

Research Article

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Chemical composition and bioactivities of the methanol root extracts of *Saussurea costus*

<https://doi.org/10.1515/chem-2024-0002>

received January 10, 2024; accepted February 19, 2024

Abstract: *Saussurea costus* (*S. costus*) is a medicinal plant from the *Asteraceae* family that is widely used in traditional medicine in Saudi Arabia. This study examines *S. costus* root extract for its chemical composition and its antioxidant, anti-cancer, and antibacterial properties. The results of the study on the methanol root extract of *S. costus* reveal a rich chemical composition, as identified by GC-MS/FID analysis. The extract also showed high levels of total phenolic content (188.2 ± 2.1 mg GAE/g DM) and total flavonoid content (129 ± 2.6 mg QE/g DM). In antioxidant tests, the extract exhibited strong activity, with the half-maximal inhibitory concentration (IC_{50}) values of $137.15 \mu\text{g/mL}$ for ABTS and $175.5 \mu\text{g/mL}$ for DPPH as compared to positive control's IC_{50} values of $45.5 \pm 0.3 \mu\text{g/mL}$ for ABTS and $55.3 \pm 0.1 \mu\text{g/mL}$ for DPPH. The cytotoxic assessment against MCF-7 and A549 cell lines showed notable effects, particularly at higher concentrations. Additionally, the extract induced apoptosis in these cell lines, evidenced by changes in gene expression. Antibacterial tests revealed significant activity against various strains, with MIC values ranging from 7.81 to $125 \mu\text{g/mL}$. The study underscores the importance of plant extracts in modern healthcare and suggests future research directions, including clinical applications and compound identification.

Keywords: apoptosis markers, antioxidant, anti-cancer, antibacterial properties, natural products

1 Introduction

Plant-based treatments have long been an integral component of traditional medicine, offering an abundance of bioactive medicinal compounds. Natural products are growing in popularity due to their ability to provide safer alternatives to synthetic pharmaceuticals, notably in the treatment of chronic diseases [1]. In recent decades, a multitude of signaling transduction pathways have generated natural compounds derived from plants with antioxidative, anti-cancer, anti-inflammatory, and antibacterial properties [2–6].

Notwithstanding the recent identification of diverse pharmacological compounds in botanical specimens, the extensive therapeutic capacity of plants as reservoirs of groundbreaking pharmaceutical agents remains largely unexplored [7–10]. Among interesting medicinal plants, the *Saussurea* (*Compositae*) genus has 400 species that are found mostly in cold areas of the world [11]. The *Saussurea* genus has several uses in traditional treatments and possesses strong pharmacological properties. Many bioactive compounds have the potential to be discovered in *Saussurea* species [11].

The Folin–Ciocalteu method is commonly utilized for the determination of phenolic compounds in plant samples. It enables researchers to quantify the presence of these compounds accurately. However, it is important to note that the molecular response of phenolic compounds can vary widely. This variation is primarily influenced by the chemical structure of the phytochemical constituents. Therefore, understanding the diverse chemical structures of these compounds is crucial for interpreting the results obtained by the Folin–Ciocalteu method. The available ascorbic acid or sugars in the seed extract interfere with the Folin–Ciocalteu assay method [12]. Although extraction techniques, particularly those used for traditional extraction, are well established, researchers are always looking for ways to increase extraction yields. To optimize the extraction conditions for each type of extraction method, it is important to utilize optimization techniques for the extraction processes, employ modeling approaches, and consider the impact of extraction and optimization

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methods on the quality of the extracted compounds and the enhancement of extraction yields. This optimization process aims to maximize the yield of desired compounds while maintaining their quality [13]. Fingerprinting the chemical profile of analytes with HPLC is regarded as an unsophisticated, repeatable, profound, and dependable technology [14]. In a recent study, various conventional methodologies were employed to investigate several aspects of interest. The research focused on examining the total phenolic and flavonoid contents, conducting HPLC-DAD analysis, evaluating antibacterial and antifungal activities, assessing cytotoxicity against the HepG2 cell line, determining hemolysis potential, and exploring antioxidant properties. These conventional methodologies were chosen to provide comprehensive insights into the characteristics and potential applications of the studied samples [15].

Saussurea costus (*S. costus*) is a member of the *Asteraceae* family, which is found globally; however, its most common places are India, Pakistan, and the Himalayas [16–18]. *S. costus* is a plant used in many traditional medical systems to treat asthma, inflammation, ulcers, and stomach disorders [19].

Although *S. costus* is not grown in Saudi Arabia, its roots are commonly utilized in traditional medicine in Saudi Arabia [20–22]. *S. costus* root is often consumed with warm water, milk, or honey, and the root paste is administered topically [19]. The plant under investigation exhibits a wealth of valuable compounds with medicinal properties. Among them are costunolide, dihydrocostunolide, 12-methoxydihydrocostunolide, dihydrocostus lactone, dehydrocostus lactone, and Shikokiols. These compounds have garnered significant attention due to their potential therapeutic applications. The presence of such diverse and bioactive compounds highlights the importance of studying this plant for its medicinal properties and exploring its potential benefits in various healthcare applications [11]. These compounds work synergistically to relieve smooth muscle spasms in both the bronchi and gastrointestinal tract, as the synergistic effect of these compounds enhances their therapeutic efficacy and underscores their potential as treatments for bronchial and gastrointestinal spasms [23]. In addition to their ability to relieve smooth muscle spasms, these compounds also demonstrate antibacterial and anti-cancer properties. They have been shown to exhibit activity against various bacterial strains, making them potential candidates for the development of antibacterial agents. Furthermore, their anti-cancer properties suggest a potential role in cancer treatment and prevention [24]. Moreover, these compounds have demonstrated the ability to prevent oxidation and remove free radicals. Oxidation and the accumulation of free radicals in the body can contribute to various diseases and aging processes. The antioxidant properties of

these compounds make them valuable in combating oxidative stress and protecting cells from damage caused by free radicals [25].

In this research, multiple solvent systems were employed to extract fractions more effectively, which may be advantageous in phytochemical separation. Various fractions revealed significant antibacterial, antioxidant, and cytotoxic properties. The exploration of *S. costus*, a plant deeply rooted in traditional medicinal practices, has increasingly captivated the scientific community. This study focuses on an in-depth analysis of the chemical composition of the methanol extract from *S. costus* roots, assessing its potential antioxidant, anti-cancer, and antibacterial properties. The significance of *S. costus* in traditional medicine systems, particularly in Ayurveda and Chinese medicine, cannot be overstated, where it is used for its myriad therapeutic benefits [26,27].

The research on the potential of plant-derived compounds to fight cancer is a rapidly growing field. Moreover, the anti-cancer potential of these compounds is an area of research that is expanding rapidly [28,29]. During the 1960s, the Food and Drug Administration granted authorization for the therapeutic application of vinblastine and vincristine, which were extracted from *Catharanthus roseus*. These plant-derived anti-cancer drugs were among the earliest ones to be approved [30]. Podophyllotoxin, extracted from *Podophyllum peltatum* and *Podophyllum emodi*, is not only an important but also a prominent plant-derived natural substance [31]. Roscovitine, also known as seliciclib, is an anti-cancer compound derived from purine. Its isolation was carried out from the cotyledons of *Raphanus sativus* L., a member of the *Brassicaceae* family [32]. Paclitaxel (Taxol®) is perhaps the best-known plant-derived anti-cancer medication. This taxane diterpene's cytotoxic action was discovered in the extracts from the *Taxus brevifolia* bark [33]. With cancer being a leading cause of death worldwide, the identification of novel anti-cancer agents from natural sources like *S. costus* is of great importance. Previous research revealed that *S. costus* extract is a possible source of secondary metabolites that might be employed as an anti-cancer drug to treat a variety of malignancies, including breast, colon, and liver [34]. The present study explores the impact of the root extract on diverse cancer cell lines, thereby contributing to the expanding body of research on natural therapies for cancer treatment.

In the field of antibacterial research, the rise of antibiotic-resistant bacteria has created an urgent need for new therapeutic agents [35,36]. The antibacterial activity of *S. costus* roots is evaluated against a spectrum of bacterial strains, potentially offering new insights into combating bacterial resistance.

By combining traditional knowledge with modern scientific techniques, this study aims to provide a comprehensive analysis of the methanol extract of *S. costus* roots. Investigating its chemical composition and assessing its antioxidant, anti-cancer, and antibacterial activities, this research could significantly contribute to the understanding of its pharmacological potential and applications.

Notably, in the realm of *S. costus* research, there have been only a few preliminary investigations, which have shed light on the cytotoxic and antimicrobial attributes of the root extract. However, a conspicuous absence of scholarly inquiry persists regarding the antimicrobial, antioxidant, and anti-cancer potential inherent in root extracts. Consequently, this study endeavors to redress this scholarly void by meticulously scrutinizing methanol extracts derived from the roots of *S. costus*, thereby contributing to the advancement of knowledge in the field. Although there is a lot of literature available on an extract of *S. costus*, our research fills a significant gap due to the limited scientific exploration of this plant despite its known traditional medicinal uses. The comprehensive chemical analysis using GC-MS is a pioneering approach, revealing a unique and rich chemical composition. This study not only quantifies the levels of total phenolic content (TPC) and total flavonoid content (TFC), but also offers compelling evidence regarding the potent antioxidant, anti-cancer, and antibacterial activities exhibited by the extract. These findings, especially the extract's effect on inducing apoptosis in cancer cell lines and its significant antibacterial action against various strains, offer new insights into the potential pharmaceutical applications of *S. costus*. The research aligns with the growing interest in plant-based natural products in healthcare and underscores the untapped therapeutic potential of such extracts in modern medicine.

2 Materials and methods

2.1 Extraction process of the methanol extract from the roots of *S. costus*

In this research endeavor, the roots of *S. costus* were procured from a local market in Riyadh, Saudi Arabia. The plant species was authenticated by Professor Dr. Mohammed Fasil from the Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia. To ensure consistency with previous studies, the extraction of

the root extract was conducted using the established methodologies outlined by Boskovic et al. in 2018. By adhering to these standardized procedures, the scientific integrity of the study was upheld, allowing for meaningful comparisons and reliable analysis [37], albeit with some minor adjustments. Concretely, we subjected 50 g of the roots to mechanical blending and pulverization. Subsequently, 10 g of the resulting dried powder was combined with 200 mL of methanol, and this mixture was introduced into a flat-bottom Erlenmeyer flask. The solution was then subjected to evaporation on a rotary evaporator, maintaining a temperature of 40°C. The phytochemical compounds thus extracted were meticulously separated using a Whatman No. filter paper. Following this, the extract underwent a further round of evaporation and drying, this time at 80°C, culminating in the acquisition of a crude extract. The ultimate methanol extract was precisely quantified and subsequently solubilized in either 0.1% dimethylsulfoxide (DMSO) or ethanol at the requisite concentrations. This prepared extract was then securely stored at a temperature of 4°C for subsequent experimentation and analysis.

2.2 Analysis of phytochemicals in the methanol root extract of *S. costus*

In order to determine the phytochemical constituents present in the methanol root extract of *S. costus*, a comprehensive analytical approach was employed. The analysis was conducted using a gas chromatography-mass spectrometry (GC-MS) system manufactured by Agilent Technologies Inc., USA, coupled with an Agilent 5977A MSD system. The volatile compounds within the methanol extract were subjected to purification through a capillary column with dimensions of 30 m in length, 0.25 mm in diameter, and a film thickness of 0.25 µm. Helium gas was utilized as the carrier gas, flowing at a rate of 0.5 mL/min. The injector temperature was maintained precisely at 250°C. The temperature program within the oven consisted of a series of distinct steps: an initial temperature of 70°C was maintained for 3 min, followed by a gradual increase to 100°C at a rate of 3°C/min (held for 3 min), and further elevated to 120°C at a rate of 10°C/min (held for 3 min). Finally, the temperature was ramped up to 220°C at a rate of 10°C/min. The mass spectrometer settings included an electron impact (EI) source, with an ionization temperature of 230°C, an electron energy of 70 eV, a quadrupole temperature of 150°C, an interface temperature of 280°C, and a scanning range spanning from 20 to 500 amu for quantity determination. This rigorous analytical approach facilitated the

identification and quantification of phytochemical compounds present in the methanol root extract of *S. costus*, thereby providing a reliable and accurate assessment of its phytochemical profile.

2.3 Determination of TPC

The determination of TPC in the methanol root extract of *S. costus* was conducted following a modified version of the protocol established by Wolfe and Liu in 2003 [38]. To quantify the TPC, 0.05 mg of the methanol root extract of *S. costus* was carefully mixed with 2.0 mL of pre-diluted Folin–Ciocalteu reagent in a 1:1 ratio with double-distilled water. The resulting mixture was vigorously vortexed and supplemented with 2 mL of a 7.5% aqueous sodium carbonate solution. Subsequently, the mixture was incubated in darkness at a temperature of $29 \pm 1^\circ\text{C}$ for 20 min. After the incubation period, the color intensity of the solution was measured at a wavelength of 765 nm using a VR-2000 spectrophotometer (JP Selecta, Barcelona, Spain). Gallic acid standards with concentrations ranging from 20 to 200 $\mu\text{g/mL}$ were prepared to establish a calibration curve. The TPC content in the sample was expressed as milligrams of gallic acid equivalent per gram (mg GAE/g) of dry matter (DM), providing a quantitative measure of the phenolic compounds present in the methanol root extract of *S. costus*.

2.4 Analysis of TFC

The quantification of TFC was performed following the methodology delineated by Ordonez *et al.* in 2006 [39]. To ascertain TFC, a precisely measured volume of 0.1 mL of the methanol root extract of *S. costus* or a gallic acid standard was meticulously amalgamated with a 3 mL solution containing 2% AlCl_3 . The resultant mixture was subsequently subjected to a carefully controlled 30-min incubation period. Following the stipulated incubation interval, the chromatic intensity of the solution was meticulously assessed at a specific wavelength of 420 nm utilizing an ELX-808 microplate reader (BioTek Laboratories, LLC, Shoreline, WA, USA). Calibration curves were meticulously established by employing quercetin standards prepared at diverse and varied concentrations, spanning the range from 20 to 200 $\mu\text{g/mL}$. The TFC results were expressed as milligrams of quercetin equivalent per gram of extracts (mg QE/g) of DM, thereby furnishing a judicious and quantitative

assessment of the flavonoid content within the methanol root extract of *S. costus*.

2.5 Assessment of antioxidant activity in the methanol extract of *S. costus*

The evaluation of the scavenging activity against 1,1-diphenyl-2-picryl hydrazyl (DPPH) was conducted using a methanol root extract of *S. costus*. The methanol extract was prepared at four distinct concentrations, ranging from 200 to 1,000 $\mu\text{g/mL}$. A volume of 0.2 mL of the diluted methanol extract was combined with 2 mL of a 0.08 mM DPPH solution. The resulting mixture was then subjected to a 30-s incubation period in darkness. Following incubation, the absorbance of the solution was measured using a spectrophotometer. To validate DPPH activity, vitamin C was used as a positive control. Subsequently, the optical density of the samples was analyzed, and both the IC_{50} value (representing the concentration required for 50% inhibition) and the percentage of DPPH free radical scavenging activity were determined. These measurements were performed in accordance with the methodology described by Tian *et al.* in 2020 [40].

2.6 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) activity

In the study, the ABTS free radical scavenging activity of the methanol root extract of *S. costus* was evaluated [41]. To prepare the ABTS solution, a concentration of 7 mM was dissolved in millipore sterile water. Subsequently, 5 mL of the ABTS solution was mixed with 1.6 μL of potassium persulfate (2.45 mM) and incubated in the dark at a controlled temperature of $28 \pm 1^\circ\text{C}$ for 12 h. The formation of radical cations was indicated by the development of a distinct blue-green color. Vitamin C was used as a reference compound in the experiment. For absorbance measurement, the reaction mixture was diluted tenfold with ethanol to ensure that the absorbance fell within the desired range of 0.5–0.6 at 750 nm. Then, 1.925 μL of the ABTS solution was combined with 25 μL of the methanol root extract of *S. costus*, and the resulting mixture was incubated in the dark at $28 \pm 1^\circ\text{C}$ for 20 min. After the incubation period, the color intensity of the sample was measured at 734 nm relative to a blank. The percentage scavenging power was calculated based on the observed absorbance values, providing insight into the ABTS free radical scavenging activity of the methanol root extract of *S. costus* [42].

2.7 Cell culture and cytotoxicity evaluation using MTT assay

To evaluate the cytotoxic effects of the methanol root extract of *S. costus*, an MTT assay was employed on MCF-7 (ATCC HTB-22) and A549 (ATCC: CCL-185) cell lines. This assay relies on the enzymatic conversion of the MTT reagent by mitochondrial dehydrogenases, ultimately forming formazan crystals. The protocol for the MTT assay was carried out following the guidelines outlined by Riss et al. in 2016 [43]. In the experimental procedure, exponentially growing cells were collected using a 0.25% Trypsin-EDTA solution. Subsequently, the cells were seeded in 96-well plates at a density of 1×10^4 cells/well (100 μ L) in fresh complete media and allowed to adhere for 24 h before treatment. Varying concentrations of the methanol root extract of *S. costus* (50, 100, 200, and 400 μ g/mL) were then applied to the cells for a 24-h duration, resulting in a total treatment volume of 100 μ L per well. The cell culture was maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, incubated at 37°C in a humidified atmosphere containing 5% CO₂. As a positive control, cisplatin (0–30 μ g/mL) was utilized. Following the incubation period, a working solution of MTT (5 mg/mL in phosphate-buffered saline, 10 μ L) was added to each well and incubated for 4 h at 37°C. The formazan crystals formed as a result were subsequently dissolved in 100 μ L of DMSO per well and incubated for an additional 10 min at 37°C with gentle shaking. The absorbance in each well was measured at 570 nm using an ELX-808 automatic microplate reader (BioTek, USA). To calculate cell viability (%), the following formula was employed: $[(A - B)/A] \times 100$, where *A* represents the absorbance of untreated control cells and *B* represents the absorbance of the treated cells. This methodology was described by Rafieian-Kopaei et al. in 2014 [44]. The IC₅₀ values were determined using the Graph Pad Prism software.

2.8 Gene expression analysis focused on apoptotic genes

In the present study, the examination of gene expression related to apoptosis in A549 cells was conducted. Initially, A549 cells (1×10^4) were cultured in six-well plates with 3 mL of DMEM culture media enriched with 10% FBS and 1% penicillin–streptomycin. Furthermore, after a 24-h incubation period, the media was replaced with 3 mL of DMEM supplemented with 1% FBS, 1% penicillin–streptomycin, and 100 μ L

of the methanol root extract of *S. costus*. Moreover, following a 48-h incubation period, the cells were trypsinized using a 0.25% trypsin solution and were subsequently centrifuged at 10,000×*g* for 10 min in a refrigerated centrifuge. The resulting cell pellet was then resuspended in PCR buffer for subsequent reverse transcription-polymerase chain reaction (rRT-PCR) analysis. RNA extraction for gene expression analysis was carried out using an RNeasy kit (Qiagen, Hilden, Germany), following the manufacturer's guidelines. The concentration and purity of the extracted RNA were determined using a nanodrop spectrophotometer. This RNA served as the template for quantitative PCR (qPCR), with a 25 μ L master mix prepared using GoTaq qPCR Master Mix and specific forward (F) and reverse (R) primers. The RT2 PCR array process was executed using a 7500 Fast Real-Time PCR System (7500 Fast; Applied Biosystems, Foster City, CA, USA). The expression levels of genes related to apoptosis were quantified using rRT-PCR, and the data were analyzed by the $2^{-\Delta\Delta Cq}$ method, as delineated by Schmittgen and Livak in 2008. Consequently, this method allows for the relative quantification of gene expression by comparing the threshold cycle (Cq) values of the target genes with those of reference genes and normalizing the data to a control group. As a result, the analysis provides insights into the changes in gene expression associated with apoptosis in response to the treatment of A549 cells with the methanol root extract of *S. costus* [45]. The delta Cq (ΔCq) values for the genes of interest were normalized against the GAPDH gene values from the same samples. Expression levels were calculated relative to a control group that was not treated with the extract. Table 1, included in the

Table 1: Primer sequences for the determination of apoptosis and anti-apoptotic genes

Gene name	Primers sequence	References
Caspase-3	F: 5'-GCTGGATGCCGTCTAGAGTC-3' R: 5'-ATGTGTGGATGATGCTGCCA-3'	[46]
Caspase-8	F: 5'-AGAAGAGGGTCATCCTGGGAGA-3' R: 5'-TCAGGACTTCCTCAAGGCTGC-3'	[47]
Caspase-9	F: 5'-ATTGCACAGCACGTTACAC-3' R: 5'-TATCCCATCCCAGGAAGGCA-3'	[46]
Bax	F: 5'-GAGCTAGGGTCAGAGGGTCA-3' R: 5'-CCCCGATTCACTACCCTGC-3'	[46]
Bcl-2	F: 5'-ACCTACCCAGCCTCCGTTAT-3' R: 5'-GAAGTGGGGGAGGATTGTGG-3'	[46]
Bcl-XL	F: 5'-CAGAGCTTTGAACAGGTAG-3' R: 5'-GCTCTCGGGTGCTGATTG-3'	[48]
GAPDH	F: 5'-CGGAGTCAACGGATTGGTC-3' R: 5'-AGCCTTCTCCATGGTCGTGA-3'	[49]

study, lists the primer sequences and corresponding genes examined in this analysis.

2.9 Screening for antibacterial activity

The present investigation aimed to evaluate the antimicrobial efficacy of the methanol extract obtained from the roots of *S. costus* against a diverse range of bacterial species, including representatives from both the Gram-positive and Gram-negative categories. The Gram-positive bacterial strains utilized in this study included *Bacillus subtilis* (MTCC-10400), *Staphylococcus aureus* (MTCC-29213), and *Staphylococcus epidermidis* (MTCC-12228). Within the Gram-negative bacterial group, strains of *Klebsiella pneumoniae* (MTCC-13883), *Escherichia coli* (ATCC-25922), and *Pseudomonas aeruginosa* (MTCC-27853) were employed. These bacterial strains were sourced from King Khalid University Hospital, located in Riyadh, Saudi Arabia, thereby ensuring the local relevance and applicability of the research findings to the regional microbial landscape.

2.9.1 Disc diffusion method

In this study, the agar disc diffusion method, a benchmark technique for evaluating antimicrobial activity, was utilized as per the modified guidelines of Salem *et al.* [50]. This investigation focused on the antibacterial properties of methanol extracts obtained from the roots of *S. costus*. The methodology involved the use of nutrient agar as the growth medium for the bacterial assays. The bacterial strains under investigation were first cultured on nutrient agar plates for 24 h at 37°C. A standardized bacterial suspension with a concentration of 1×10^6 colony-forming units per milliliter (CFU/mL) was prepared in saline. This inoculum was then uniformly spread over fresh nutrient agar plates using a sterile L-shaped spreader to ensure even distribution. To evaluate the antimicrobial efficacy of the *S. costus* root extracts, discs of filter paper (6 mm diameter) were impregnated with 20 µg of the respective extracts. These discs were then strategically placed on the agar plates inoculated with the bacterial cultures. For comparative purposes, ciprofloxacin at a concentration of 25 µg/mL was used as a positive control, whereas a solution of 0.1% DMSO in nutrient broth served as the negative control. Following a 24-h incubation period, the antibacterial activity of the *S. costus* root extracts

was determined by measuring the diameter of the inhibition zones surrounding the discs. These zones of inhibition provided a quantitative measure of the antibacterial potency of the extracts [51].

2.10 Examination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) levels

The investigation into the MIC and MBC of methanol extracts derived from the flower and leaf of *S. costus* utilized a modified broth dilution method, following the procedure outlined by Basri and Sandra (2016) [52]. Various concentrations of extracts ranging from 1.56 to 800 µg/mL were prepared in sterile Mueller-Hinton broth. Subsequently, each well of a 96-well microtiter plate received 10 µL of bacterial culture standardized to 1×10^6 CFU/mL density, along with different concentrations of the methanol extracts. Following a 24-h incubation at 37°C to promote bacterial growth, 20 µL of triphenyl tetrazolium chloride (TTC) working solution (2 mg/mL in PBS) was added to each well and incubated for an additional 20 min at 37°C to assess bacterial viability. The presence of bacterial growth was indicated by a pink coloration in the wells, resembling that of the positive control, while colorless wells signified no bacterial growth. The MIC was determined to be the lowest concentration of methanol extracts where bacterial growth was inhibited, as evidenced by the absence of pink coloration. To determine the MBC, which denotes the lowest concentration at which bacterial growth was entirely eradicated, samples from wells indicating no growth at the MIC were further cultured. Ultimately, the MBC was identified as the lowest concentration at which no bacteria could be recovered, indicating the bactericidal efficacy of the extract [53].

2.11 Statistical analysis

The anti-cancer, antibacterial, and antioxidant activities were assessed through three separate experiments. In order to ensure robustness and reliability, cytotoxicity and gene expression analyses were each conducted on three separate occasions. The resulting data were then expressed as mean $\pm$ SD, subsequently undergoing a comprehensive statistical analysis conducted using one-way ANOVA. The significance level was set at ($p < 0.05$), ensuring robustness in the interpretation of the findings.

3 Results

3.1 Chemical composition of the methanol root extract of *S. costus*

The examination of phytochemical components within the methanol extract from the rhizome was performed utilizing gas chromatography-mass spectrometry/flame ionization detection (GC-MS/FID). Figure 1 depicts the GC-MS spectrum of the methanol root extract of *S. costus*, revealing a range of phytochemical compounds. Derived from a 30-min GC-MS analysis, this spectrum exhibits numerous peaks, each representing distinct compounds, with the most prominent peak indicating the primary phytochemical constituent. Table 2 provides a detailed list of volatile components identified in the extract. This table is a comprehensive representation of the chemical profile of the methanol root extract of *S. costus*, highlighting the complexity and diversity of compounds it contains.

Within this extract, the predominant phytochemical identified was naphtho (2,3-b)furan-2(3*H*)-one, decahydro-8*a*-methyl-3,5-bis(methylene)-, (3*aR*-(3*a*,4*a*,8*a*β,9*a*))- which constituted 40.42% of the extract. This was followed by eudesma-5,11(13)-dien-8,12-olide, accounting for 26.3%, and *cis,cis,cis*-7,10,13-hexadecatrienal, which made up 9.1% of the extract.

3.2 TPC and TFC of methanolic root extract

The TPC and TFC of the methanol root extract from *S. costus* were analyzed, revealing significant differences ($p < 0.05$) between the extracts. This study found that methanol is an exceptionally effective solvent for extracting phenols and flavonoids. The methanol root extract of *S. costus* showed the highest TPC of 188.2 ± 2.1 mg GAE/g of DM, as indicated by a correlation coefficient (R^2) of 0.938. Similarly, the TFC was recorded at 129 ± 2.6 mg QE/g DM, with an R^2 value of 0.999.

Abundance

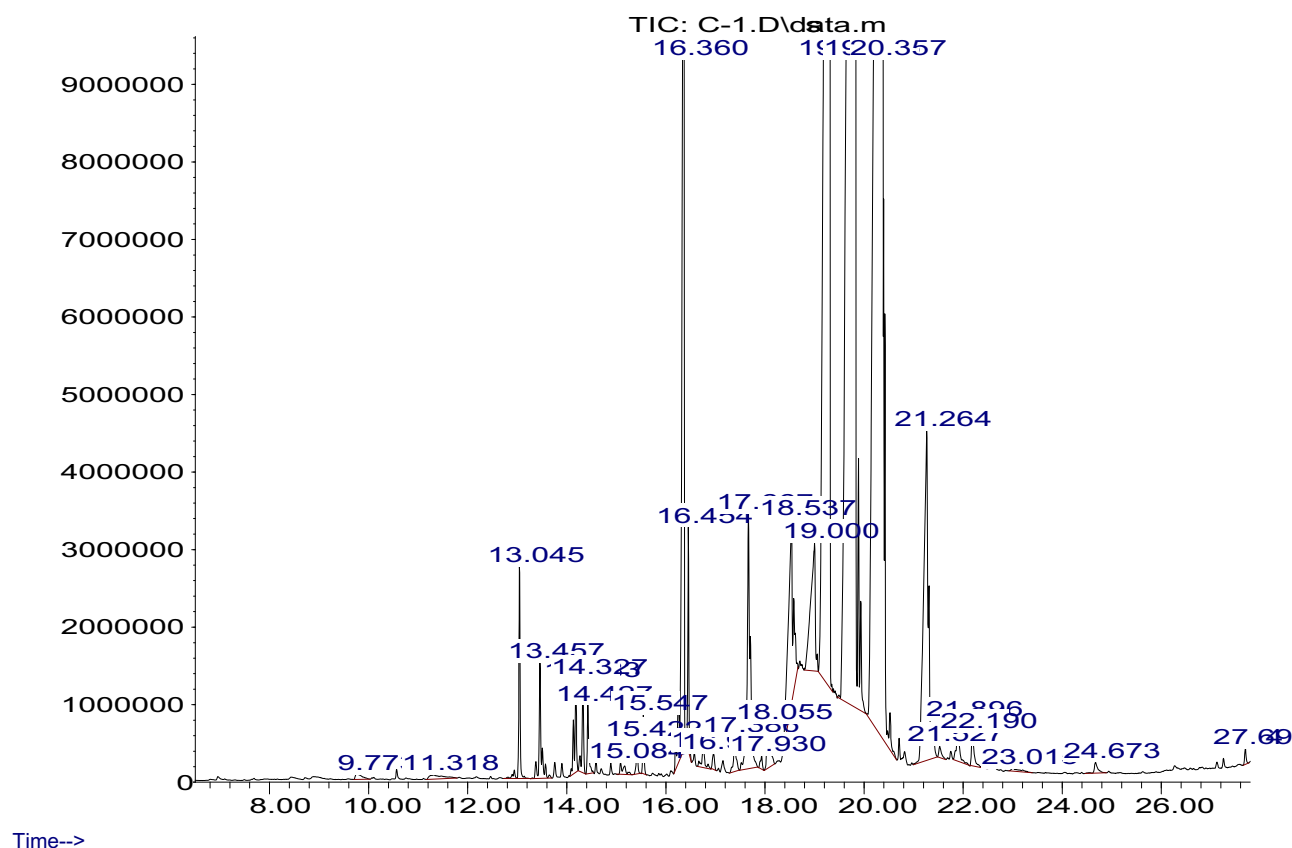


Figure 1: The GC-MS spectrum illustrates the phytochemical composition of the methanol extract derived from the root of *S. costus*. Analysis was performed using a 30-min program on the GC-MS instrument, with each peak denoting an identified compound, while the predominant peak signifies a notable concentration.

Table 2: Volatile components identified via GC-MS in the methanol root extract of *S. costus*

No.	Hit name	RT (min)	Area (Ab*s)	Area (%)	Total area	Molecular weight (amu)
1	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	9.773	6,406,70	0.15	429,781,109	144.042
2	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	11.318	1,025,977	0.24	429,781,109	126.032
3	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-	13.045	5,200,816	1.21	429,781,109	204.188
4	Caryophyllene	13.457	4,262,546	0.99	429,781,109	204.188
5	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	14.183	3,208,629	0.75	429,781,109	202.172
6	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.alpha.)]-	14.327	2,784,393	0.65	429,781,109	204.188
7	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4a.alpha.,7.alpha.,8a.beta.)]-	14.427	1,878,964	0.44	429,781,109	204.188
8	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)-, [1R-(1.alpha.,3.alpha.,4.beta.)]-	15.084	808,343	0.19	429,781,109	222.198
9	cis,cis,cis-7,10,13-Hexadecatrienal	16.36	38,995,390	9.07	429,781,109	234.198
10	Bicyclo[4.3.0]nonane, 7-methylene-2,4,4-trimethyl-2-vinyl-	16.454	4,304,776	1.00	429,781,109	204.188
11	Tricyclo[6.3.3.0]tetradec-4-ene,10,13-dioxo-	16.748	1,033,568	0.24	429,781,109	218.131
12	Oxacyclododeca-6,9-dien-2-one, 7-methyl-, (Z,E)-	16.96	570,401	0.13	429,781,109	194.131
13	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-	17.386	1,549,050	0.36	429,781,109	220.183
14	Caryophyllene oxide	17.667	13,115,373	3.05	429,781,109	220.183
15	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	17.93	476,647	0.11	429,781,109	204.188
16	Santolina triene	18.055	2,557,039	0.59	429,781,109	204.188
17	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.alpha.,7.beta.,7a.beta.)]-	18.537	16,562,170	3.85	429,781,109	232.146
18	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.alpha.,7.beta.,7a.beta.)]-	19	10,592,086	2.46	429,781,109	232.146
19	Eudesma-5,11(13)-dien-8,12-olide	19.312	113,023,061	26.30	429,781,109	232.146
20	Naphtho(2,3-b)furan-2(3H)-one, decahydro-8a-methyl-3,5-bis(methylene)-, (3aR-(3a.alpha.,4a.alpha.,8a.beta.,9a.alpha.)-	19.825	173,708,622	40.42	429,781,109	232.146
21	9,12-Octadecadienoic acid (Z,Z)-	21.264	28,092,721	6.54	429,781,109	280.24
22	9,12-Octadecadienoic acid (Z,Z)-	21.527	525,611	0.12	429,781,109	280.24
23	Diazoprogerone	21.896	2,105,085	0.49	429,781,109	338.247
24	Bicyclo[4.1.0]heptane,-3-cyclopropyl,-7-hydroxymethyl-, (cis)	22.19	1,575,952	0.37	429,781,109	166.136
25	9,17-Octadecadienal, (Z)-	23.015	475,130	0.11	429,781,109	264.245
26	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	24.673	708,089	0.16	429,781,109	330.277

3.3 Antioxidant activity

The research examined the antioxidative capabilities of the phytochemicals found in the methanol extract derived from the roots of *S. costus*. Additionally, this was accomplished through DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS radical scavenging assays. Furthermore, the research aimed to elucidate the antioxidative properties of the extract. Moreover, the DPPH assay provided insight into the free radical scavenging ability of the phytochemicals, while the ABTS assay complemented these findings. In addition, the study sought to determine the potential health benefits associated with the antioxidant activity of *S. costus* roots. Additionally, besides evaluating antioxidant activity, the study investigated other bioactive properties of the methanol extract. Furthermore, not only did the research focus on antioxidant potential, but it also explored the extract's potential applications in various fields. The efficacy

of the extract was benchmarked against established antioxidants, such as vitamin C. The outcomes, as depicted in Figure 2, reveal that the extract demonstrated notable antioxidant properties, outperforming vitamin C. Furthermore, a positive correlation was observed between the concentration of the extract and its antioxidant activity in both assays. The IC₅₀ values were computed as 137.15 ± 1.45 µg/mL for ABTS and 175.5 ± 0.7 µg/mL for DPPH as compared to the positive control's IC₅₀ values of 45.5 ± 0.3 µg/mL for ABTS and 55.3 ± 0.1 µg/mL for DPPH, underscoring the extract's strong antioxidant capability.

3.4 Cytotoxic activity

The study extensively examined the cytotoxic properties of the methanol root extract of *S. costus*. Figure 3 presents the

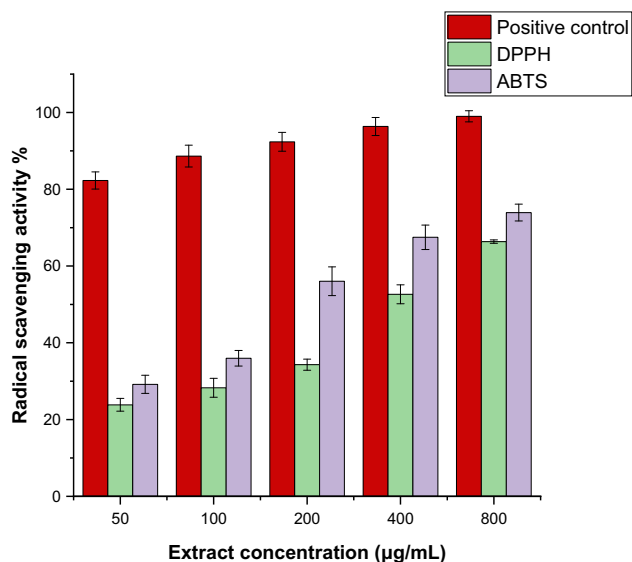


Figure 2: The methanol root extract of *S. costus* was assessed for antioxidant activity using DPPH-reducing power and ABTS scavenging activity assays. Different concentrations were tested, and results are reported as mean $\pm$ SD of three replicates.

results, which revealed a concentration-dependent impact on cell viability. In comparison to the positive control, doxorubicin, a well-known chemotherapeutic agent, the *S. costus* root extract demonstrated significant cytotoxicity toward MCF-7 (breast cancer) and A549 (lung carcinoma) cell lines. Interestingly, the introduction of the extract at a concentration of 100 µg/mL initially showed reduced cytotoxic effects. However, as the extract concentration increased progressively, the cytotoxicity became more pronounced. Significantly, the A549 cell line, with an IC_{50} of 92.4 ± 3.2 µg/mL,

was more susceptible to the extract than the MCF-7 cells, which had an IC_{50} value of 122.5 ± 1.2 µg/mL.

3.5 Analysis of the *S. costus* root extract on MCF-7 and A549-induced apoptosis signaling

To examine the impact of *S. costus* root extract on apoptosis signaling, a 48-h treatment period was administered to MCF-7 and A549 cells, followed by an assessment of the mRNA expression levels of caspases using real-time reverse transcription polymerase chain reaction (rRT-PCR). The study findings unveiled a substantial augmentation in the expression levels of all three types of caspase mRNA in cells treated with the *S. costus* root extract, juxtaposed to the control group that remained untreated. Moreover, the extract-treated MCF-7 and A549 cells exhibited heightened levels of pro-apoptotic Bax mRNA, alongside a concomitant reduction in the expression of anti-apoptotic genes, specifically Bcl-xL and Bcl-2. Significantly, these disparities in gene expression demonstrated statistical significance, as evidenced by a p -value < 0.05 , as depicted in Figure 4.

3.6 Antibacterial activity of the *S. costus* root extract

This section focuses on the assessment of the antibacterial properties of the methanol extract derived from the root of

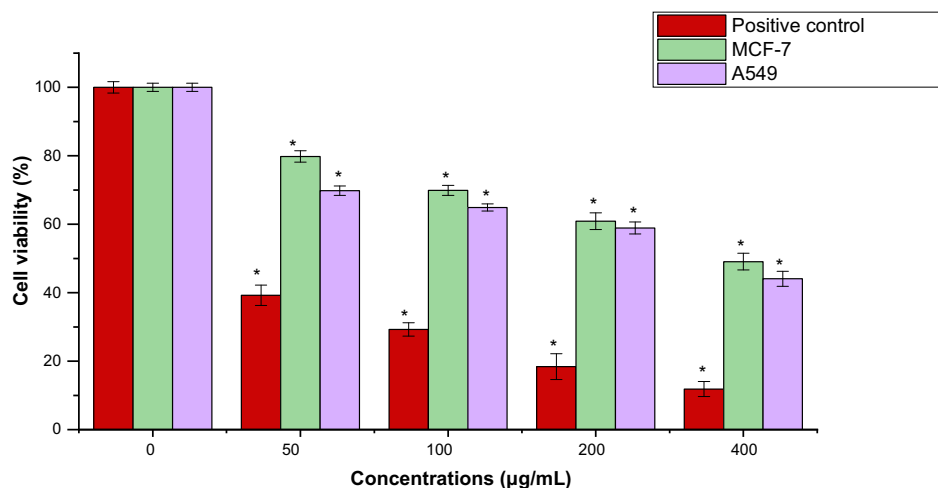


Figure 3: The cytotoxicity of the *S. costus* root extract was evaluated at different concentrations (ranging from 0 to 400 µg/mL) after a 24-h treatment period, and the cell viability was expressed as a percentage. The reported results represent mean $\pm$ SD of three independent experiments. Importantly, it was observed that at higher concentrations, both the flower and leaf extracts significantly (*) decreased cell viability ($p < 0.05$).

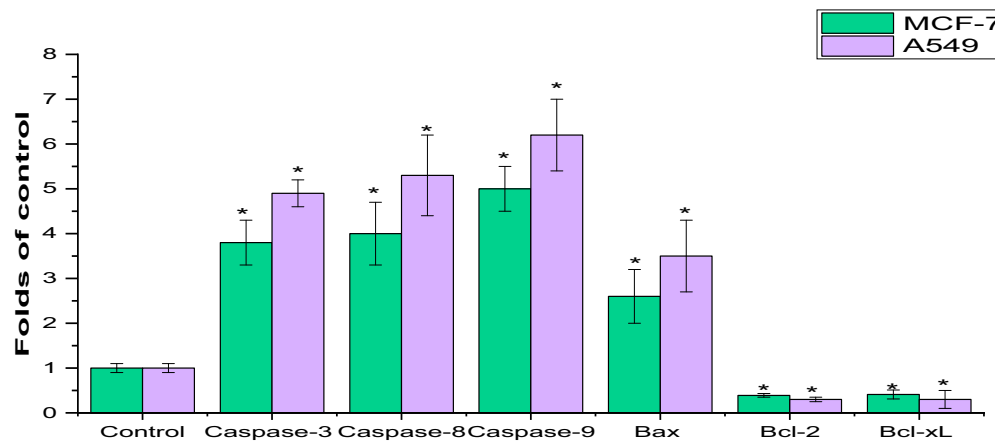


Figure 4: The impact of the *S. costus* root extract on MCF-7 and A549 cells was investigated, focusing on the analysis of pro- and anti-apoptosis marker genes, including caspase-3, caspase-8, caspase-9, Bax, Bcl-2, and Bcl-XI genes. The reported values represent the mean of three separate experiments, with the standard deviation (SD) indicating the variability within the data. The asterisk (*) denotes statistical significance, with $p < 0.05$.

S. costus. Specifically, the study targeted six bacterial strains. The obtained results are meticulously documented in Table 3. In order to establish a reference for efficacy, a rigorous comparison was conducted against the widely used antibiotic ciprofloxacin. The disc diffusion assay was employed to evaluate the antibacterial potential of the *S. costus* root extract. The assay revealed its noteworthy effectiveness in inhibiting the growth of the tested bacterial strains, surpassing the positive control, ciprofloxacin.

Notably, the extract exhibited its most profound antibacterial activity, as evidenced by the MIC values ranging from 7.81 ± 1.8 to 125 ± 3.7 $\mu\text{g/mL}$. Among the strains examined, strains such as *S. aureus*, *S. epidermidis*, and *B. subtilis* demonstrated higher susceptibility to the extract. Conversely, strains such as *P. aeruginosa*, *K. pneumoniae*, and *E. coli* exhibited relatively lower sensitivity. This observation implies a tendency for Gram-negative bacteria to manifest greater resistance towards the *S. costus* root extract compared to Gram-positive bacteria.

In summary, the findings of this study underscore the pronounced antibacterial efficacy of the *S. costus* root

extract against the tested bacterial strains, surpassing the effectiveness of ciprofloxacin.

4 Discussion

For centuries, botanical sources have been extensively utilized for their medicinal properties, and *S. costus*, commonly referred to as costus or Indian costus, exemplifies this trend. Despite previous investigations shedding light on the cytotoxic and antimicrobial attributes of *S. costus* root extract, there exists a noticeable research void pertaining to its antimicrobial, antioxidant, and anti-cancer capabilities. Consequently, this study endeavors to bridge this knowledge gap by undertaking a methodical examination of methanol extracts derived from the roots of *S. costus*. Particularly noteworthy is the limited number of prior inquiries conducted in this area, which underscores the significance of this study in elucidating the chemical composition and potential pharmacological activities of the

Table 3: Inhibitory zone, MIC, and MBC of the root extract of *S. costus*

Bacterium/dilution	Positive control	500 $\mu\text{g/mL}$	250 $\mu\text{g/mL}$	125 $\mu\text{g/mL}$	62.5 $\mu\text{g/mL}$	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
<i>S. aureus</i> (MTCC 29213)	25 ± 1.3	18 ± 1.6	16 ± 0.6	13 ± 1.4	11 ± 1.4	7.81 ± 1.8	15.6 ± 2.9
<i>S. epidermidis</i> (MTCC 12228)	24 ± 1.2	20 ± 1.3	18 ± 2.4	14 ± 1.9	10 ± 1.3	15.6 ± 1.4	31.3 ± 4.2
<i>B. subtilis</i> (MTCC 10400)	22 ± 0.4	20 ± 2.9	19 ± 1.4	13 ± 1.7	11 ± 1.69	15.6 ± 1.5	31.3 ± 1.3
<i>E. coli</i> (ATCC 25922)	24 ± 2.2	19 ± 1.6	17 ± 1.6	14 ± 2.3	6 ± 0.3	62.5 ± 1.8	125 ± 2.9
<i>K. pneumoniae</i> (MTCC 13883)	23 ± 1.35	20 ± 132	15 ± 1.25	13 ± 1.45	7 ± 0.93	125 ± 3.7	250 ± 1.5
<i>P. aeruginosa</i> (MTCC 27853)	27 ± 2.27	19 ± 0.6	15 ± 0.4	11 ± 1.2	7 ± 0.8	62.5 ± 3.6	125 ± 3.7

methanol extract procured from *S. costus* roots. The principal objectives of this study encompass the comprehensive analysis of the antioxidant, anti-cancer, and antibacterial properties inherent in the root methanol extract of *S. costus*. Through a meticulous investigation, this study uncovers substantial therapeutic potential inherent in the methanol root extract of *S. costus*, thereby aligning with the prevailing body of evidence that underscores the medicinal value attributed to plant-derived extracts. The phytochemical profile of the root methanol extract of *S. costus* was assessed using GC-MS/FID analysis. This analytical approach revealed a diverse array of compounds present in the extract, which is in line with the observations made by a previous study [54]. The aforementioned study emphasized the presence of a wide range of secondary metabolites with established bioactivity in medicinal plants. The findings of our GC-MS/FID analysis align with the previous findings, further substantiating the diverse and bioactive nature of the secondary metabolites present in *S. costus* root extract, highlighting the diversity and bioactivity of secondary metabolites in medicinal plants.

Phenolics represent the largest category of phytochemicals, playing a pivotal role in the antioxidant activity exhibited by plants and plant-derived products. Among the various subclasses of phenolics, flavonoids emerge as the most abundant and diverse group of naturally occurring phenolic compounds. These compounds are found in a wide range of plant tissues, existing in both free and glycoside forms. The presence of flavonoids in diverse plant sections further underscores their significance in contributing to the overall phenolic content and antioxidant capacity of plants [55]. The methanol extract derived from the roots of *S. costus* exhibited notable levels of TPC measuring at 188.2 ± 2.1 mg gallic acid equivalents (GAE) per gram of dry weight (DM). Additionally, the extract demonstrated a significant TFC of 129 ± 2.6 mg QE per gram of DM. These findings are in line with previous research, which reported the presence of phenols and flavonoids in the methanolic extract of *Saussurea lappa* L. roots within the ranges of 12.34–75.02 mg GAE/g and 16.2–67.60 mg QE/g, respectively. The consistency between these studies further supports the presence of considerable phenolic and flavonoid compounds in the methanol root extract of *S. costus*.

The findings regarding the high levels of phenolic and flavonoid compounds in the methanol root extract of *S. costus* are consistent with the research conducted by Jones and King [56]. Their study established methanol as an efficient solvent for extracting these bioactive compounds from plant materials. Furthermore, the literature extensively documents the effectiveness of phenolic and flavonoid compounds in combating various ailments, as demonstrated in the work of Patel and Majumdar and

others. These studies provide additional support for the potential therapeutic value of the phenolic and flavonoid compounds present in the methanol root extract of *S. costus* [57].

The remarkable antioxidant capacity exhibited by the *S. costus* extract, surpassing that of vitamin C, represents a noteworthy finding. This observation aligns with previous studies, such as the work conducted by Lee and Huang [58], which have highlighted the potent antioxidant activities of herbal extracts. These studies attribute the antioxidant potential to the rich composition of phytochemicals present in these extracts. Of particular significance in our study is the dose-dependent antioxidant activity observed, indicating the possibility of tailoring dosages to achieve specific therapeutic outcomes. This concept is supported by the pharmacodynamic principles elucidated by Park and underscores the potential for optimizing the therapeutic efficacy of *S. costus* extract through careful dosage adjustments [59].

The cytotoxicity assessment of the *S. costus* extract revealed significant efficacy in inhibiting the growth of MCF-7 and A549 cancer cell lines. This finding is consistent with the research conducted by Liu et al. (2021), which highlighted the anti-cancer potential of plant extracts. The anti-cancer effects of such extracts are mediated through diverse cellular mechanisms, including apoptosis induction, cell cycle arrest, and inhibition of metastasis. These findings further support the potential of the *S. costus* extract as a promising candidate in the development of anti-cancer therapies [60]. Collectively, the cytotoxicity evaluation of the *S. costus* extract against MCF-7 and A549 cancer cell lines, along with the supporting findings from Liu et al. (2021) and related studies, underscores the promising anti-cancer potential of plant extracts. The multifaceted mechanisms through which these extracts exert their effects, including the induction of apoptosis, regulation of the cell cycle, and inhibition of metastasis, highlight their significance as potential therapeutic agents in combating cancer. The concentration-dependent nature of this cytotoxicity underscores the importance of dosage in therapeutic applications, a principle widely recognized in pharmacological studies.

Apoptosis, a highly regulated and essential process in cellular biology, serves as a programmed mechanism for cell death in response to various environmental stimuli. Mammalian cells possess two major apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic pathway is initiated by the engagement of death receptors, leading to the activation of caspase-8, while the intrinsic pathway involves the release of mitochondrial factors and subsequent activation of

caspase-9. The activation of caspases, a family of proteases, is a pivotal event in both the extrinsic and intrinsic apoptotic cascades, ultimately leading to cellular demise [61]. It is noteworthy that caspase-8 predominantly plays a critical role in the extrinsic apoptotic route. On the other hand, the activation of caspase-9 has been associated with both the mitochondrial and intrinsic apoptotic pathways [62]. Caspases may be activated in anti-cancer treatment by either triggering the extrinsic or intrinsic pathways at the mitochondria [63]. Increased Bax expression enhances sensitivity to apoptotic stimuli and prevents tumor formation in breast cancer cells [64]. Another different anti-apoptotic genes such as Bcl-xL can serve to prevent apoptosis in response to Bax [65]. Bcl-xL expression has been linked to the development of breast cancer [66]. While Bcl-xL reduces apoptosis, it is regarded to be a critical molecule in chemoresistance development [67]. The extract's impact on apoptosis signaling, evidenced by the modulation of caspase mRNA and the balance between pro-apoptotic and anti-apoptotic genes, provides a window into its potential mechanism of action against cancer cells. This mechanism aligns with the findings of Zhang and Wong [68], who reported similar apoptotic pathways being targeted by phytochemicals in cancer therapy.

Moreover, the observed antibacterial activity of the extract, particularly against Gram-positive bacteria, serves to further enhance its pharmacological profile. This noteworthy finding aligns with the research conducted by Gupta and Chen [69], who emphasized the potential of plant extracts as viable alternatives to conventional antibiotics. The emergence and proliferation of antibiotic resistance, as extensively discussed in the comprehensive reviews by Sharma and Agrawal [70], have raised serious concerns regarding the efficacy of existing therapeutic options. This critical situation accentuates the pressing need for the development of novel antibacterial agents. In this regard, plant extracts, such as the extract derived from *S. costus*, present a promising avenue for exploration due to their potential as alternative sources of antibacterial compounds.

The pharmacological features of *S. costus* root extract, which include anti-cancer, antioxidant, and antibacterial properties, show its potential as a multifaceted therapeutic agent. These results not only confirm the traditional use of *S. costus* in herbal medicine but also provide new opportunities for the creation of innovative medicines. The work, therefore, adds to a better knowledge of the therapeutic characteristics of plant extracts, highlighting the need for further research into their potential uses in current healthcare systems. The findings of this study provide a basis for future research endeavors aimed at exploring the clinical applications of these extracts, optimizing the extraction methods employed, and identifying the specific compounds

responsible for the observed bioactivities. These investigations hold considerable promise in facilitating the development of novel drugs and therapeutic approaches.

A limitation of this study is the lack of *in vivo* experiments, as they were not the primary focus of our research. While *in vitro* investigations provide valuable insights, conducting *in vivo* trials is crucial for a comprehensive exploration of the potential bioactivity of *S. costus* L. in combating infectious diseases. Further research encompassing both *in vitro* and *in vivo* studies is warranted to deepen our understanding of the efficacy and mechanisms of action of *S. costus* L. and its potential applications in the treatment of infectious diseases.

5 Conclusions

The present study undertook an extensive investigation into the pharmacological characteristics of the methanol root extract of *S. costus*, employing a diverse array of analytical techniques, notably GC-MS/FID. The findings revealed a multifaceted phytochemical composition, prominently featuring compounds such as naphtho(2,3-*b*)furan-2(3*H*)-one and eudesma-5,11(13)-dien-8,12-olide. Importantly, the extract demonstrated a notably high TPC and TFC, thereby underscoring its potent antioxidant properties, as corroborated by rigorous DPPH and ABTS assays. These results substantiate the extract's potential efficacy in managing oxidative stress-related disorders. Furthermore, the extract exhibited substantial cytotoxic effects against MCF-7 and A549 cancer cell lines, thereby accentuating its potential utility in cancer therapy. The observed dose-dependent cytotoxicity and its consequential impact on apoptosis signaling pathways, as evidenced by discernible changes in caspase mRNA expression and the intricate equilibrium between pro- and anti-apoptotic genes, furnish valuable insights into its mechanism of action against cancer cells. In addition, the extract exhibited noteworthy antibacterial activity, particularly against Gram-positive bacteria, thereby suggesting its potential application in combating bacterial infections, a matter of escalating concern in light of the burgeoning antibiotic resistance crisis. These findings engender a deeper comprehension of the medicinal properties intrinsic to plant extracts and firmly advocate for further exploration into their potential applications within contemporary healthcare systems. Subsequent research endeavors must concentrate on the clinical implications of these extracts, optimization of extraction methodologies, and identification of specific bioactive compounds responsible for the observed bioactivities, which hold the potential to engender the development of novel pharmaceuticals and therapeutic modalities for diverse disease states.

Acknowledgments: The authors thank the Researchers Supporting Project number (RSP2024R418), King Saud University, Riyadh, Saudi Arabia.

Funding information: The research was financially supported by Researchers Supporting Project number (RSP2024R418), King Saud University, Riyadh, Saudi Arabia.

Author contributions: The research was conceptualized by M.A.B. and I.M.A., who contributed to the development of the study's overall framework and objectives. I.M.A. was responsible for designing the methodology and selecting the appropriate software tools for data analysis. The validation process involved the input and expertise of M.A.B. and S.M.I., ensuring the accuracy and reliability of the research findings. I.M.A. conducted the formal analysis of the collected data, while also overseeing the investigation and managing the available resources. I.M.A. curated the data, organizing and preparing it for analysis and interpretation. The initial draft of the manuscript was written by S.M.I., with I.M.A. contributing to the subsequent review and editing process. I.M.A. was also responsible for visualizing the data in a clear and concise manner. M.A.B. provided supervision throughout the research project, ensuring its smooth progression. The project administration, including coordination and logistical aspects, was overseen by R.M.A. Finally, R.M.A. played a role in securing the necessary funding for the research. Importantly, all authors have thoroughly reviewed the manuscript and have given their consent for its publication in its current form.

Conflict of interest: The authors have stated that they have no conflict of interest related to the research conducted in the study. This declaration signifies that there are no competing financial or personal interests that could potentially influence the objectivity, integrity, or interpretation of the research findings.

Ethical approval: The research conducted does not pertain to the utilization of humans or animals.

Data availability statement: All the data generated and analyzed during the course of this study have been comprehensively documented and are fully available in the published article.

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