Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

GC–MS Characterization and Biological Evaluation of Costus speciosus Rhizomes: Antibacterial Activity against MDR and XDR Bacteria, Ultrastructural and Morphological Alterations, and Anticancer Potential

Somaya S. A. Ahmed
Al-Azhar University
Nagwa M. Sidkey
Al-Azhar University
Amal A. I. Mekawey
Al-Azhar University
Asmaa S. Mansour
as.mansour@nrc.sci.eg

National Research Center

Article

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

Posted Date: November 10th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11321001/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

GC–MS Characterization and Biological Evaluation of *Costus speciosus* Rhizomes: Antibacterial Activity against MDR and XDR Bacteria, Ultrastructural and Morphological Alterations, and Anticancer Potential

Somaya S. A. Ahmed[1], Nagwa M. Sidkey[2], Amal A. I. Mekawey[3], Asmaa S. Mansour[4]

Faculty of Science (Girls branch), Al-Azhar University, Cairo, Egypt [1]

Department of Botany and Microbiology, Faculty of Science (Girls branch), Al-Azhar University, Cairo, Egypt [2]

Department of Microbiology, Regional Center of Mycology and Biotechnology, Al-Azhar University. Cairo, Egypt [3]

Department of Microbiology and Immunology, National Research Center, Dokki, Giza, Egypt [4]

Corresponding author* asmaasami85@yahoo.com; as.mansour@nrc.sci.eg

Orcid: Asmaa S. Mansour: 0000-0002-2307-0760

Abstract

The rapid emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) 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. A total of 29 bacterial isolates obtained from clinical specimens and meat products were subjected to antimicrobial susceptibility testing (AST) to determine their resistance profiles. Based on the observed resistance patterns, 11 representative MDR/XDR isolates, including *Staphylococcus aureus* (n=18), *Escherichia coli* (n=8), *Klebsiella pneumoniae* (n=1), *Serratia rubbidea* (n=1), and *Hafnia alvei* (n=1), were selected for further investigation. Rhizome extracts were prepared using ethanol, methanol, ethyl acetate, and cold water, and their antibacterial activities were initially screened using the agar well diffusion assay. The ethyl acetate extract showed the strongest antibacterial activity and was subsequently fractionated using column chromatography, yielding 43 fractions. The fractions were screened for antibacterial activity, and nine fractions exhibited activity, with fraction 4 showing the strongest antibacterial effect. The MIC and MBC values of the active fraction ranged from 0.063 –0.250 mg/mL each, respectively. Gas chromatography–mass spectrometry (GC–MS) analysis of fraction 4 identified 3 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%). Transmission electron microscopy (TEM) revealed severe ultrastructural alterations in treated bacterial cells, including cell wall disruption and cytoplasmic leakage. The cytotoxic activity of the most active fraction was subsequently evaluated against HepG2, A-549, and WI-38 cell lines. The fraction demonstrated potent cytotoxic activity against HepG2 and A-549 cells, with IC_{50} values of 5.17 ± 0.49 and 8.27±0.61 µg/mL, respectively, while the CC_{50} value against WI-38 cells was 23.92 ±0 .86 µg/ml, yielding selectivity indices (SI) of 4.63 and 2.89, respectively. In conclusion, the active fraction of *Costus speciosus* rhizome 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 [1]. 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) [2]**.**

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 [3,4].

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 acquisition of resistance determinants. Consequently, infections caused by these pathogens are associated with treatment difficulties, prolonged disease duration, and increased clinical burden [5].

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 transmitted through direct contact with contaminated hands, surfaces, or infected wounds [6]**.**

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 [7]. 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 [8].

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 [9].

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 [10, 11]. *C. speciosus* contains a high concentration of bioactive metabolites of commercial value, such as diosgenin and azulene, as well as considerable therapeutic potential [12].

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.

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, as shown in Table 1 and Figure 1. The XDR isolates showed susceptibility to only two out of eight antibiotic classes tested.

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

The antibacterial activity of the different *C. speciosus* rhizome extracts was evaluated agaisnt the tested drug-resistant bacterial isolates using the agar well diffuson assay. All extracts exhibited variable inhibitory activity against the tested isolates, with differences observed according to both the extract type and bacterial isolate. The ethyl acetate extract generally showed the srongest antibacterial activity, followed by the other tested extracts, whereas the aqueous extract exhibited compartively lower activity. The inhibition zones are presented in Table 2 and Figure 2.

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

The MIC and MBC of the *C. speciosus* ethyl acetate extract were determined against the tested drug-resistant bacterial isolates. The extract exhibited variable inhibitory and bactericidal activities among the tested isolates, with MIC values ranging from 0.031 to 0.125 mg/mL and corresponding MBC values ranging from 0.063 to 0.250 mg/mL. The MIC and MBC values are presented in Table 3.

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

The ethyl acetate extract of *C. speciosus* was subjected to column chromatography to separate its constituents into distinct fractions. A total of 43 fractions were obtained and subsequently evaluated for their antibacterial activity. The fractionation profile and the antibacterial activity of the obtained fractions are presented in Table 4. Data in Figure 3 (a, b, and c) shows that Fraction 4 achieves maximum efficiency against *S. aureus* (S13m and S1a), *E. coli* (E1), *K. pneumoniae* (K), *H. alvei* (H), and *S. rubbidea* (C). However, Fraction 8 has no effect, and this confirms the boundary between the two peaks.

Determination of MIC and MBC of the ethyl acetate Fraction 4 of *C. speciosus* against drug-resistant bacterial isolates

The MIC and MBC of Fraction 4 were determined against the tested drug-resistant bacterial isolates. The fraction showed variable inhibitory and bactericidal activities among the tested isolates, with the obtained MIC and MBC values summarized in Table 5.

GC-MS analysis of the ethyl acetate Fraction 4 of *C. speciosus*

As shown in Table 6 and Figures 4 and 5, GC–MS analysis of the selected ethyl acetate 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.

Ultrastructural and morphological alterations in *S. aureus* and *E. coli* following treatment with the ethyl acetate Fraction 4 of *C. speciosus*.

TEM was used to examine ultrastructural and morphological changes in *S. aureus* and *E. coli* following treatment with the ethyl acetate Fraction 4 of *C. speciosus*. Compared with the untreated cells, treated bacterial cells exhibited noticeable morphological alterations, including irregular cell shape, disruption of the cell membrane, and

changes in cellular integrity. Representative TEM images of untreated and Fraction 4-treated bacterial cells are presented in Figures 6 and 7.

Cytotoxicity assessment of the ethyl acetate Fraction 4 of *C. speciosus*

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

The ethyl acetate Fraction 4 of *C. speciosus* exhibited CC_{50} value of 23.92 ± 0.86 µg/mL against human lung fibroblast normal cell line, as shown in Table 7, Figure 8, Supplementary Table 1, and Supplementary Figure 1. CC_{50} of cisplatin drug against WI-38 cells = 20.67 ± 1.08 µg/ml.

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

As shown in Table 8, Figure 9&10, Supplementary Table 2, and Supplementary Figure 2, the ethyl acetate Fraction 4 exhibited cytotoxic activity against human liver carcinoma cell line with an IC_{50} value of 5.17 µg/mL, selectivity index (SI) = IC_{50} normal cells/IC_{50} cancer cells = 23.92/5.17 = 4.63, whereas IC_{50} of cisplatin drug against HepG-2 cells = 1.99 ± 0.12 µg/mL.

Morphological evaluation of cytotoxic activity against human liver carcinoma cell line (HepG2 cells)

Morphological changes of human liver carcinoma cells are displayed in Table (10).

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

Data presented in Table 9, Figure 11&12, Supplementary Table 3, and Supplementary Figure 3 showed a concentration-dependent inhibitory effect of the ethyl acetate Fraction 4 of *C. speciosus* against human lung carcinoma (A-549) cells, with an IC_{50} value of 8.27 ± 0.61 µg/mL, whereas, IC_{50} of cisplatin drug against A-549 cells = 6.41 ± 0.63 µg/mL.

Morphological evaluation of cytotoxic activity against human lung carcinoma cell line (A-549 cells)

Morphological changes of human lung carcinoma cells are displayed in Table (12).

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 [26]. 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 [27-29].

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 [30]. These antibacterial findings are in agreement with those reported by authors [31,32] 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* [33].

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 [31,32]. 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 ethyl acetate extract exhibited the highest antibacterial activity, which may be attributed to its intermediate polarity and its ability to extract and enrich moderately polar bioactive secondary metabolites.

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 0.031 to 0.125 and 0.063 to 0.250 mg/mL, respectively, demonstrating that the ethyl acetate extract possessed effective inhibitory and bactericidal activities against the tested bacterial isolates.

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 [34,35]. 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 biological effects and subsequent impacts on cellular physiology [36]. 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 findings of [37,38] 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 [39]. Moreover, the methanolic leaf extract significantly reduced the viability of HepG2

cells and induced apoptosis through activation of caspase-3 [40]. 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 [41]. In contrast to the modest anticancer activity reported for the methanolic extract of *C. speciosus* against MCF-7 cells [42].

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 [43,44].

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 [45]. 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 [46]. 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.

The present study has some limitations. The GC–MS analysis of the silylated ethyl acetate fraction characterized mainly derivatizable and GC-amenable constituents and may not represent the complete phytochemical profile, particularly non-volatile compounds. In addition, while TEM demonstrated ultrastructural and morphological alterations, further mechanistic studies are required, and the promising cytotoxicity results obtained using normal and cancer cell lines require further in vivo studies.

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 [13].

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 [14].

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 [15].

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

Agar well diffusion method

Antibacterial activity of different extracts of *C. speciosus* 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 [16,17]. 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 rhizome extract was studied at different concentrations. The lowest concentration with no visible turbidity in the test tube was determined as the MIC value [18].

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 [19].

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. speciosus* 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, 43 fractions were collected from *C. speciosus.* 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 [20].

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) [18].

TEM analysis of ultrastructural and morphological alterations

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 [21].

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. Cisplatin drug was used as a standard antitumor drug.

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 (IC_{50}), 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) [22-25].

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.

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.

Ethics Declarations

Data Availability

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.

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

References

1. Zouine N, El Ghachtouli N, El Abed S, Koraichi SI (2024) A comprehensive review on medicinal plant extracts as antibacterial agents: Factors, mechanism insights and future prospects. Scientific African 26:e02395. https://doi.org/10.1016/j.sciaf.2024.e02395
2. Tanwar J, Das S, Fatima Z, Hameed S (2014) Multidrug resistance: an emerging crisis. Interdisciplinary perspectives on infectious diseases 541340. https://doi.org/10.1155/2014/541340
3. Syame SM, Mansour AS, Khalaf DD, Ibrahim ES, Gaber ES (2020) Green synthesis of silver nanoparticles using lactic acid bacteria: Assessment of antimicrobial activity. World Vet J 10(4):625-633. https://doi.org/10.54203/scil.2020.wvj75

4. Belay WY, Getachew M, Tegegne BA, Teffera ZH, Dagne A, Zeleke TK, Abebe RB, Gedif AA, Fenta A, Yirdaw G, Tilahun A and Aschale Y (2024) Mechanism of antibacterial resistance, strategies and next-generation antimicrobials to contain antimicrobial resistance: a review. Front Pharmacol 15:1444781. https://doi.org/10.3389/fphar.2024.1444781
5. Samy AA, Mansour AS, Khalaf DD, Khairy EA (2022) Development of multidrug-resistant *E. coli* in some Egyptian veterinary farms. Vet World 15(2):488–495. https://doi.org/10.14202/vetworld.2022.488-495
6. Alghamdi BA, Al-Johani I, Al-Shamrani JM, Alshamrani HM, Al-Otaibi BG, Almazmomi K, Yusof NY (2023) Antimicrobial resistance in methicillin-resistant *Staphylococcus aureus*. Saudi journal of biological sciences 30(4):103604. https://doi.org/10.1016/j.sjbs.2023.103604
7. Frieri M, Kumar K, Boutin A (2017) Antibiotic resistance. Journal of Infection and Public Health. 10(4):369-378. https://doi.org/10.1016/j.jiph.2016.08.007
8. Mirpour M, Siahmazgi ZG, Kiasaraie MS (2015) Antibacterial activity of clove, gall nut methanolic and ethanolic extracts on *Streptococcus mutans* PTCC 1683 and *Streptococcus salivarius* PTCC 1448. Journal of oral biology and craniofacial research, 5(1):7-10. https://doi.org/10.1016/j.jobcr.2015.02.002
9. Ahmed HY, Kareem SM, Atef A, Safwat NA, Shehata RM, Yosri M, Youssef M, Baakdah MM, Sami R, Baty RS et al (2022) Optimization of Supercritical Carbon Dioxide Extraction of *Saussurea costus* Oil and Its Antimicrobial, Antioxidant, and Anticancer Activities. Antioxidants 11(10):1960. https://doi.org/10.3390/antiox11101960
10. Maji P, Dhar DG, Misra P, Dhar P (2020) *Costus speciosus* (Koen ex. Retz.) Sm.: Current status and future industrial prospects. Industrial crops and products 152:112571. https://doi.org/10.1016/j.indcrop.2020.112571
11. Ravikumar S, Pandiyan V, Alam M, Ahmad N, Nithya V, Krishnakumar B, Sobral AJFN (2021) *Costus speciosus* koen leaf extract assisted CS-ZNX (X= O or S) nanomaterials: synthesis, characterization and photocatalytic degradation of rr 120 dye under UV and direct sunlight. Journal of Molecular Structure 1225:129176. https://doi.org/10.1016/j.molstruc.2020.129176
12. Aslam RW, Shu H, Yaseen A (2023) Monitoring the population change and urban growth of four major Pakistan cities through spatial analysis of open source data. Annals of GIS 29(3):355-367. https://doi.org/10.1080/19475683.2023.2166989
13. Helmy YA, Abdelaziz KT, Hawwas HAE, Ghosh S, AlKafaas SS, Moawad MMM, Saied EM et al (2023) Antimicrobial resistance and recent alternatives to antibiotics for the control of bacterial pathogens with an emphasis on foodborne pathogens. Antibiotics 12(2):274. https://doi.org/10.3390/antibiotics12020274
14. Gajic I, Kabic J, Kekic D, Jovicevic M, Milenkovic M, Mitic Culafic D, Trudic A et al (2022) Antimicrobial Susceptibility Testing: A Comprehensive Review of Currently Used Methods. Antibiotics 11(4):427. https://doi.org/10.3390/antibiotics11040427

15. Mazimba O, Wale K, Kwape TE, Mihigo SO, Kokengo BM (2015) Cinnamomum verum: Ethyl acetate and methanol extracts antioxidant and antimicrobial activity. Journal of Medicinal Plants Studies 3(3):28-32. https://api.semanticscholar.org/CorpusID:9498770
16. Syame SM, Mohamed SM, Elgabry EA, Darwish YAA, Mansour AS (2022) Chemical characterization, antimicrobial, antioxidant, and cytotoxic potentials of *S. mahagoni*. AMB Expr 12:77. https://doi.org/10.1186/s13568-022-01406-w
17. Montaser AS, Abdelhamed M, Elshahid ZA, Mansour AS, Abdelrahman SS, Rehan M (2026) Multifunctional Collagen–Hyaluronic Acid Scaffolds Loaded with Propranolol as Platforms for Accelerated Diabetic Wound Healing. Industrial & Engineering Chemistry Research. https://api.semanticscholar.org/CorpusID:286613741
18. Ragab TIM, Mansour AS, Khalaf DD, Elshamy AI, El-Gendy AG (2023) Antimicrobial effects of essential oils of *Artemisia annua*, *Mentha longifolia*, and *Vitex agnus-castus* and their nanoemulsions against pathogenic microbes causing cattle mastitis. Egypt J Chem 66(3):483–493. https://doi.org/10.21608/ejchem.2022.147800.6430
19. Khalaf DD, Ragab TIM, Mansour AS, Elshamy AI, El-Gendy AENG (2022) Chemically characterized essential oils of *Citrus reticulata* and *Thymus vulgaris* L. and their nanoemulsions inhibited the pathogenic microbes causing mastitis in cattle. J of Essent Oil Bear Plants 25(5):953–966. https://doi.org/10.1080/0972060X.2022.2133975
20. Srivastava N, Singh A, Kumari P, Nishad JH, Gautam VS, Yadav M, Bharti R et al (2021) Chapter 21 - Advances in extraction technologies: isolation and purification of bioactive compounds from biological materials. Natural Bioactive Compounds Technological Advancements 409-433. https://doi.org/10.1016/B978-0-12-820655-3.00021-5
21. Amin BH, Amer A, Azzam M, Abd Elsattar NEA, Mahmoud D, Al-Ashaal S, Al-khalaf AA, Hozzein WN (2022) Antimicrobial and anticancer activities of *Periplaneta Americana* tissue lysate: An *in vitro* study. Journal of King Saud University-Science 34:102095. https://doi.org/10.1016/j.jksus.2022.102095
22. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55-63.
23. Gomha SM, Riyadh SM, Mahmoud EA, Elaasser MM (2015) Synthesis and Anticancer Activities of Thiazoles, 1,3-Thiazines, and Thiazolidine Using Chitosan-Grafted-Poly (vinyl pyridine) as Basic Catalyst. Heterocycles 91(6):1227-1243. 10.3987/COM-15-13210
24. Huda SA, Moh S (2022) Survey on computation offloading in UAV-Enabled mobile edge computing. Journal of Network and Computer Applications 201:103341. https://doi.org/10.1016/j.jnca.2022.103341
25. Abdelsalam EA, Abd El-Hafeez AA, Eldehna WM, El Hassab MA, Marzouk HMM, Elaasser MM et al (2022) Discovery of novel thiazolyl-pyrazolines as dual EGFR and VEGFR-2 inhibitors endowed with in vitro antitumor activity towards non-small lung cancer. Journal of Enzyme Inhibition and Medicinal Chemistry 37(1):226. https://doi.org/10.1080/14756366.2022.2104841

26. Abdelmoneim SA, Ghazy RM, Sultan EA, Hassan MA, Mahgoub MA (2024) Antimicrobial resistance burden pre and post-COVID-19 pandemic with mapping the multidrug resistance in Egypt: a comparative cross-sectional study. Sci Rep 14:7176. https://doi.org/10.1038/s41598-024-56254-4
27. Bahshwan SM, Aljehany BM (2020) A review on the therapeutic and medicinal activities of *Costus Speciosus*. Pharmacophore 11(3):124-129.
28. Nadda RK, Ali A, Goyal RC, Khosla PK, Goyal R (2020) *Aucklandia costus* (syn. *Saussurea costus*): Ethnopharmacology of an endangered medicinal plant of the Himalayan region. Journal of Ethnopharmacology 263:113199. https://doi.org/10.1016/j.jep.2020.113199
29. Singh VK, Yadav K, Thomas SC, Gupta A, Luqman S, Shanker K et al (2024) An Update on Pharmacological and Phytochemical Aspects of Costus pictus D. Don-A Promising Anti-diabetic Plant. Current Topics in Medicinal Chemistry 24(9):810-829. https://api.semanticscholar.org/CorpusID:267318705
30. Arya P, Vaidya D, Kaushal M et al (2025) Effects of different solvents on phytochemical constituents, in-vitro antimicrobial activity, and volatile components of *Boehmeria rugulosa* Wedd. wood extract. Sci Rep 15:29135. https://doi.org/10.1038/s41598-025-14506-x
31. Duraipandiyan V, Al-Harbi NA, Ignacimuthu S, Muthukumar CH (2012) Antimicrobial activity of sesquiterpene lactones isolated from traditional medicinal plant, *Costus speciosus* (Koen ex.Retz.) Sm. BMC Complement and Altern Med 12(13). https://doi.org/10.1186/1472-6882-12-13
32. Reda NM, Salim HGH, Hassan KKAA, Habeeb SA (2024) Study The Effect of The Alcoholic and Hot Aqueous Extract of *Costus specious* on Some Bacterial Species. Research & Reviews: A Journal of Life Sciences 14 (03):13-18. https://journals.stmjournals.com/rrjols/article=2024/view=169987
33. Adesegun OO (2021) Isolation and Characterization of Secondary Metabolites and Essential Oils from *Costus lucanusianus* J. Braun & K. Schum and Their Antimicrobial Activity (Doctoral dissertation).
34. Deabes MM, Fatah AE, Sally I, Salem SHE, Naguib KM (2021) Antimicrobial activity of bioactive compounds extract from *Saussurea costus* against food spoilage microorganisms. Egyptian Journal of Chemistry 64(6):2833-2843. 10.21608/ejchem.2021.69572.3528
35. Gheraibia S, Belattar N, Diab KA, Hassan ME, El-Nekeety AA, Abdel-Aziem SH et al (2022) *Costus speciosus* extract protects against the oxidative damage of zearalenone via modulation of inflammatory cytokines, Nrf2, and INOS gene expression in rats. Toxicon 214:62-73. https://doi.org/10.1016/j.toxicon.2022.05.002
36. Azad MA, Wright GD (2012) Determining the mode of action of bioactive compounds. Bioorganic & medicinal chemistry 20(6):1929-1939. https://doi.org/10.1016/j.bmc.2011.10.088
37. Swamy MK, Akhtar MS, Sinniah UR (2016) Antimicrobial Properties of Plant Essential Oils against Human Pathogens and Their Mode of Action: An Updated Review. Evidence-Based Complementary and Alternative Medicine 3012462:1-21. https://doi.org/10.1155/2016/3012462

38. Choudhary N, Rai A, Kuniyal JC, Srivastava P, Lata R, Dutta M, Kotnala RK (2023) Chemical characterization and source apportionment of PM10 using receptor models over the Himalayan Region of India. Atmosphere 14(5):880. https://doi.org/10.3390/atmos14050880
39. Baskar AA, Al Numair KS, Alsaif MA, Ignacimuthu S (2012) In vitro antioxidant and antiproliferative potential of medicinal plants used in traditional Indian medicine to treat cancer. Redox Rep: Communications in Free Radical Research 17(4):145-156. https://doi.org/10.1179/1351000212Y.0000000017
40. Nair KP, Nair AB, Joseph R (2014) Carboxylated acrylonitrile butadiene rubber latex/kaolin nanocomposites: preparation and properties. Composite Interfaces 21(6):571-583. https://doi.org/10.1080/15685543.2014.899198
41. Selim S, Al Jaouni S (2015) Anticancer and apoptotic effects on cell proliferation of diosgenin isolated from *Costus speciosus* (Koen.) Sm. BMC complementary and alternative medicine 15(1):301. https://doi.org/10.1186/s12906-015-0836-8
42. Thabit ZA (2018) Evaluation of some bioactive effects of phenolic compounds in *Costus speciosus* rhizome extract. Iraqi Journal of Science 59(1A):38-43. 10.24996/ijs.2018.59.1A.6
43. Hsu YL, Wu LY, Kuo PL (2009) Dehydrocostuslactone, a medicinal plant-derived sesquiterpene lactone, induces apoptosis coupled to endoplasmic reticulum stress in liver cancer cells. The Journal of pharmacology and experimental therapeutics 329(2):808-819. https://doi.org/10.1124/jpet.108.148395
44. Thambi M, Shafi MP (2015) Rhizome essential oil composition of *Costus speciosus* and its antimicrobial properties. Int J of Pharm Res & All Sci 4(1):28-32. www.ijpras.com
45. Omar AS, Nasraldeen RA, Albiheyri R, Kadi RH, Abo-Aba SEM (2022) The Effects of *Costus speciosus* Root Extract on Cultured Human Lung Cancer Cells, A549. Biosc Biotech Res Comm 15(1). http://dx.doi.org/10.21786/bbrc/15.1.25
46. Bawakid NO, Abdel-Lateff A, El-Senduny FF, Alarif WM (2021) *Costus speciosus* J Koenig (Costaceae) exerts anti-proliferative effect on breast cancer cells via induction of cell cycle arrest and inhibition of activity of metalloproteinase-2. Tropical Journal of Pharmaceutical Research 20(7):1365-1372. http://dx.doi.org/10.4314/tjpr.v20i7.7

Figure legends

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

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

Fig. 3a Antibacterial activity of different fractions obtained from column chromatography of the ethyl acetate extract of *C. speciosus* against *S. aureus* (S1a) and *S. aureus* (S13m), expressed as mean inhibition zone diameter (mm).

Fig. 3b Antibacterial activity of different fractions obtained from column chromatography of the ethyl acetate extract of *C. speciosus* against *E. coli* (E1) and *K. pneumoniae* (K), expressed as mean inhibition zone diameter (mm).

Fig. 3c Antibacterial activity of different fractions obtained from column chromatography of the ethyl acetate extract of *C. speciosus* against *Hafnia alvei* (H) and *S. rubbidea* (C), expressed as mean inhibition zone diameter (mm).

Fig. 4 GC–MS chromatogram illustrating the identified phytochemical compounds in the selected ethyl acetate Fraction 4 of *C. speciosus*, 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.

Fig. 6a Transmission electron microscopy (TEM) image of untreated *S. aureus* (S1a); arrows indicate 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 ethyl acetate Fraction 4 of *C. speciosus*, showing ultrastructural alterations compared to untreated control cells; arrows indicate cell wall disruption, membrane leakage, cytoplasmic leakage, and loss of cellular integrity.

Fig. 7a Transmission electron microscopy (TEM) image of untreated *E. coli* (E1); arrows indicate 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 ethyl acetate Fraction 4 of *C. speciosus*, showing ultrastructural alterations compared to untreated control cells; arrows indicate cell wall disruption, membrane leakage, cytoplasmic leakage, and loss of cellular integrity.

Fig. 8 Dose-response curve of the ethyl acetate Fraction 4 of *C. speciosus* against human lung fibroblast normal cell line

Fig. 9 Dose-response curve of the ethyl acetate Fraction 4 of *C. speciosus* against human liver carcinoma cell line

Fig. 10 Microscopic examination of HepG2 cell line cancer cells treated with different concentrations of the ethyl acetate Fraction 4 of *C. speciosus*. Compared with the untreated control, a concentration-dependent reduction in cell density was observed, with progressively fewer adherent cells at higher concentrations.

Fig. 11 Dose-response curve of the ethyl acetate Fraction 4 of *C. speciosus* against human lung carcinoma cell line

Fig. 12 Microscopic examination of A-549 cell line cancer cells treated with different concentrations of the ethyl acetate Fraction 4 of *C. speciosus*. Compared with the untreated control, a concentration-dependent reduction in cell density was observed, with progressively fewer adherent cells at higher concentrations.

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.

Competing Interests

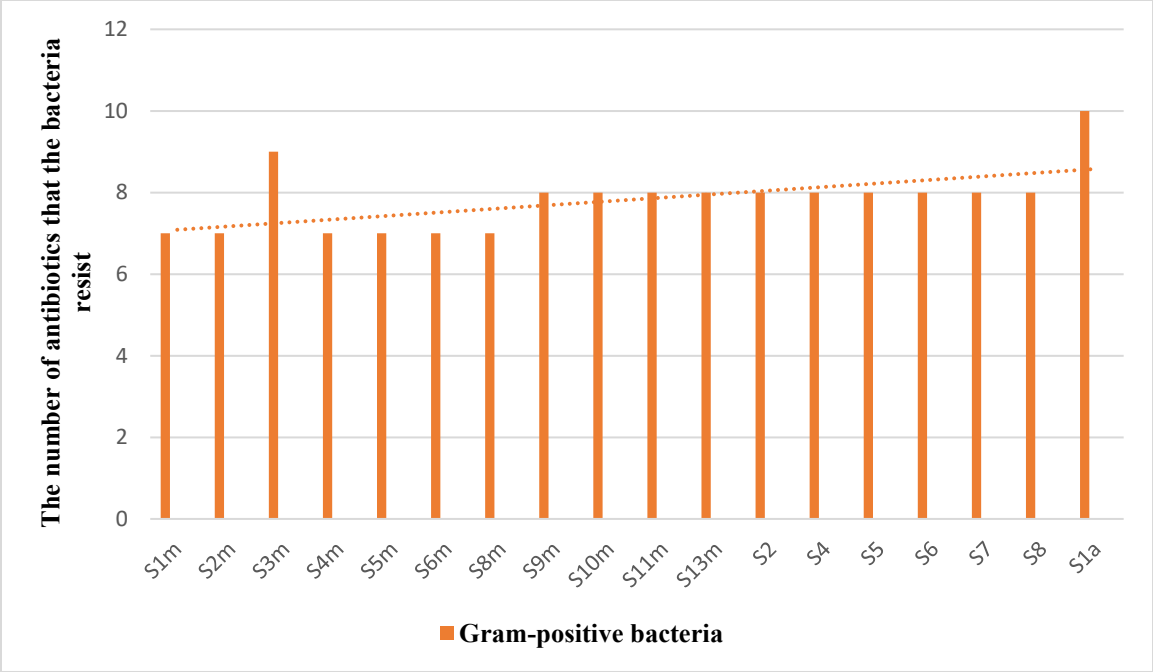
The authors have no relevant financial or non-financial interests to disclose.

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.



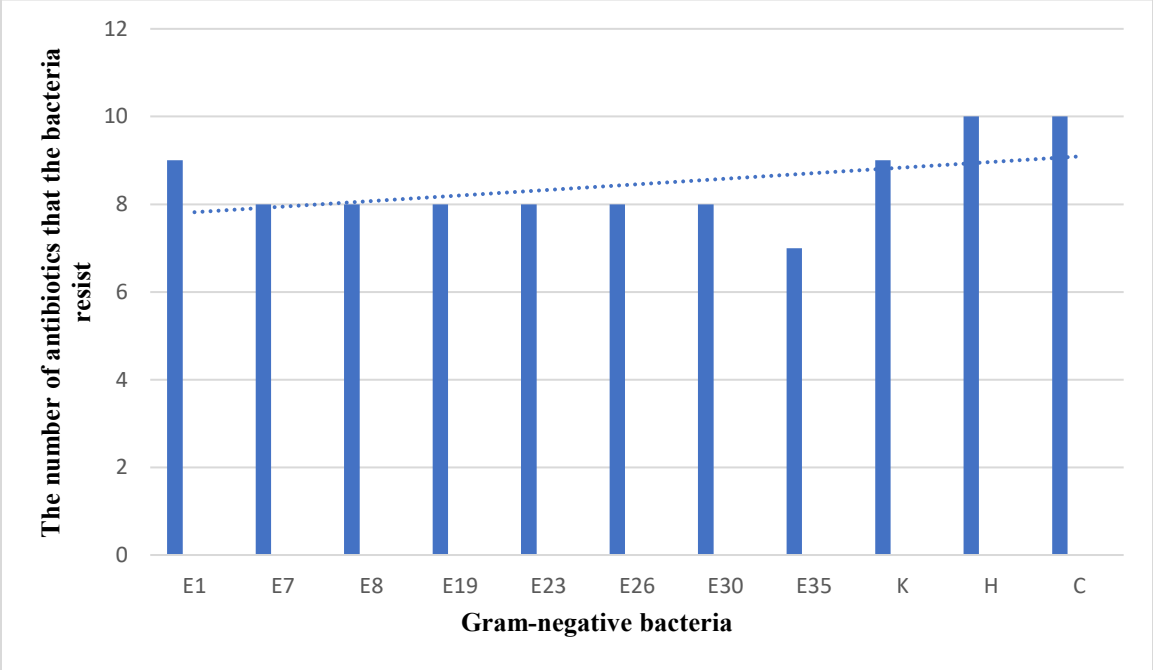


Figure 1. Antimicrobial susceptibility testing (AST) of the tested bacterial isolates using the Kirby-Bauer disk diffusion method on Muller-Hinton agar. Representative plates showing zones of inhibition around different antibiotic discs after incubation at 37º C for 18-24 h, indicating sensitive, intermediate,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

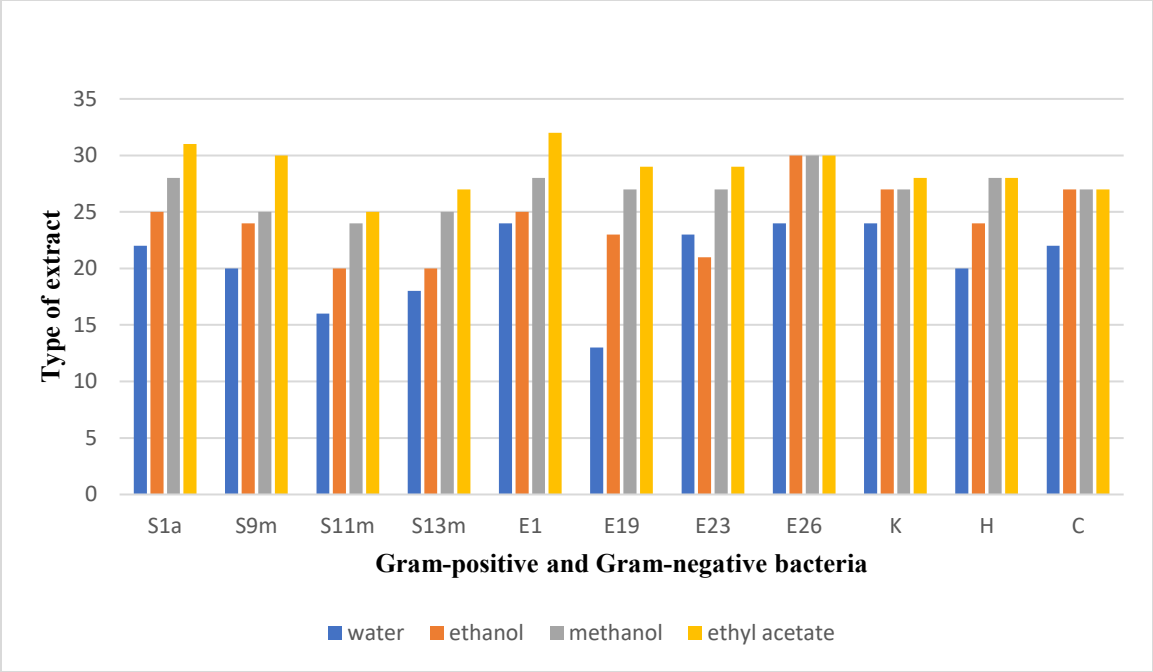


Figure 2. Antibacterial activity of different plant extracts of *C. speciosus* against drug-resistant bacterial isolates, expressed as zones of inhibition (mm) after incubation at 37°C for 18-24 h. Results are shown as mean ±SD

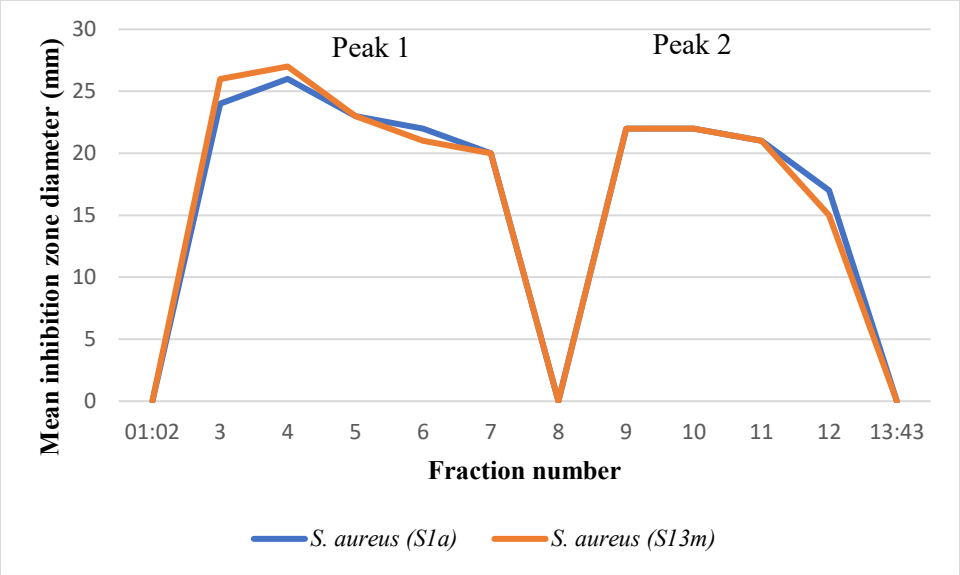


Figure 3a. Antibacterial activity of different fractions obtained from column chromatography of the ethyl acetate extract of *C. speciosus* against *S. aureus* (S1a) and *S. aureus* (S13m), expressed as mean inhibition zone diameter (mm).

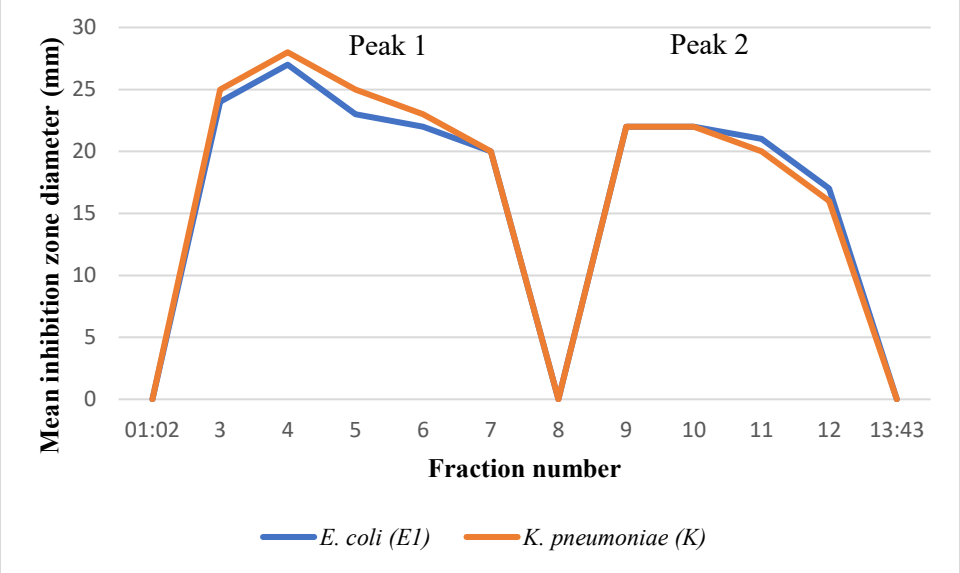


Figure 3b. Antibacterial activity of different fractions obtained from column chromatography of the ethyl acetate extract of *C. speciosus* against *E. coli* (E1) and *K. pneumoniae* (K), expressed as mean inhibition zone diameter (mm).

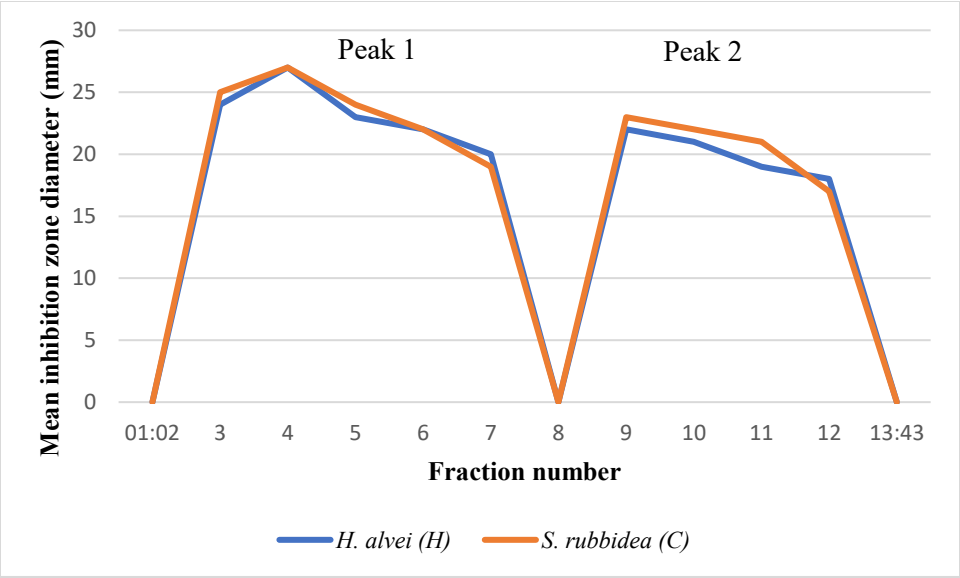


Figure 3c. Antibacterial activity of different fractions obtained from column chromatography of the ethyl acetate extract of *C. speciosus* against *Hafnia alvei* (H) and *S. rubbidea* (C), expressed as mean inhibition zone diameter (mm).

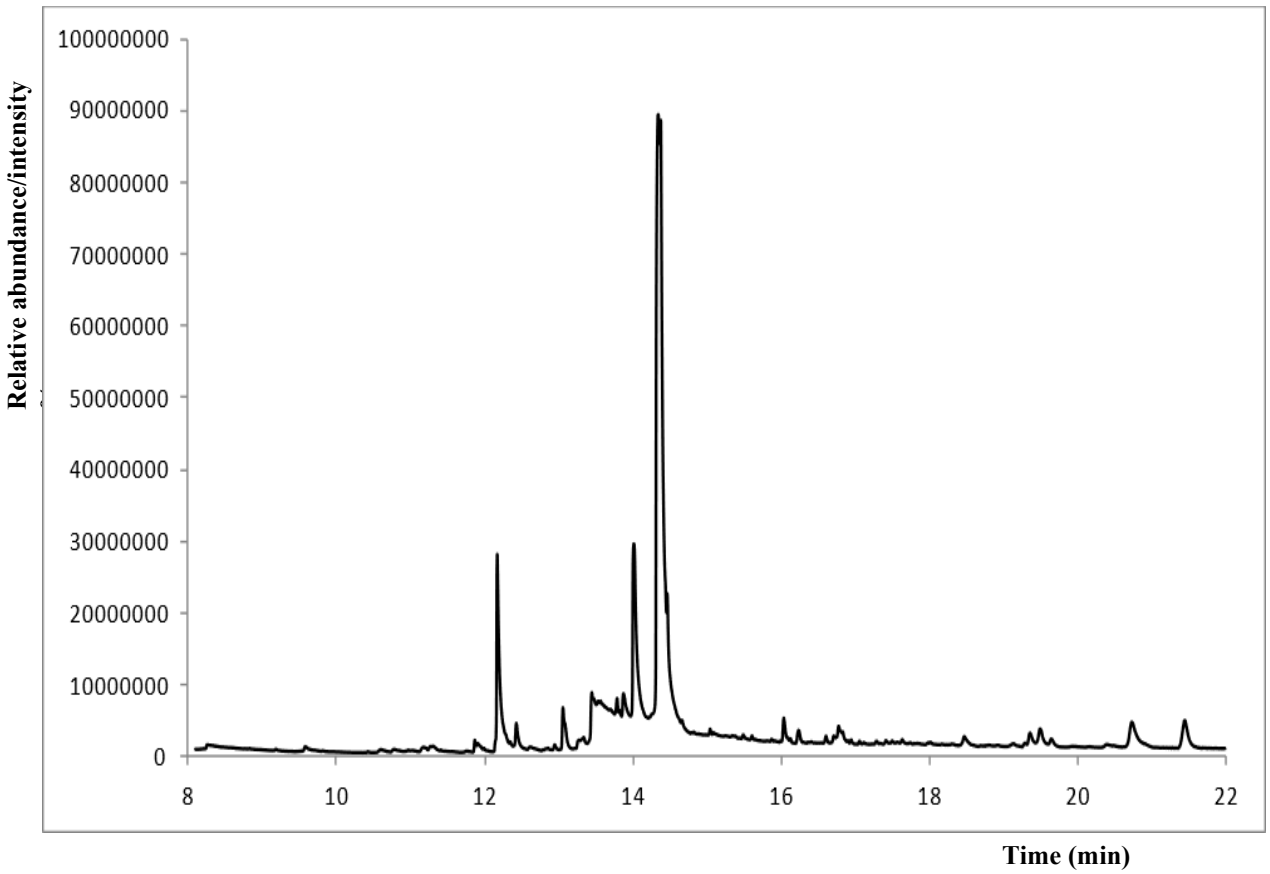


Figure 4. GC–MS chromatogram illustrating the identified phytochemical compounds in the selected ethyl acetate Fraction 4 of *C. speciosus*, including major constituent such as azuleno[4,5-b]furan-2(3H)-one derivative and other terpenoids, fatty acid esters, and aromatic compounds.

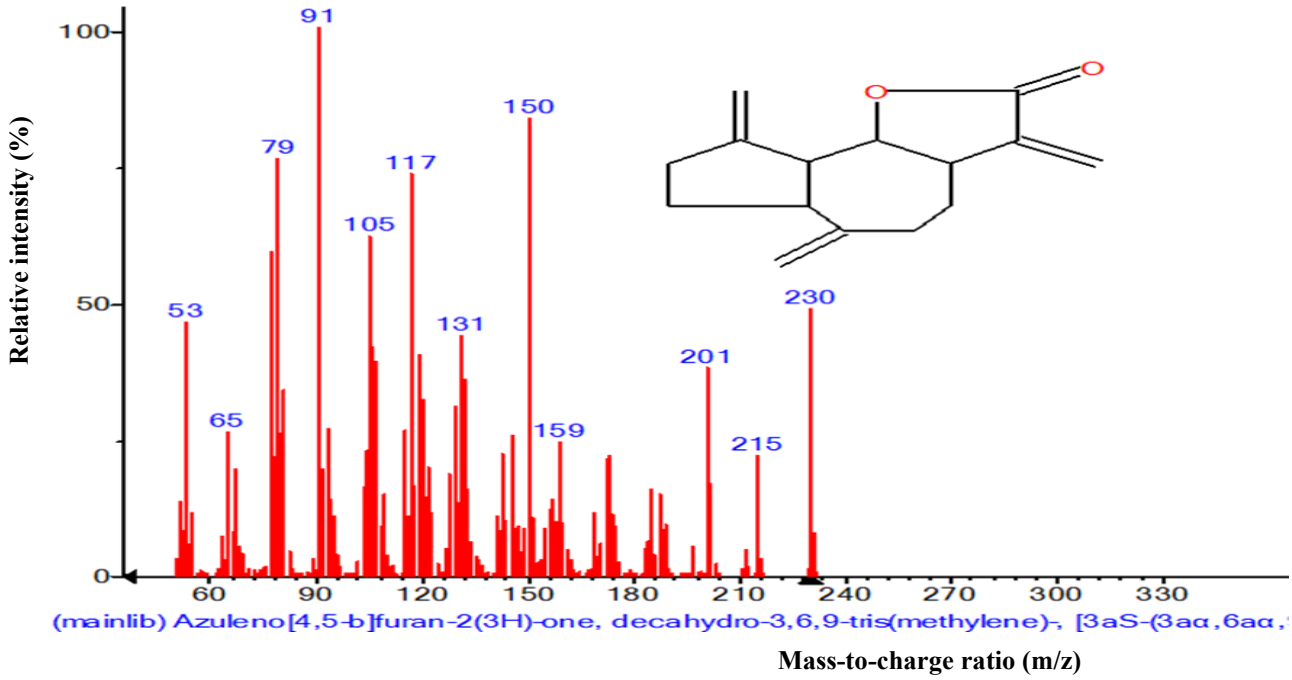


Figure 5. Mass spectrum showing fragmentation pattern of azuleno[4,5-b]furan derivative obtained from GC–MS analysis.

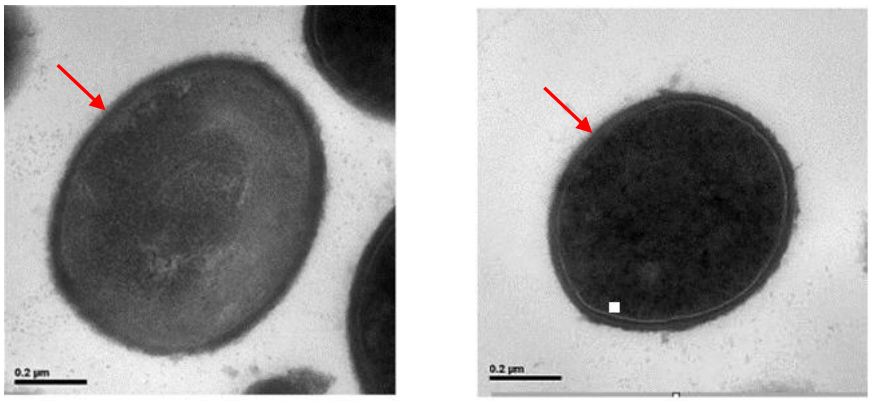


Figure 6a. Transmission electron microscopy (TEM) image of untreated *S. aureus* (S1a); arrows indicate normal spherical (cocci) morphology with intact cell wall and membrane structure under standard growth conditions.

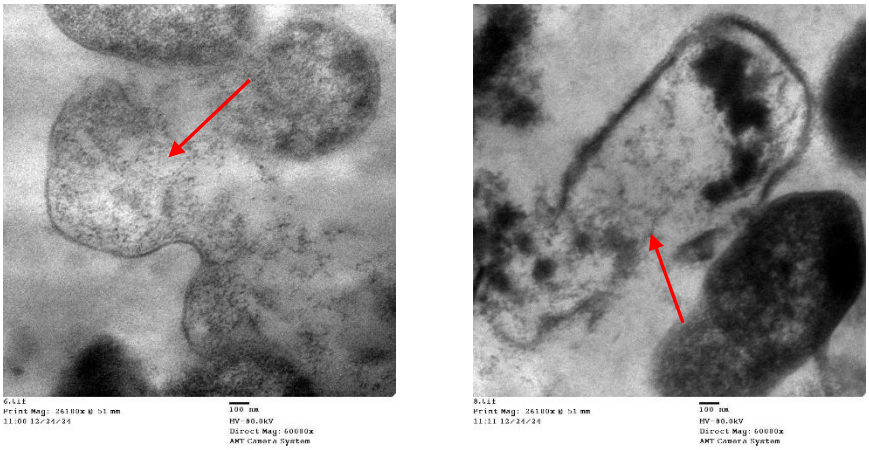


Figure 6b. Transmission electron microscopy (TEM) image of *S. aureus* (S1a) treated with ethyl acetate Fraction 4 of *C. speciosus*, showing ultrastructural alterations compared to untreated control cells; arrows indicate cell wall disruption, membrane leakage, cytoplasmic leakage, and loss of cellular integrity.

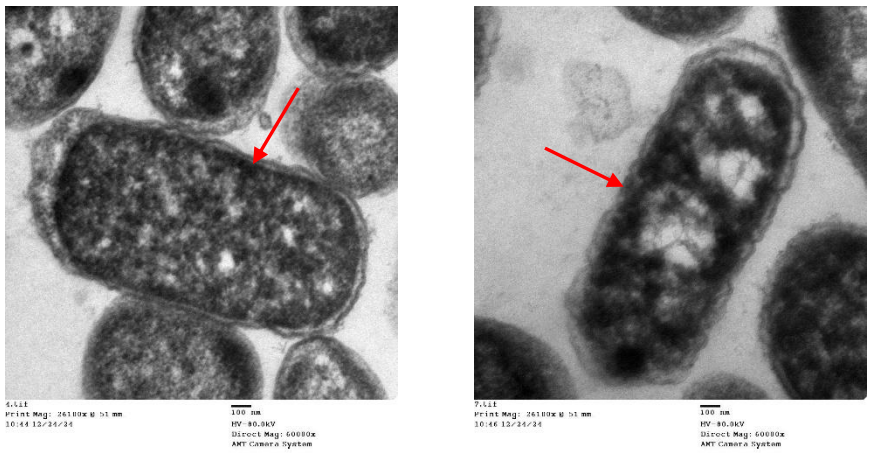


Figure 7a. Transmission electron microscopy (TEM) image of untreated *E. coli* (E1); arrows indicate normal rod-shaped (bacilli) morphology with intact cell wall and membrane structure under standard growth conditions.



Figure 7b. Transmission electron microscopy (TEM) image of *E. coli* treated with ethyl acetate Fraction 4 of *C. speciosus*, showing ultrastructural alterations compared to untreated control cells; arrows indicate cell wall disruption, membrane leakage, cytoplasmic leakage, and loss of cellular integrity.

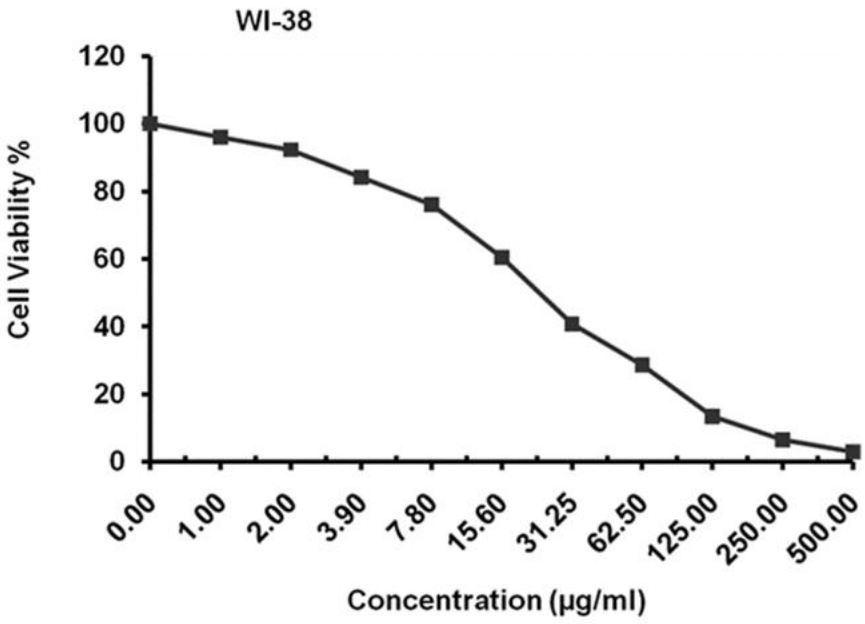


Figure 8. Dose-response curve of the ethyl acetate Fraction 4 of *C. speciosus* against human lung fibroblast normal cell line

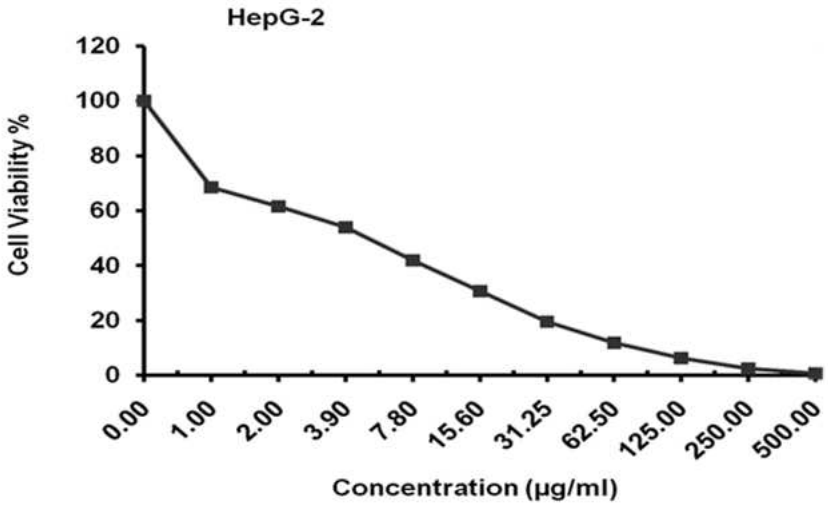


Figure 9. Dose-response curve of the ethyl acetate Fraction 4 of *C. speciosus* against human liver carcinoma cell line

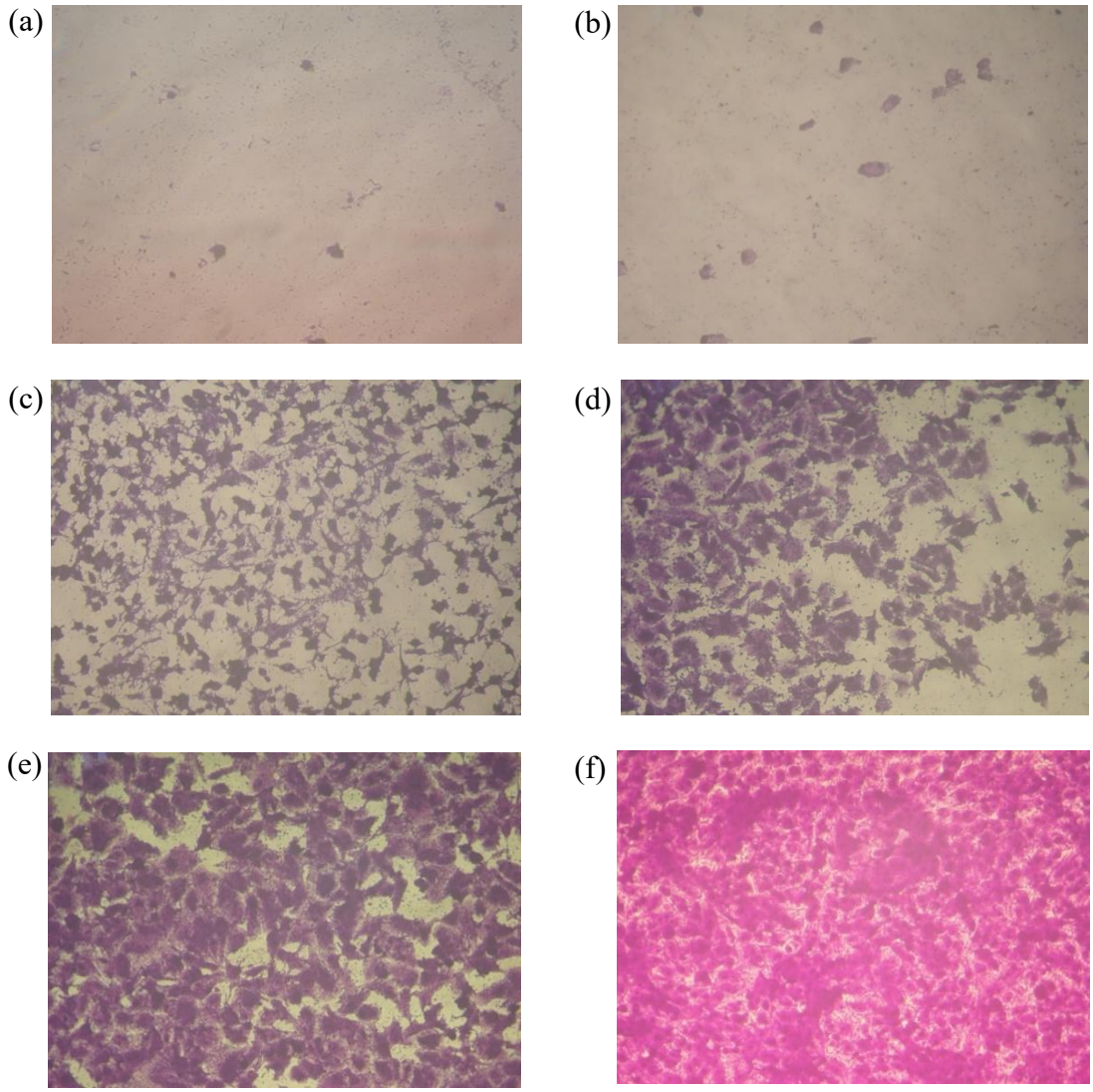


Figure 10. Microscopic examination of HepG2 cell line cancer cells treated with different concentrations of the ethyl acetate Fraction 4 of *C. speciosus*. Compared with the untreated control, a concentration-dependent reduction in cell density was observed, with progressively fewer adherent cells at higher concentrations.

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)

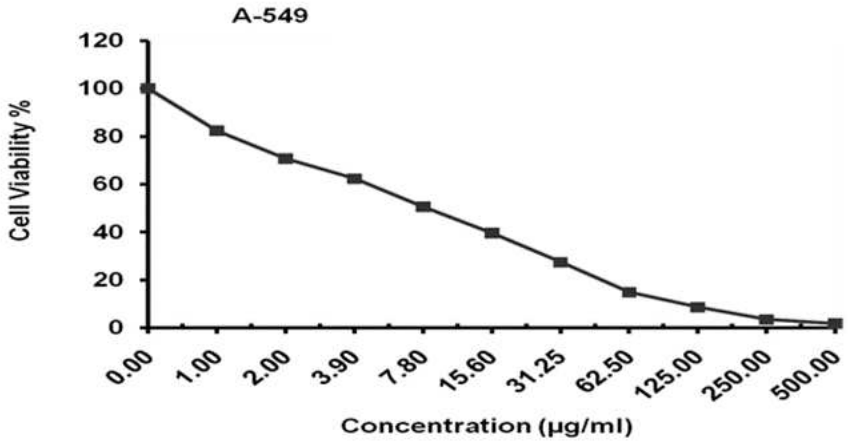


Figure 11. Dose-response curve of the ethyl acetate Fraction 4 of *C. speciosus* against human lung carcinoma cell line.

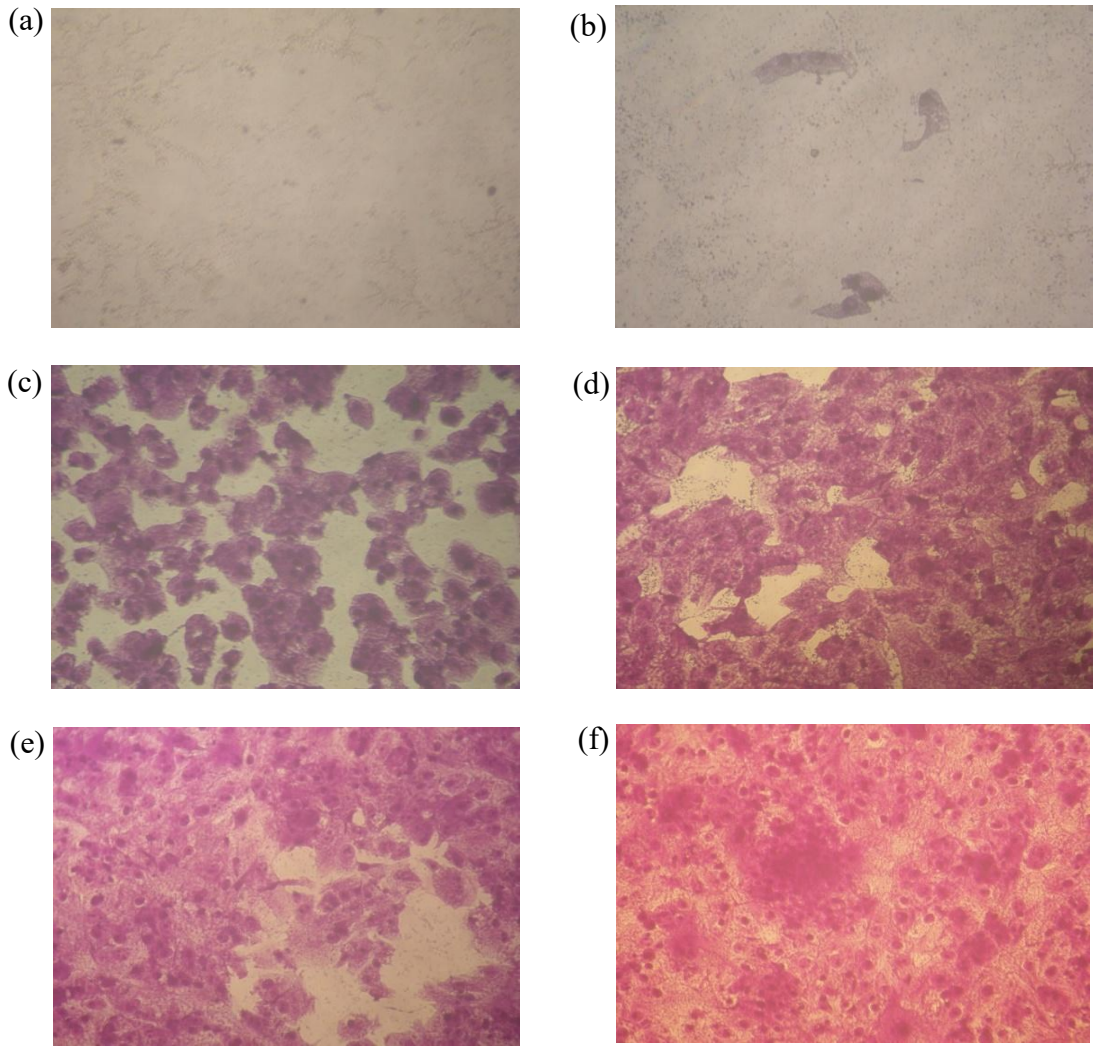


Figure 12. Microscopic examination of A-549 cell line cancer cells treated with different concentrations of the ethyl acetate Fraction 4 of *C. speciosus*. Compared with the untreated control, a concentration-dependent reduction in cell density was observed, with progressively fewer adherent cells at higher concentrations.

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

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

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

Isolate	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.

Table (3). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethyl acetate extract of *C. speciosus* against drug-resistant bacterial isolates.

Isolate	Bacteria	MIC (mg/mL)	MBC (mg/mL)
Gram-positive bacteria			
1	*S. aureus* (S1a)	0.031	0.063
2	*S. aureus* (9m)	0.063	0.063
3	*S. aureus* (11m)	0.063	0.125
4	*S. aureus* (13m)	0.031	0.063
Gram-negative bacteria			
5	*E. coli* (E1)	0.063	0.125
6	*E. coli* (E19)	0.031	0.063
7	*E. coli* (E23)	0.031	0.063
8	*E. coli* (E26)	0.063	0.125
9	*K. pneumoniae* (K)	0.063	0.063
10	*H. alvei* (H)	0.063	0.125
11	*S. rubbidea* (C)	0.125	0.250

No statistically significant differences were observed in MIC and MBC values among the studied groups of different bacterial species, with p-values of 0.369 and 0.287, respectively. In contrast, a statistically significant difference was observed between MIC and MBC values (Wilcoxon signed-rank test, $p = 0.007$), with MBC values being significantly higher than MIC values.

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.

Table (5). Minimum inhibitory concentration (MIC) and minimum inhibitory concentration (MBC) of the ethyl acetate extract Fraction 4 of *C. speciosus*

Isolate	Bacteria	MIC (mg/mL)	MBC (mg/mL)
1	*S. aureus* (S1a)	0.063	0.125
2	*S. aureus* (9m)	0.063	0.063
3	*S. aureus* (11m)	0.063	0.125
4	*S. aureus* (13m)	0.063	0.125
5	*E. coli* (E1)	0.125	0.125
6	*E. coli* (E19)	0.063	0.125
7	*E. coli* (E23)	0.125	0.125
8	*E. coli* (E26)	0.063	0.125
9	*K. pneumoniae* (K)	0.125	0.250
10	*H. alvei* (H)	0.250	0.250
11	*S. rubbidea* (C)	0.125	0.250

No statistically significant differences were observed in MIC values among the tested bacterial isolates (Kruskal-Wallis test, $p = 0.074$). Similarly, no statistically significant differences were observed in MBC values among the same isolates (Kruskal-Wallis test, $p = 0.129$).

Table (6). Phytochemical constituents identified in the ethyl acetate Fraction 4 of *C. speciosus* 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

Table (7). Cytotoxic activity of the ethyl acetate Fraction 4 of *C. speciosus* expressed as CC_{50} values (µg/mL) against human lung fibroblast normal cell line

Sample concentration (µg/ml)	Cell viability (%)	Growth inhibition (%)	SD
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

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

Sample concentration (µg/ml)	Cell viability (%)	Growth inhibition (%)	SD
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

Table (9). Cytotoxic activity of the ethyl acetate Fraction 4 of *C. speciosus* against human lung carcinoma cell line (A-549 cells)

Sample concentration (µg/ml)	**Cell viability (%)**	**Growth inhibition (%)**	**SD**
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	

a.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.doc