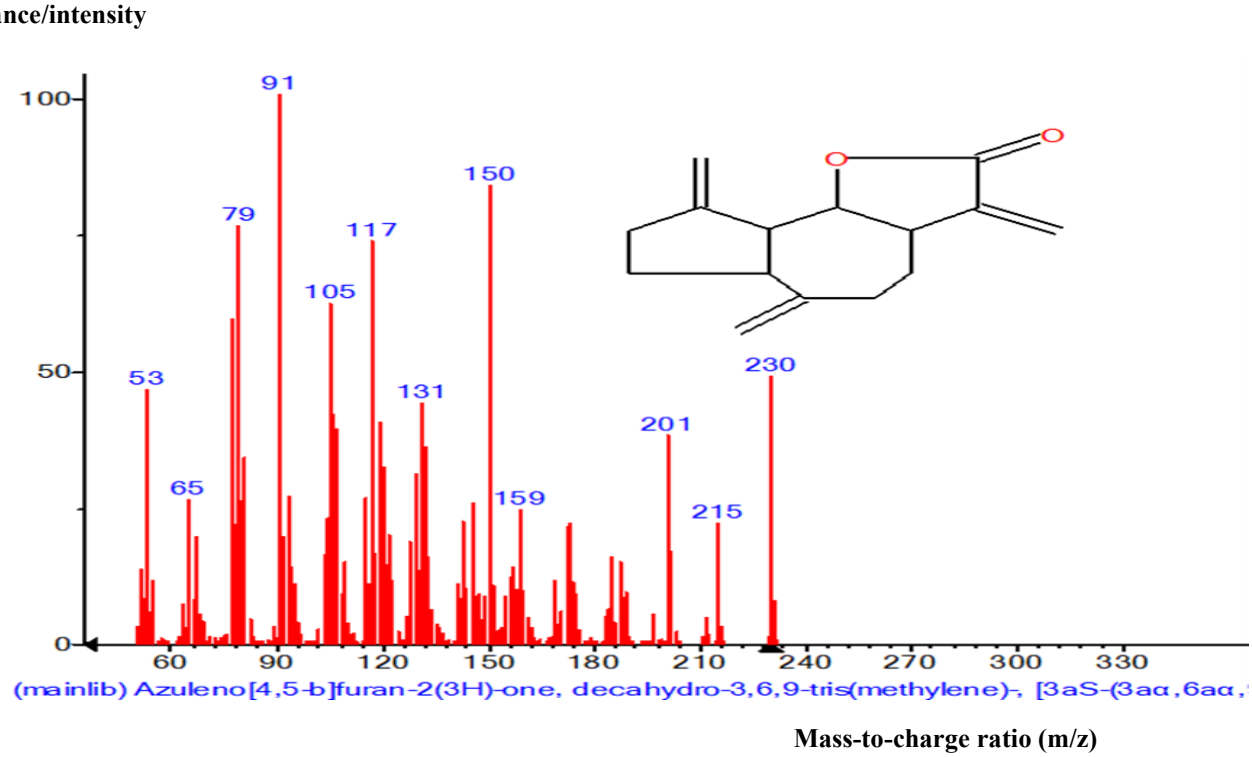


Fig. 5 Mass spectrum showing fragmentation pattern of azuleno[4,5-b]furan derivative obtained from GC–MS analysis.

Mode of action of the ethyl acetate extract of *C. speciosus* (Fraction 4) against *S. aureus*

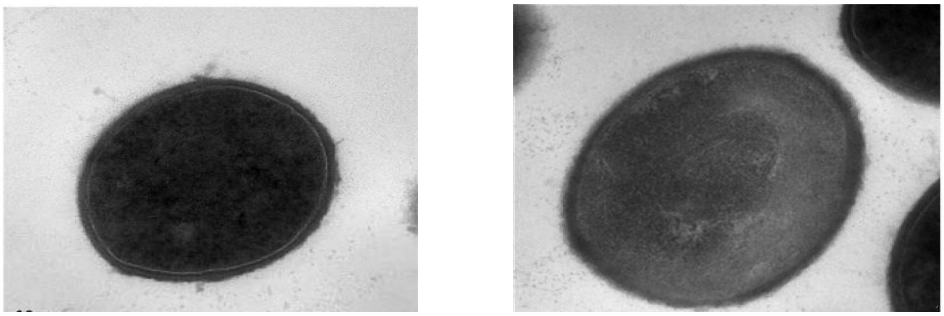


Fig. 6a Transmission electron microscopy (TEM) image of untreated *S. aureus* (S1a) cells showing normal spherical (cocci) morphology with intact cell wall and membrane structure under standard growth conditions

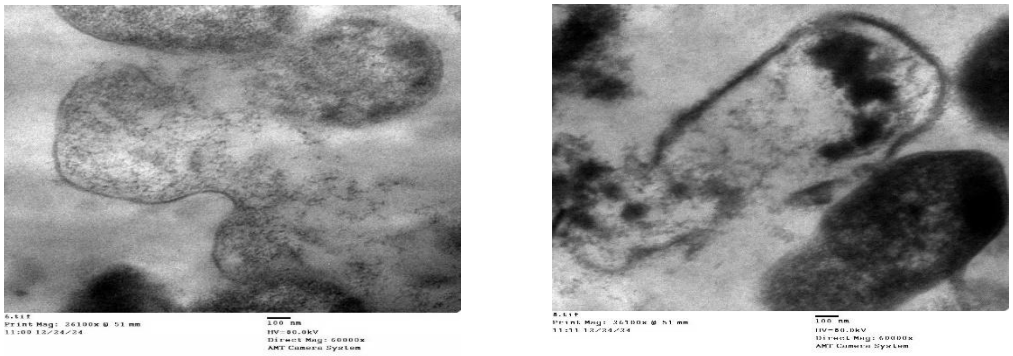


Fig. 6b Transmission electron microscopy (TEM) image of *S. aureus* (S1a) treated with fraction (4) of the ethyl acetate extract, showing ultrastructural alterations compared to untreated control cells

Mode of action of the ethyl acetate extract of *C. speciosus* (Fraction 4) against *E. coli*

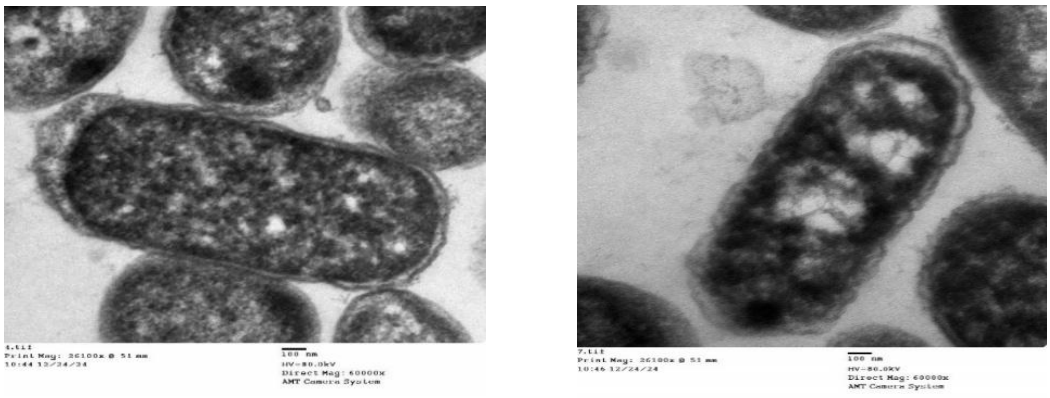


Fig. 7a Transmission electron microscopy (TEM) image of untreated *E. coli* (E1) cells showing normal rod-shaped (bacilli) morphology with intact cell wall and membrane structure under standard growth conditions

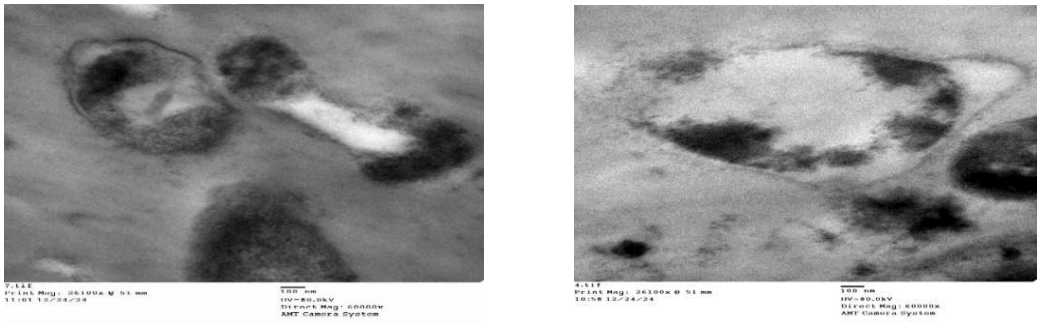


Fig. 7b Transmission electron microscopy (TEM) image of *E. coli* treated with fraction (4) of the ethyl acetate extract, showing ultrastructural alterations compared to untreated control cells

Cytotoxicity assessment of the ethyl acetate extract

Cytotoxicity against human lung fibroblast normal cell line (WI-38 cell line)

The ethyl acetate extract (Fraction 4) exhibited CC50 value of 23.92±0.86 µg/ml against human lung fibroblast normal cell line, as shown in Table (7) and Figure (11).

Table 7. Cytotoxic activity of the ethyl acetate extract of *C. speciosus* (Fraction 4) expressed as CC50 values (µg/mL) against human lung fibroblast normal cell line

Sample conc. (µg/ml)	Viability %	Inhibitory %	S.D. (±)
500	2.98	97.02	0.16
250	6.53	93.47	0.21
125	13.42	86.58	0.64
62.5	28.67	71.33	1.59
31.25	40.82	59.18	1.44
15.6	60.49	39.51	1.73
7.8	76.03	23.97	0.85
3.9	84.17	15.83	0.91
2	92.31	7.69	0.23
1	96.05	3.95	0.47
0	100	0	0

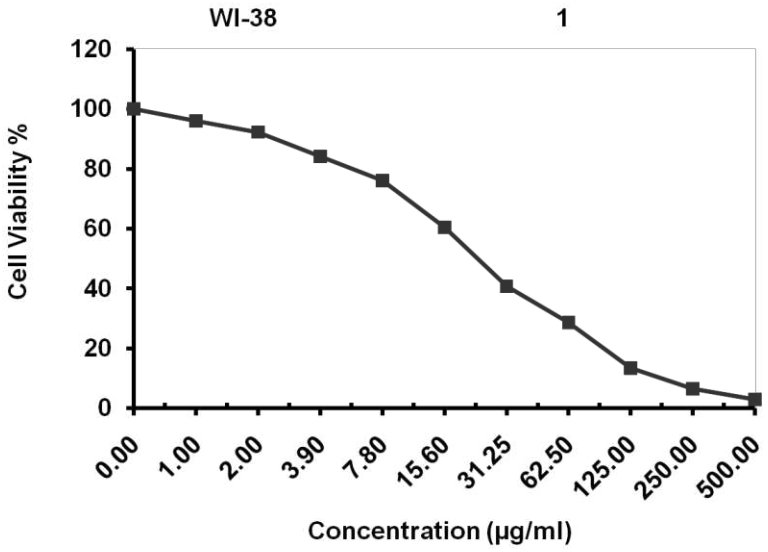


Fig. 8 Dose-response curve of the ethyl acetate extract (Fraction 4) against human lung fibroblast normal cell line

Anticancer activity against human liver carcinoma cell line (HepG-2 cells)

As shown in Table (8) and Figure (12), the ethyl acetate extract (Fraction 4) exhibited cytotoxic activity against human liver carcinoma cell line with an IC_{50} value of 5.17 µg/mL, while showing lower toxicity against normal cells with an IC_{50} value of 23.92 µg/mL. The selectivity index (SI) was calculated to be 4.63.

Selectivity index (SI) = IC_{50} normal cells/IC_{50} cancer cells = 23.92/5.17 = 4.63

Table 8. Anticancer activity of the ethyl acetate extract of *C. speciosus* (Fraction 4) expressed as IC_{50} (µg/mL) values against human liver carcinoma cell line (HepG-2 cells)

Sample conc. (µg/ml)	Viability %	Inhibitory %	S.D. (±)
500	0.75	99.25	0.06
250	2.46	97.54	0.08
125	6.29	93.71	0.14
62.5	11.84	88.16	0.38
31.25	19.58	80.42	0.76
15.6	30.64	69.36	1.08
7.8	41.92	58.08	2.34
3.9	53.89	46.11	1.05
2	61.47	38.53	1.29
1	68.52	31.48	0.84
0	100	0	0

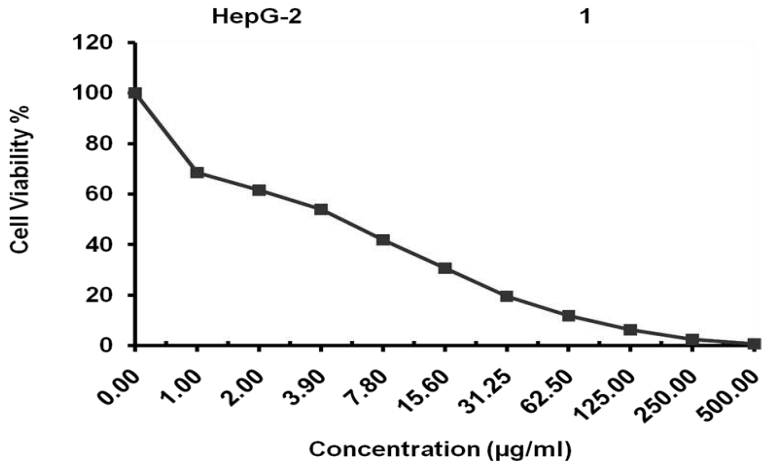


Fig. 9 Dose-response curve of the ethyl acetate extract (Fraction 4) against human liver carcinoma cell line

Morphological Evaluation of anticancer activity against human liver carcinoma cell line (HepG2 cells)

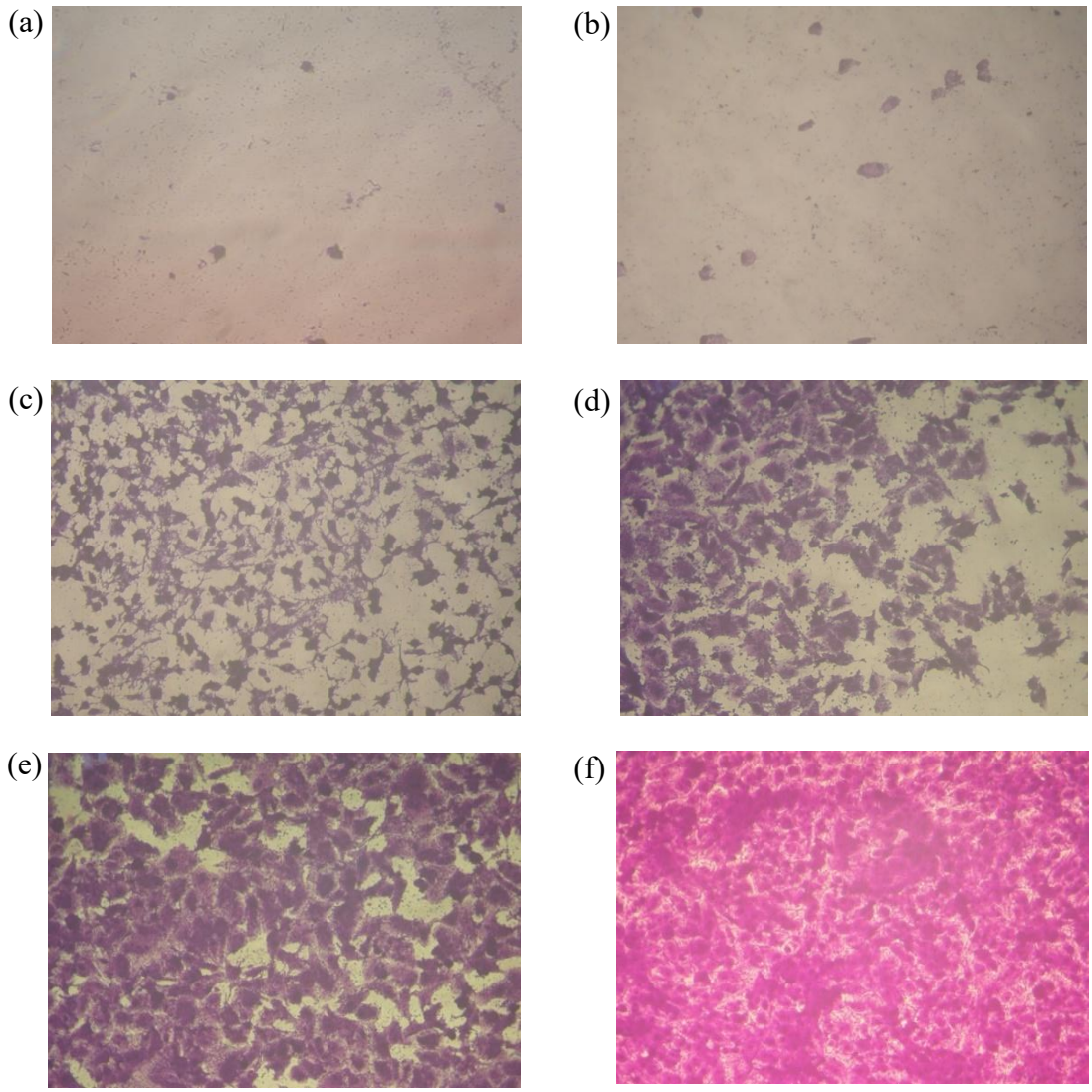


Fig. 10 Microscopic assessment of morphological alterations in HepG2 cell line cancer cells treated with ethyl acetate extract of *C. speciosus* (Fraction 4)

a. HepG2 cells treated with sample (1) at 500 µg/mL concentration
b. HepG2 cells treated with sample (1) at 125 µg/mL concentration
c. HepG2 cells treated with sample (1) at 31.25 µg/mL concentration
d. HepG2 cells treated with sample (1) at 7.8 µg/mL concentration
e. HepG2 cells treated with sample (1) at 2 µg/mL concentration
f. HepG2 cells non-treated (control)

Anticancer activity against human lung carcinoma cell line (A-549 cells)

Data in Table 13 showed that the inhibitory activity against human lung carcinoma cells was detected under these experimental conditions with IC_{50} = 8.27±0.61 µg/ml. In addition to the direct relation between the concentration of the sample and the inhibitory activity. Also, when the concentration of the active substance increases, the viability and inhibitory activity decrease.

Table 9. Anticancer activity of ethyl acetate extract of *C. speciosus* against human lung carcinoma cell line (A-549 cells)

Sample conc. (µg/ml)	Viability %	Inhibitory %	S.D. (±)
500	1.83	98.17	0.09
250	3.52	96.48	0.14
125	8.65	91.35	0.37
62.5	14.92	85.08	0.46
31.25	27.46	72.54	1.28
15.6	39.58	60.42	2.69
7.8	50.67	49.33	2.31
3.9	62.34	37.66	1.62
2	70.65	29.35	0.98
1	82.39	17.61	0.75
0	100	0	

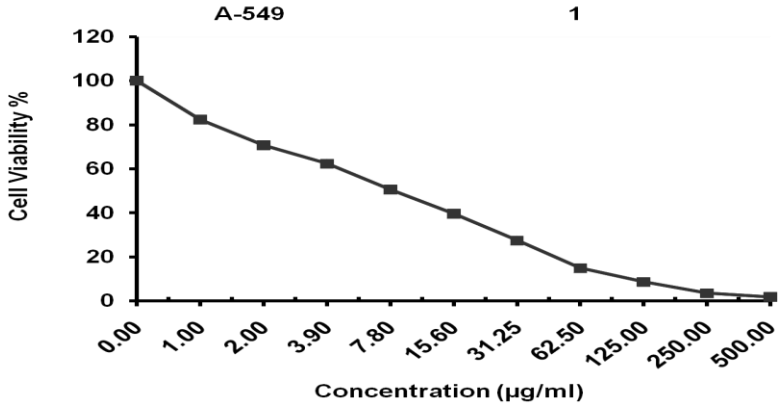


Fig. 11 Dose-response curve of the ethyl acetate extract (Fraction 4) against human lung carcinoma cell line

Morphological Evaluation of anticancer activity against human lung carcinoma cell line (A-549 cells)

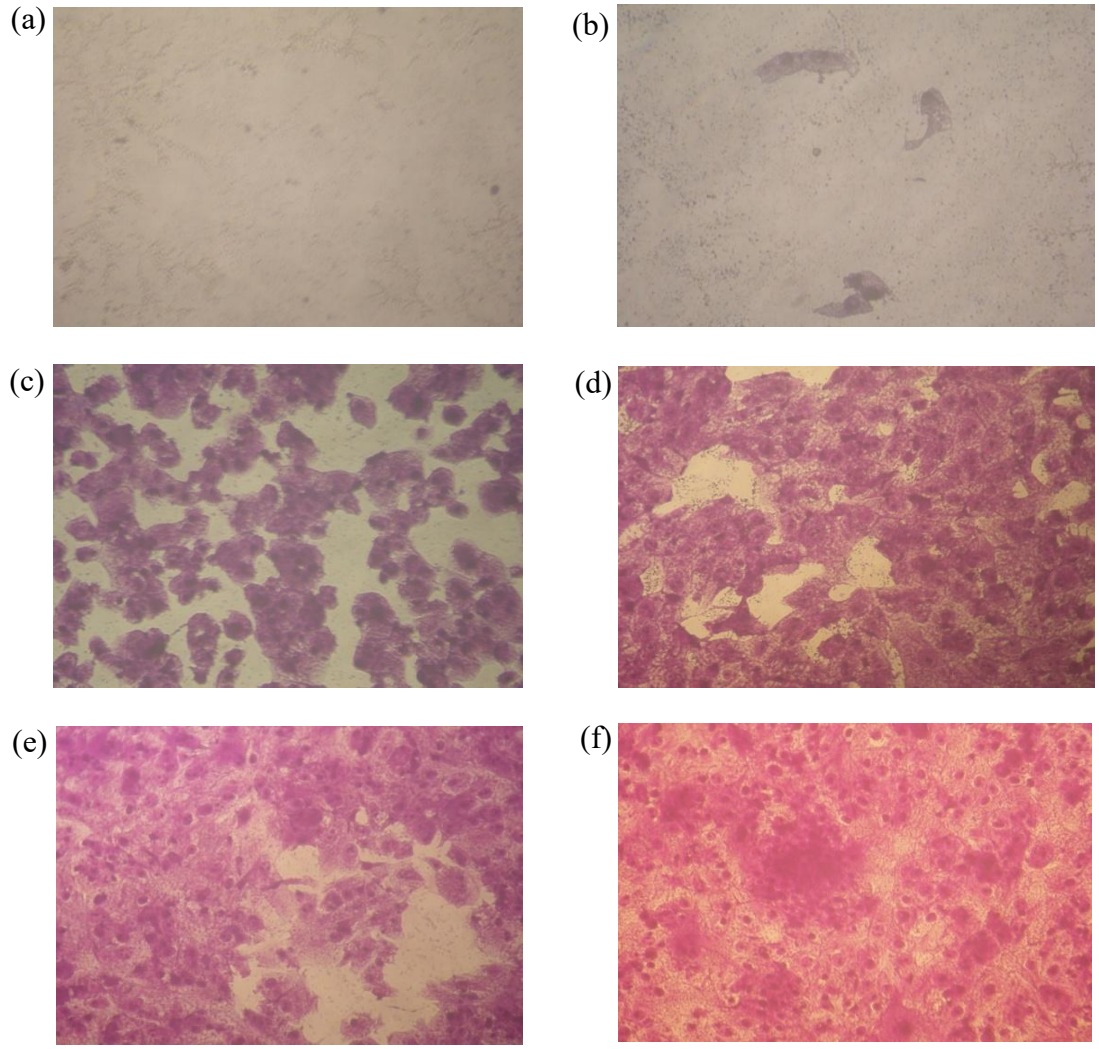


Fig. 12 Microscopic assessment of morphological alterations in A549 cell line cancer cells treated with ethyl acetate extract of *C. speciosus* (Fraction 4) compared to untreated control

a. A-549 cells treated with sample (1) at 500 µg/mL concentration
b. A-549 cells treated with sample (1) at a 125 µg/mL concentration
c. A-549 cells treated with sample (1) at 31.25 µg/mL concentration
d. A-549 cells treated with sample (1) at 7.8 µg/mL concentration
e. A-549 cells treated with sample (1) at 2 µg/mL concentration
f. A-549 cells non-treated control

Discussion

The increasing prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria represents a serious public health challenge due to the limited efficacy of conventional antibiotics and increasing the risk of treatment failure. Therefore, plant-derived compounds have attracted attention as potential alternative antimicrobial agents. In the present study, the tested plant extract exhibited antibacterial activity against MDR and XDR bacterial isolates, indicating its potential against resistant pathogens.

The antimicrobial susceptibility testing revealed that *K. pneumoniae*, *H. alvei*, and *S. rubbidea* were resistant to all tested antibiotic classes. In addition, *S. aureus* (S1a) and *E. coli* (E8) were resistant to seven out of eight antibiotic classes, while *E. coli* (E1) was resistant to all tested antibiotic classes and showed intermediate resistance to one antibiotic. Moreover, *S. aureus* isolates (S3m, S9m, S10m, S11m, S13m, S2, S4, S5, S6, S7, and S8) and *E. coli* isolates (E7,E19, E23, E26, E30, and E35) were resistant to all tested antibiotic classes except two classes. Accordingly, these isolates were classified as extensively drug-resistant (XDR) bacteria. The remaining *S. aureus* isolates were identified as methicillin-resistant *Staphylococcus aureus* (MRSA) based on cefoxitin and oxacillin resistance and were additionally classified as multidrug-resistant (MDR) isolates.

The high prevalence of MDR and XRD bacterial isolates in the current study indicates the growing challenge of antimicrobial resistance among pathogenic bacteria. Such resistance may be attributed to the excessive and inappropriate use of antibiotics, in addtion to the ability of bacteria to acquire resistance genes and form biofilm, which enhance their survival against antimicrobial agents. Similar findings have been reported in previous studies, which demosntrated the increasing emergence of resistant bacterial strains worldwide **(Abdelmoneim et al., 2024)**. Therefore, the observed antibacterial activity of the tested plant extract against MDR and XDR isolates highlights its potential as a promising natural alternative for combating resistant pathogens.

Numerous studies have demonstrated the many therapeutic benefits of *C. speciosus* extract, including antioxidant, antidiabetic, anticholinesterase, antibacterial, anthelmintic, antifungal, anticancer, analgesic, anti-inflammatory, antipyretic, antihyperlipidemic, anxiolytic, mosquito larvicidal, estrogenic, nephroprotective, and hepatoprotective activities **(Bahshwan, 2020; Nadda et al. 2020** and **Singh et al. 2024).**

In the present study, the three solvent-based extracts (ethanol, methanol, and ethyl acetate) of *C. speciosus* showed comparable antibacterial activity against the tested bacterial isolates, while the aqueous extract exhibited slightly lower activity. This difference may be attributed to the variation in the solubility and extraction efficiency of bioactive compounds depending on solvent polarity. Organic solvents such as methanol and ethanol are more efficient in extracting a broad range of phytochemicals, including phenolics, flavonoids, and alkaloids, which are known for their strong antimicrobial properties. In contrast, water mainly extracts highly polar compounds, which may not possess the same antibacterial activity or may be present in lower concentration **(Arya et al., 2025)**. These antibacterial findings are in agreement with those reported by **(Duraipandiyan et al. 2012** and **Reda et al., 2024)** especially in Gram-positive bacteria. In addition, the cold extracts, essential oils, and isolated compounds of *C. speciosus* exhibited antimicrobial activity against *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *S. aureus* **(Adesegun, 2021).**

The organic extracts exhibited stronger antibacterial activity against Gram-negative bacterial compared with Gram-positive bacteria. This finding is somewhat contrary to the commonly reported pattern, where Gram-positive bacteria are generally more susceptible due to the absence of an outer membrane **(Duraipandiyan et al. 2012** and **Reda et al., 2024)**. The observed higher susceptibility of Gram-negative bacteria in this study may be attributed to the nature and mode of action of the bioactive compounds present in the extracts, which may possess the ability to penetrate or disrupt the outer membrane of Gram-negative bacteria effectively.

The determination of MIC and MBC provides valuable information regarding the antibacterial potency of the tested plant extract. Ethyl acetate extract revealed MIC and MBC values, ranging from 62 to 250 mg/mL, demonstrating that the ethyl acetate extract possessed effective inhibitory and bactericidal activities against the tested bacterial isolates. Lower MIC and MBC values indicate higher antimicrobial efficacy, suggesting that relatively low concentrations of the extracts were sufficient to inhibit bacterial growth and exert bactericidal effects.

Due to the significant antibacterial activity demonstrated by the ethyl acetate extract, it was selected for further fractionation in order to identify and evaluate the biologically active fractions responsible for the observed antimicrobial effect. Among the fractions, fraction 4 showed the strongest antibacterial activity, followed by fractions 3, 5, 6, 9, 10, and 11, whereas fraction 12 exhibited the lowest antibacterial effects. Fractions 1, 2, 8, and fractions 13-43 showed no antibacterial activity against the tested bacterial isolates. However, the antibacterial activity of the fractions increased gradually and reached its highest level in fraction 4. Thereafter, the activity showed a slight decrease in the subsequent fractions until no antibacterial effect was observed from fraction 13 onward. Since fraction 4 was selected for GC–MS analysis to identify the bioactive compounds responsible for its antimicrobial effect. GC–MS analysis revealed the presence of several phytochemical compounds, particularly azuleno[4,5-b]furan-2(3H)-one derivative, dihydrodehydrocostus lactone, and (Z,Z, Z)-1,8,11,14-heptadecatetraene. These compounds are mainly sesquiterpene lactone, representing approximately 76% of the identified constituents. This finding is consistent with previous studies, which reported that *C. speciosus* extract contains azuleno[4,5-b] furan-2(3H)-one derivative with a highest abundance from 66-77.37%. These compounds are widely recognized for their antimicrobial applications due to their strong bioactive properties **(Ibrahim et al., 2019; Deabes et al. 2021** and **Gheraibia et al. 2022)**. The mass spectrum of the compound showed a characteristic fragmentation pattern with major ions at m/z 53, 65, 79, 91, 105, 117, 131, 150, 159, 201, 215, and 230. The fragment ions at m/z 105, 117, and 131 suggest sequential cleavage of side chains and loss of small hydrocarbon units. The molecular ion peak at m/z 230 confirms the proposed molecular weight of the compound, supporting its identification as an azulene-derived sesquiterpene lactone. This fragmentation pattern is consistent with that reported in GC–MS libraries.

Matching bioactive compounds with their biological targets is therefore critical for understanding their modes of action (MOA) and their subsequent effects on physiology **(Azad and Wright, 2012)**. Transmission electron microscopy (TEM) images showed ultrastructural changes for *S. aureus* and *E. coli* bacterial cells treated with fraction 4 of ethyl acetate extract, including rupture of the cell, and lysis of the cell wall and cellular organelles. These findings matched with those of **Swamy et al. (2016)** and **Choudhary et al. (2023)** who reported that bioactive compounds affect microbial cells through several mechanisms, including disruption of membrane function and structure (including efflux systems), induction of coagulation of cytoplasmic constituents, interference with intermediary metabolism,

inhibition of DNA/RNA synthesis and function, and disruption of normal cell-to-cell communication (quorum sensing). In particular, many compounds target the cytoplasmic membrane, altering its structure, integrity, permeability, and overall functionality in different ways**.**

The cytotoxicity assessment of the tested fraction against the normal human lung fibroblast (WI-38) cell line revealed a CC_{50} value of 23.92±0.86 µg/ml. There is a direct relation between the concentration of the sample and the inhibitory activity, when the concentration increases, the inhibitory effect increases, and cell viability decreases.

In the present study, the tested fraction exhibited potent cytotoxic activity against HepG2 hepatocellular carcinoma cells, with an IC_{50} value of 5.17±0.49 µg/ml. This finding indicates a strong antiproliferative effect and suggests the presence of highly active bioactive constituents within the fraction. The observed activity is consistent with previous studies that have shown that hexane, ethyl acetate, and methanol extracts of *C. speciosus* rhizomes possess significant antiproliferative activity against human colon adenocarcinoma (COLO 320 DM) cell lines. The authors reported that all tested extracts exhibited considerable antioxidant and antiproliferative activities in both dose- and time-dependent manners **(Baskar et al., 2012)**. Moreover, the methanolic leaf extract significantly reduced the viability of HepG2 cells and induced apoptosis through activation of caspase-3 **(Nair et al., 2014)**. Similarly, diosgenin, a major bioactive constituent of *C. speciosus*, exhibited notable anticancer activity against HepG2 cells with an IC_{50} value of 32.62 µg/mL and promoted apoptosis through the regulation of apoptotic pathways. In addition, diosgenin displayed stronger activity against breast adenocarcinoma (MCF-7) cells, with an IC_{50} value of 11.03 µg/mL. The apoptotic effect of diosgenin was associated with upregulation of death receptor-4 (DR4) and caspase-3 expression, indicating activation of apoptosis-related signaling pathways in MCF-7 cells **(Selim and Al Jaouni, 2015)**. In contrast to the modest anticancer activity reported for the methanolic extract of *C. speciosus* against MCF-7 cells (**Thabit, 2018)**.

Notably, the IC_{50} value obtained in the current study (5.17±0.49 µg/ml) was lower than the previously reported for diosgenin against HepG2 cells, suggesting that the investigated fraction possesses enhanced cytotoxic potency. This activity may be attributed to the high abundance of the major constituent identified in the fraction, azuleno derivative and dehydrocostuslactone, and/or possible synergistic interactions among the fraction components. This result agrees with **Hsu et al., (2009)** and **Thambi** & **Shafi, (2015)**.

In addition, the tested fraction exhibited cytotoxic activity against human lung carcinoma (A-549) cell line, with IC_{50} value of 8.27±0.61 µg/ml, suggesting that the bioactive constituents present in the fraction may contribute to its broad-spectrum anticancer potential of *C. speciosus*. These findings are consistent with previous studies demonstrating the anticancer activity of *C. speciosus* against lung cancer cells. Ethyl acetate root extract of *C. speciosus* significantly reduced the viability of A549 cells in a time- and dose-dependent manner and effectively suppressed lung cancer cell growth. The authors further suggested that the extract exerted its anticancer effects through the induction of cellular damage and inhibition of processes associated with cancer progression. Collectively, these findings support the potential of *C. speciosus* as a promising source of bioactive compounds for the development of novel anticancer agents **(Omar et al., 2022)**. The cytotoxic impact of the non-polar fraction of *C. speciosus* was seen on both positive ER (MCF-7) and triple-negative breast cancer cells (MDA-MB-231). It prevented the migration and colony development of cancer cells. Remarkably, non-polar fraction stopped the breast cancer cell cycles in the sub-

G1 phase. Through DNA damage, downregulation of mutant p53, and overexpression of cell cycle inhibitors p21 and p27, it caused apoptosis **(Bawakid et al., 2021).** The obtained results indicate that the extract possesses a selective cytotoxic effect toward cancer cells compared to normal cells, as evidenced by a selectivity index (SI) of 4.63. This suggests that the extract is more toxic to cancer cells while exhibiting relatively lower toxicity to normal cells, highlighting its potential as a promising anticancer agent. Such selectivity is a desirable feature in anticancer drug development, as it reduces the risk of adverse effects on healthy tissues.

In conclusion, the present study provides evidence that the plant extract possesses significant antibacterial and anticancer activities, supporting its potential application as a valuable natural source for the development of new antimicrobial and anticancer drugs.

Conclusion

In conclusion, the present study demonstrated the antibacterial potential *C. speciosus* against multi-drug resistant (MDR) and extensively drug-resistant (XDR) bacterial isolates obtained from different clinical and non-clinical sources. The ethyl acetate extract exhibited the most potent antibacterial effect among the tested extracts, indicating its richness in bioactive constituents responsible for the observed activity. Bioassay-guided fractionation followed by GC–MS analysis of the most active fraction revealed eight identified compounds, with azulene-related compounds as a the major constituent based on relative peak area (66%). In addition, the active fraction showed low cytotoxicity toward normal human cells, while exhibiting notable anticancer activity against liver and lung cancer cell lines. Overall, these findings suggest that *C. speciosus* is a promising natural source of bioactive compounds with potential therapeutic applications against MDR and XDR bacterial pathogens, as well as cancer cell lines.

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Author Contributions

NMS conceived the study, designed the research plan, and supervised the work. AAIM and ASM contributed to the supervision of the experimental work, provided technical and laboratory support, and participated in data interpretation. SSA carried out the experimental work, analyzed the data, and prepared the original draft of the manuscript. ASM critically revised, edited, and finalized the manuscript. All authors reviewed and approved the final version of the manuscript.

Ethical Approval

Not applicable. This manuscript doesn't refer to or imply the use of any animal or human data or tissues. The research is based on the use of bacterial isolates obtained from Microbiology and Immunology Department (National Research Centre) and Biotechnology Laboratory (Faculty of Science)

Data Availability Statements

The datasets generated and/or analyzed during the current study are included in this published article and its supplementary files. Additional data are available from the corresponding author on reasonable request.