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Evaluation of antimicrobial, cytotoxicity effects and antioxidant potential of *Stemodia verticillata* (Mill.) Hassl extract

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ABSTRACT

The aim of this study was to evaluate the antimicrobial, cytotoxic, and antioxidant activities of *Stemodia verticillata* extract. Antimicrobial activity was assessed using agar well diffusion assay and Broth microdilution method, cytotoxicity effects using the 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide assay, the 2,2-diphenylpicrylhydrazyl and ferric reducing antioxidant power assays were used to measure antioxidant. The various bioactive components were identified with the gas chromatography-mass spectrometer (GC-MS) technique. The highest antimicrobial activity was observed against *Staphylococcus aureus* with a minimum inhibitory concentration (MIC) of 4,000 µg/ml and a minimum bactericidal concentration of 16,000 µg/ml. In comparison, the least activity was observed for *Aspergillus niger* with a MIC of 16,000 µg/ml and minimum fungicidal concentration of 64,000 µg/ml. The cytotoxicity results revealed the presence of anticancer activity with an IC_{50} of 79.03 µg/ml against COLO205 colon cancer cell lines. The highest antioxidant activity was exhibited by acetone extract (IC_{50} = 29.112 µg/ml), followed by 80% ethanolic extract (IC_{50} = 34.72 µg/ml). The quantitative phytochemical analysis indicated that acetone and 80% ethanolic extracts contained higher amounts of flavonoids and phenolic compounds. Furthermore, five major bioactive compounds were identified by GC-MS. The Findings from the present investigation represent the high potency of *S. verticillata* extract as a source of more valuable bioactive compounds for developing future phytotherapeutic products.

INTRODUCTION

Infectious diseases are contagious illnesses or diseases brought on by pathogenic microorganisms, such as bacteria, fungi, viruses, protozoans, and helminthes (Shukla *et al*., 2014). In low-income societies, it is one of the primary causes of fatalities and morbidity (WHO, 2018a, 2018b). The diseases can cause suffering and death of the people but also have significant economic impacts that are not often recognized (Lindahl and Grace, 2015). The emergence and increasing rates of antimicrobial resistance to modern antibiotics are the main challenges to eradicating microbial infections, and the worst aspect is the development of antimicrobial resistance as a natural protective process among microorganisms; even rational use of antibiotics provides for antimicrobial resistance development (Review on Antimicrobial Resistance, 2016). The World Health Organization (WHO) has identified several priority pathogens against which newer antimicrobials should be developed to simplify the search for appropriate antimicrobials; these include *Mycobacterium tuberculosis*, *Escherichia coli*, *Candida albicans*, *Streptococcus pneumoniae*, *Enterobacter* spp., *Staphylococcus aureus*, and *Streptococcus pneumonia* and others (WHO, 2017). Most of these microorganisms have the capacity to produce biofilms, which are mostly made of DNA, proteins and polysaccharides. The biofilms created by these pathogenic bacteria and fungi are of serious concern because they give the underlying microbes a broad range of resistance (Bakkiyaraj *et al*., 2013). Novel agents are therefore required to combat these drug-resistant pathogens.

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Utilizing natural sources is one of many strategies for finding novel antimicrobial agents in developing nations, including India. About 70–90% of the population still uses traditional medicines made of plant extracts (WHO, 2013). The largest biochemical reserves are found in plants, which can produce several compounds with medicinal properties, including antibacterial, antifungal, anticancer, antioxidant, antimycobacterial, and anti-inflammatory effects (Abdallah, 2011; Katiyar *et al*., 2012; Savoia, 2012). According to the WHO, plants are the most abundant source of numerous pharmaceuticals, and both microbial and non-microbial diseases can be treated with these traditional medicines (Gupta *et al*., 2016). An estimated 7,500 species of medicinal plants serve as the primary source of medicine for the rural population in India and are utilized for preventative, promotional, and therapeutic purposes (Patil, 2016).

Stemodia verticillata (Mill.) Hassl is an annual herbaceous plant belonging to the family Plantaginaceae; it grows on the banks of rivers and streams (Liang *et al*., 2011). In India, the plant species are widely distributed in Maharashtra, Kerala, Arunachal Pradesh, Karnataka, and Tamil Nadu state (Sharma *et al*., 2016). The plant species reported from various states as an agricultural weed used by rural peoples in the Western Ghats of India as a sedative and for managing and treating various infections and their symptoms (Thomas, 2020). The plant species is famously known as Dhangar by the local community of Northern Western Ghats; they use it to treat inflammation and breast cancer (Pers. Communication). However**,** despite its widespread use, antimicrobial, antioxidant activity and cytotoxicity effects were uninvestigated. Therefore, the study aimed to evaluate antimicrobial, cytotoxicity effects and antioxidant activity of *S. verticillata* extracts. Furthermore, chemical profiling and quantification of phytochemicals were studied (Awalekar *et al*., 2021).

MATERIALS AND METHODS

Solvent and chemicals

Ethanol, ascorbic acid, acetone, phenol from Folin-Ciocalteu, dichloromethane (DCM), ethyl acetate, methanol, sodium nitrite, 2, 2-Diphenyl picrylhydrazyl (DPPH), aluminum chloride, dimethylsulphoxide (DMSO), distilled water, sodium carbonate, gallic acid are the reagents and chemicals used. The analytical grade chemicals were all purchased from Loba Chem Pvt. Ltd. in Kolhapur, Maharashtra, India.

Plant sample collection and preparation

The whole plant species of *S. verticillata* was collected from the Kolhapur District in December 2021. The qualified taxonomist identified the plant species, and the verified plant materials were submitted for herbarium sheets and voucher specimens (VBS 6312); they were then placed in The New College's herbarium in Kolhapur, Maharashtra, India. The materials were delivered to the Medicinal Chemistry Research Laboratory at Shivaji University's Chemistry Department for the study. Distilled water was used to clean the plant materials, which were then shade-dried. The dried materials were then ground into powder and stored in a clean labeled container at room temperature until extraction.

Extraction

Cold maceration method using 80% ethanol, aqueous, acetone, ethyl acetate, and DCM separately was used to extract dry powdered plant materials (200 g per solvent) for 48 hours with occasional agitation. The filter paper (Whatman No. 1, England) was used for filtration; the resulting filtrate was concentrated between 40°C and 50°C in a rotary evaporator. The dry extracts were packed in airtight containers and kept in a refrigerator (5°C–8°C) until when required for biological and chemical experimentation.

Antimicrobial activities evaluation

Test microorganism used

A total of six pathogen strains were used, including two Gram-positive bacteria (*Bacillus subtilis* MTCC 1687 and *Staphylococcus aureus* MTCC 96), two Gram-negative bacteria (*Escherichia coli* MTCC 1687 and *Pseudomonas aeruginosa* MTC 1628), and two fungal strain (*Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 9029), all were purchased from the NCCS, Pune, Maharashtra, India.

Agar-well diffusion assay

With slight modifications, the 80% ethanolic of *S. verticallata* was first screened for antimicrobial activity using the agar well diffusion method (Mohamed, 2017). Growth mediums were incorporated into the experimental design, and the standard methods for sample preparation and zone of inhibition assessment were used (Gupta *et al*., 2016). In potato dextrose and nutritional agar media, the microorganisms were grown overnight at 37°C, and 100 µl of suspension containing 106 CFU ml^{-1} of microbes was dispersed at the top of the surface of prepared agar plates. Wells was created with a cork borer and filled with 100 µl of plant extracts with a 100 mg concentration; kanamycin and surfactin drugs were used as a positive control for comparison. After that, 24 hours were spent incubating the plates at 37°C; the diameters of the inhibition zones (mm) on the surface of the agar surrounding the well were measured using a vernier caliper to determine the antibacterial activity.

Determination of minimum inhibitory concentration (MIC)

Using 96-well microplates, the broth microdilution technique was used to determine the MIC of the 80% ethanolic extracts of *S. verticillata* (Romulo *et al*., 2018). Briefly, in 1% DMSO, plant extracts were dissolved and placed into the plate in two-fold repeated dilutions (100 µl each) and diluted in Sabouraud Dextrose or Mueller Hinton Broth (MHB), resulting in concentrations of 500 to 128,000 µg/ml**.** The positive control (Streptomycin/ Miconazole, MHB, and test organism) and negative control (MHB, test organism, and 1% DMSO) were maintained. Afterward, the appropriate microorganisms were suspended and inoculated to the plates, resulting in a final density of 5 × 105 CFU/ml for bacteria and 1.5 × 103 CFU/ml for fungi, respectively. Plates were then incubated for 24 hours at 37°C (48 hours for *C. albicans*). ELISA microplate reader was used to measure the turbidity of microorganism growth at 500 nm (Naz *et al*., 2017). The MIC was defined as the lowest concentration that inhibits the growth of microorganisms greater than 50%. The experiment was done in triplicate.

Minimum bactericidal/fungicidal concentration (MBC/MFC) determination

MBC/MFC was determined by taking 100 µl of culture medium from each well not showing growth and inoculating into

Mueller Hinton or Sabouraud Dextrose agar plates and incubating for 24 hours at 37°C, after that, the growth of microorganisms was examined. The extract concentration found to kill 99.9% of bacteria was found to be MBC/MFC.

Evaluation of anti-biofilm formation

Crystal violet assay was used to evaluate the anti-biofilm potential of 80% ethanolic extract of *S. verticillata* as described by O'toole and Kolter (1998) with some modifications (Fathi *et al*., 2022). Briefly, biofilms were grown in 96-well plates; 200 µl of TSB was added in each well of the microplate, followed by 100 µl of each selected bacteria (1×10^6 cells/ml). 100 µl of plant extract of various concentrations (1,000, 3,000, 5,000, 7,000 and 10,000 µg/ml) was added in each test wells. As a positive control, 100 ml of standard streptomycin (500 µg/ml) solution was applied to standard test wells for bacteria, Micanozole for fungi, and three wells without test extract or Streptomycin/Micanozole were used as a negative control. The plates were incubated at 37°C for 24 hours. After incubation, the supernatant was taken out. Each well was thoroughly cleaned with 200 µl of sterile saline to remove bacteria/fungi floating around, plates were then dried in the air for 30 minutes. The biofilms created by adhering cells in the plate were stained for 20 minutes at room temperature using 0.1% crystal violet. After incubation, the excess stain was removed, and any unabsorbed stain was eliminated by washing the plate three times with sterile saline. Finally, 200 µl of 96% ethanol was added to solubilize the dye attached to the cells. Using ELISA microplate reader, the optical densities (OD) of stained adhering bacteria/fungi were measured at 630 nm. Each assay was performed in triplicate. The mean absorbance of the samples was determined. The percentage inhibition was calculated as per the formula below,

$$\% \text{ Inhibition} = \frac{\text{Opticak Density of negative control} - \text{Optical Density of test sample}}{\text{Optical Density negative control}} \times 100\%$$

Determination of antioxidant activity

Ferric reducing power

With a minor modification, the method reported by Alavi and Karimi (2018) was used to assess the reducing power of crude extracts. 2.6 ml of [K_3 Fe $(CN)_6$] (1% W/v) and buffer phosphate buffer (0.2 M, pH 6.6) were mixed with 1.0 ml of extracts (5, 10, 15, 20, 25, and 30 µg/ml). The mixed solution was placed in an incubator at 50°C for 20 minutes. 2.6 ml of Trichloroacetic acid (10%) was added to the mixture to stop the chemical reaction and then centrifuged for 15 minutes at 3,000 rpm. The top layer (2.6 ml) of the centrifuged solution was removed and combined with 2.6 ml of water and 0.6 ml (0.1%) Iron (III) chloride. Using ms-Vis Spectrophotometer, the absorbance was recorded at 700 nm in triplicate versus blank solution and expressed as mean ± standard deviation SD. Ascorbic acid was used as standard.

DPPH radical scavenging assay

Using a slightly modified of Shekhar *et al*. (2014)'s DPPH free radical scavenging test, the sample's antioxidant activity was examined (Tailor Chandra Shekhar and Goyal Anju, 2014). The 3.0 ml of 1.0 M solutions of DPPH in methanol were added to 3.0 ml of different extracts in methanol and ascorbic acid in water (5, 10, 15, 20, 25, and 30 µg/ml). After shaking, the mixture was let to stand for 30 minutes, and the absorbance was taken at 517 nm. The DPPH solution served as control, methanol was used as a blank, and three times the experiment was repeated ($n = 3$). The percentage radical scavenging was calculated using the equation below,

$$\text{Radical Scavenging (\%)} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100\%$$

Cytotoxicity assay

According to Mosmann (1983), with minor modifications, the MTT colorimetric assay was employed to screen the cytotoxic activities of plant extracts (Ogbole *et al*., 2017). The normal kidney cell line NRK52E and colon cancer cell line COLO205 were incubated at 1×10^4 cells/ml in culture medium for 24 hours at 37°C and 5% CO_2. At a density of (100 µl) 104 cells/well) in 100 µl culture medium, the cells were seeded in microplates and then treated to solutions containing 80% ethanolic plant extracts (20, 40, 60, 80, and 100 µg/ml) (tissue culture grade and 96 wells). DMSO [0.2% in phosphate buffer solution (PBS)] and the cell line were used as the controls in the wells; the samples were subsequently incubated in duplicate. In order to determine the control cell survival and the percentage of live cells after culture, controls were maintained. Cell cultures were incubated for 24 hours at 37°C and 5% CO_2 in a CO_2 incubator (Thermo Scientific BB150); after that, the medium was removed and replaced with 20 µl of the MTT reagent (5 mg/minute PBS). After MTT was added, cells were incubated for 4 hours at 37°C in a CO_2 incubator; forming formazan crystals in the wells was seen under a microscope. Only live cells could convert the yellowish MTT into a dark formazan. After completely removing the medium, 200 µl of DMSO was added, kept for 10 minutes, and then incubated at 37°C. (Wrapped with aluminium foil). Using a microplate reader (Benesphera E21), each sample's absorbance at 550 nm was measured. Standard 5- Fluorouracil of different concentrations (20, 40, 60, 80, 100 µg) was used as a positive control. The % cell inhibition was determined by using the formula below. By using a linear approximation regression of the percentage of inhibition versus the concentration of the test extract, IC_{50} values were calculated.

$$\% \text{ Cell Inhibition} = 100 - [(A1 - A2) / (A3 - A2)] \times 100\%$$

where $A1$ = absorbance of the test extract, $A2$ = blank absorbance, $A3$ = absorbance of the control.

Qualitative phytochemical analysis

To determine whether different secondary metabolites were present in *S. verticillata*, the chemical techniques described by other researchers were used, and the extracts were submitted to conventional preliminary phytochemical examination (Akinyemi *et al*., 2006; Rao *et al*., 2016).

Quantification of phytochemical constituents

Quantification of protein content

With some modifications, the traditional Lowry's method was used to determine total protein content (Sarkar *et al*., 2020). Briefly, six different concentrations of 5, 10, 15, 20,

25, and 30 µg/ml of standard Bovine serum albumin (BSA) were prepared. 4 ml of an alkaline copper sulphate solution was added to 1,000 µl of diluted sample/standard and was thoroughly mixed. 500 µl of Folin Ciocalteu Reagent was added and mixed at room temperature; the mixture was left for 30 minutes, and the absorbance was taken at 500 nm against a blank made by water and the reagents without test extract. All samples were run three times ($n = 3$). The total protein concentration was measured using the BSA calibration curve, which was then reported in terms of µg Bovine serum albumin Equivalents (BSAE)/mg dry weight (DW) extracts.

Flavonoids content determination

The samples' flavonoid quantities were assessed using an aluminum chloride spectrophotometric assay with slight modification (Tabasum *et al*., 2016). Briefly, distilled water (2 ml) and 0.15 mL of 5% $NaNO_2$ solution were added to 0.5 ml aliquots of each extract (3,400 µg/ml) and quercetin solution (5,000–200,000 µg/ml) separately and thoroughly mixed. After the mixture had been left for 6 minutes, 0.15ml of aluminum chloride (10% $AlCl_3$ w/v) solution was added, and the mixture was kept for 6 minutes. A 2 ml solution of 4% NaOH was added to the mixture, adding more distilled water until the mixture had a total volume of 5 ml for 15 minutes; the mixture was kept. The absorbance of each mixture was recorded at 500 nm and compared to one that didn't contain quercetin or plant extract. All experiment was made in triplicate ($n = 3$). The amount of flavonoid was estimated and reported from the quercetin calibration curve as µg of QE/mg of DW extract.

Quantification of tannin content

The vanillin/HCl method was slightly modified to determine the amount of tannin (Medini *et al*., 2014). Six different concentration of catechin was prepared. 4 ml of 4% vanillin dissolved in methanol and 1.6 ml of strong HCl were mixed separately with 500 µl of diluted sample and catechin. After that, the mixture was left to stand for 15 minutes, and the absorbance was measured at 500 nm using methanol as a blank. All experiment was done in triplicate ($n = 3$), and µg of catechin equivalent (QE)/mg DW extract was used to express the amount of tannin.

Phenolic content determination

As per the method described by Singleton *et al*. (1965), with a few minor alterations, the number of phenolics was determined using the Folin-Ciocalteu method (Tabasum *et al*., 2016). The 1.5 mL (2,000 µg/ml) of each extract solution and 3.5 ml of Folin Ciocalteu diluted 10 times, and 4.5 ml of Na_2CO_3 (7.5%) solution were mixed. The mixtures were held at room temperature for 30 minutes, with absorbance at 765 nm being measured. All the experiments were done in triplicate ($n = 3$). Gallic acid's calibration curve was used to quantify the total phenolic content, which was then represented as gallic acid equivalents (GAE)/mg DW extracts.

Identification of bioactive compounds

At the Common Facility Center USIC, Shivaji University, Kolhapur, the 80% ethanolic extract was subjected to a gas chromatography-mass spectrometer (GC-MS) analysis using a Shimadzu, Japan, model TQ8050 plus with HS20 equipment. A silica capillary column SH-Rxi-5silMS (dimensions 30.0 m 0.25 mm, film thickness 0.25 m) was coupled to the instrument and used as the stationary phase, while the Helium was used as the carrier gas with 54.1 ml/minute flow rate. The 70 eV was used for electron ionization with a 200°C ion source temperature. The oven temperature was kept at 80°C, held for 2 minutes, and ramped to 250°C at the rate of 3.5°C/minute and held for 6 minutes. A 1 µg sample was injected into the column at 300°C; the entire run time for GC was 53.33 minutes, with the splitting ratio being 50:050. The samples' phytochemical content was identified based on a comparison of the mass spectral patterns and retention times of the test samples with the spectral databases of real compounds kept in the library (Jagadhane *et al*., 2022a, 2022b).

Statistical analysis

Each experiment was carried out in duplicate and at least three times. Means ± SD were used to express the results; all the experiments were subject to statistical analysis by *t*-test. The levels of significance were set at $p < 0.05$.

RESULTS AND DISCUSSION

The extraction yield obtained in this study ranged from 24% to 36%, and the type of solvent used significantly impacted the extraction yield recorded. It has been seen that the extraction yield of 80% ethanol is higher (36%) than that of other solvents, it was followed by acetone (30.5%), aqueous (28.5%), ethyl acetate (25.5%), and DCM (24%). The higher extraction yield recorded in 80% ethanol is caused by the combination of organic solvent and water that facilitates the extraction of all soluble compounds in both water and organic solvents. In the preliminary phytochemicals analysis of *S. verticillata* extracts, the results demonstrated the presence of tannins, glycosides, flavonoids, amino acids/proteins, phenolics, saponnins, and terpenoids; however, alkaloids were absent in all extracts Table 1, these phytochemicals reported to have a diversity of biological activity such as anti-biofilm, antifungal, anticancer, anti-inflammatory, anti-oxidative, and antibacterial (Akinyemi *et al*., 2006).

In the determination of phytochemicals, standard curves shown in Figure 1 was used, comparing the phenolic content of the different extracts; acetone extract had the highest amount (89.314 ± 0.001 µg GAE/mg DW), followed by 80% ethanol (42.9715 ± 0.012 µg GAE/mg DW), aqueous (40.6011 ± 0.003 µg GAE/mg DW), ethyl acetate (24.1451 ± 0.002 µg GAE/mg DW) and DCM (19.9585 ± 0.001µg GAE/mg DW; Table 2). In the determination of flavonoids content, the acetone extract also contained more flavonoids than the other extracts, with a concentration of 589.46 ± 0.010 µg QE/mg DW followed by ethyl acetate (464.65 ± 0.014 µg QE/mg DW), DCM (286.47 ± 0.097 µg QE/mg DW), 80% ethanolic (111.62 ± 0.055 µg QE/mg DW) and aqueous (71.47 ± 0.030 µg QE/mg DW; Table 2). In the case of tannin, the ethyl acetate extract had the highest level of total tannin (44.959 ± 0.001 µg CE/mg DW), followed by DCM (44.838 ± 0.003 µg CE/mg DW), acetone (33.379 ± 0.001 µg CE/mg DW), 80% ethanolic (11.689 ± 0.001 µg CE/mg DW), and aqueous (3.900 ± 0.002 µg CE/mg DW; Table 2**)**. While in protein, the acetone extract continued to exhibit higher protein content (30.025 ± 0.002 µg BSAE/mg DW), followed by that 80% ethanolic (24.038 ±

Table 1. Qualitative phytochemical analysis of *S. verticillata* extracts.

Phytochemicals	Aqueous	80% Ethanol	Acetone	Ethyl acetate	DCM
Flavonoids	+	+	+	+	+
Phenolics	+	+	+	+	+
Alkaloids	-	-	-	-	-
Terpenoids	+	+	-	+	+
Saponin	-	+	+	-	+
Tannins	+	+	+	-	+
Amino acid/Protein	+	+	+	-	+
Glycosides	+	+	+	+	+

(-) not detected/present; (+) present.

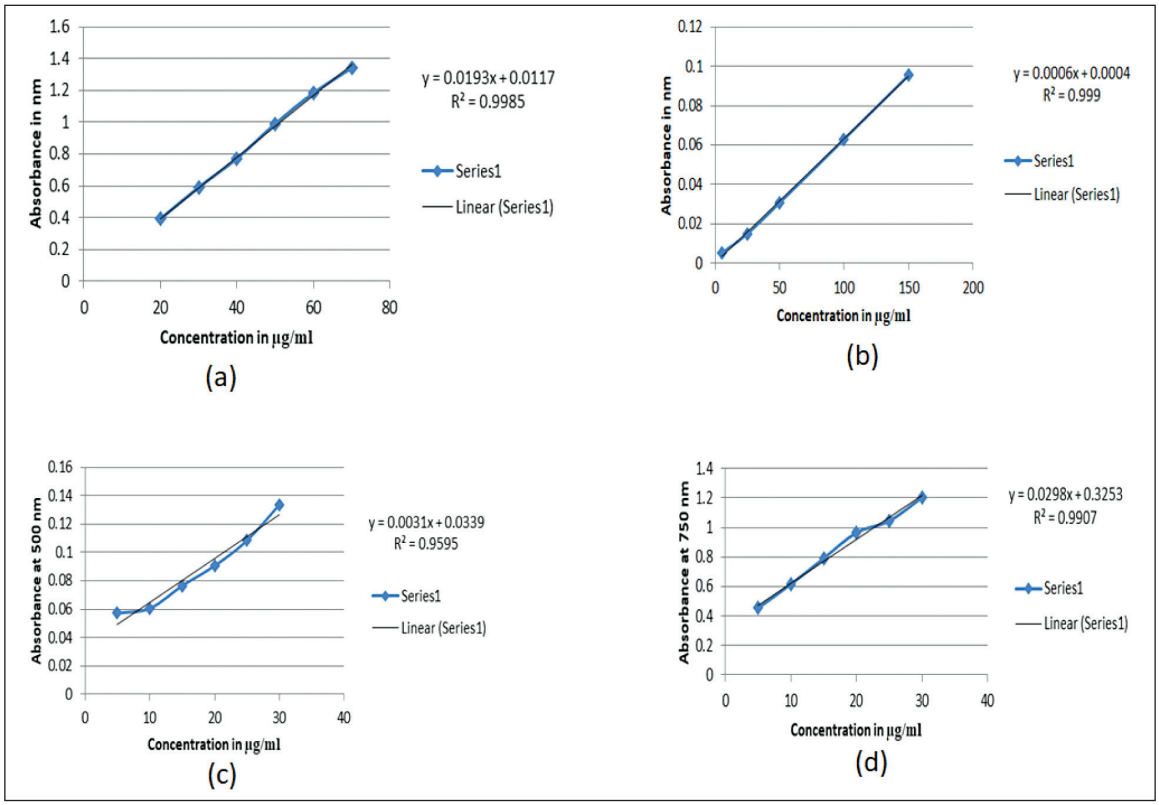

Figure 1. Standard curves used for quantification of phytochemicals; a) curve of gallic acid for quantification of phenolics, b) curve of quercetin used to quantify flavonoids, c) curve of catechin used to quantify tannin, d) curve of BSA used to quantify protein.

0.000 µg BSAE/mg DW), ethyl acetate (21.638 ± 0.001 g BSAE/mg DW), aqueous (12.342 ± 0.003 µg BSAE/mg DW), and DCM (4.060 ± 0.004 µg BSAE/mg DW; Table 2). These results demonstrate unequivocally that the polarity of the solvent has a bigger impact on the extraction of secondary metabolites.

In DPPH analysis, the acetone extract exhibited greater antioxidant activity than other solvent extracts with IC_{50} of 29.112 µg/ml, followed by 80% ethanolic (34.72 µg/ml), aqueous (35.92 µg/ml), ethyl acetate (49.17 µg/ml) and DCM (112 µg/ml; Table 2). It was observed that the percentage scavenging of different solvent extracts increases with the increase of concentration, as displayed in Figure 3b; therefore, free radical scavenging activity is concentration dependent. While in the reducing power assay, the acetone extract exhibited higher reducing power than other solvent extracts (Table 5). The reducing power of test extracts increases as sample concentration increases, as shown in Figure 3a. The greater antioxidant activity of acetone extract may be explained by the ability of acetone solvent to extract more phenolic and flavonoid components, and these chemical compounds are believed to possess reducing and chelating capabilities; it is thought that they have more potent antioxidant properties (Tabasum *et al*., 2016); therefore, acetone solvent is effective for extracting antioxidant compounds. According to other research, higher phenolic content is correlated with stronger DPPH scavenging ability (Bakchiche, 2017). Antioxidant compounds are essential in the fight against several diseases, including diabetes, cancer, hypertension, and cerebral cardiovascular disease (Rebaya *et al*., 2015). Flavonoids, which serve as anti-inflammatory and anti-allergenic substances,

Table 2. DPPH radical scavenging activity total phenolic, flavonoid, tannin and protein content of the different solvent extracts of *S. verticillata* solvent.

Extracts	Phenolic content ± SD in µg GAE/mg DW	Flavonoids content ± SD in µg QE/mg DW	Tannin content ± SD in µg CE/mg DW	Protein content ± SD in µg BSAE/mg DW	DPPH IC_{50} (µg/ml)
80% ethanolic	42.972 ± 0.012	111.62 ± 0.055	11.689 ± 0.001	24.038 ± 0.000	34.72
Aqueous	40.601 ± 0.003	71.47 ± 0.030	3.900 ± 0.002	12.342 ± 0.003	35.92
Acetone	48.811 ± 0.001	589.46 ± 0.010	33.379 ± 0.001	30.025 ± 0.002	29.11
Ethyl acetate	24.145 ± 0.002	464.65 ± 0.014	44.959 ± 0.001	21.638 ± 0.001	49.17
DCM	19.959 ± 0.001	286.47 ± 0.097	44.838 ± 0.003	4.060 ± 0.004	112.99
Ascorbic acid					7.153

The values in the table are given as Mean ± S. D (n = 3).

are significant health-protective molecules, and they lower the risk of developing chronic illnesses, such as cancer and heart disease (Rebaya *et al*., 2015). Also, it is reported that phenolic substances can prevent human mutagenesis and carcinogenesis, while polyphenols play an important role in protecting against free radicals which attack cells (Ghadigaonkar *et al*., 2021). Previous research has demonstrated that phenols and flavonoids have potent antioxidant properties and work as powerful anticancer agents, inhibiting angiogenesis and promoting apoptosis (Nguyen *et al*., 2020).

Regarding antimicrobial activity, the agar well diffusion method was used to screen the antibacterial activity of an 80% ethanolic extract of *S. verticillata* against a variety of microorganisms, the extract demonstrated good antimicrobial activity, and the results are presented in Table 3 and illustrated in Figure 2a. The findings showed that plant extract was effective in preventing bacteria growth; *E. coli* showed the highest antimicrobial activity with a zone of inhibition of 26 ± 0.75 mm, followed by *S. aureus* (12 ± 0.85), *P. aeruginosa* (11 ± 1.05 mm), *C. albicans* (10 ± 0.61 mm), *B. subtilis* (9 ± 0.86 mm), and *A. niger* (8 ± 0.45 mm); however, also it was observed that bacterial strains were more affected by plant extract than fungal strains. The findings of the evaluation of the extract's minimum bactericidal/fungicidal concentration and MIC are displayed in Table 3. The MIC of *S. verticillata* extract against all tested microorganisms varied and ranged from 4,000 to 16,000 µg/ml, while the MBC/MFC was between 16,000 and 64,000 µg/ml. The highest activity was observed against *S. aureus* with MIC and MBC of 4,000 and 16,000 µg/mL, respectively, but *A. niger* showed the least activity, with MIC and MBC values of 16,000 and 64,000 µg/ml, respectively. Compared with a zone of inhibition results, the *S. verticillata* extract appeared more effective in suppressing bacteria growth than fungi. These results strongly support the presence of bioactive components with antibacterial and antifungal effects in the *S. verticillata* 80% ethanolic extract and can be used to treat and manage infectious diseases. The different activity levels observed could result from the microbes' unique intrinsic characteristics and the substances found in the extracts (Aldakheel *et al*., 2020). An earlier study showed that phenolic compounds have stronger antibacterial activity because of their chelating characteristics and capacity to trap substances required for bacterial development (Kamdem *et al*., 2022). Due to their hydrophobic properties, phenolic compounds have a high affinity for lipids, which is related to how their antibacterial actions work; they are directly involved in rupturing the bacterial cell membrane, which inhibits cell metabolism and allows for the loss of cellular contents (Aldakheel *et al*., 2020). Inhibition of activities connected to the cell membrane, such as phosphorylation, electron transport, and protein translocation, is caused by phenolic compounds (Nizio *et al*., 2018). The antimicrobial properties of phytochemical constituents present in *S. verticillata* extracts are the main cause of the activity observed and may engage in multiple modes of action; the disruption of bacteria's cell membrane is reportedly caused directly by phenolic compounds, resulting in the inhibition of cell metabolism and, ultimately, emptying the cellular content (Andal *et al*., 2018), flavonoids compounds involves direct in the inhibition of nucleic acid synthesis, cytoplasmic membrane damage caused by process of perforation, and a decrease in membrane fluidity (Slobodníková *et al*., 2016). Proteins and amino acids may contribute to the antibacterial activity of *S. verticillata*; peptides can directly affect microorganisms and restrict their growth by interfering with the production of vital enzymes in the cell membrane (Andal *et al*., 2018).

Anti-biofilm activities of 80% ethanolic extract of *S. verticillata* were evaluated against fungi, Gram-negative and Gram-positive bacteria; the extract showed good anti-biofilm activity in a concentration-dependent manner against both Gram-negative and Gram-positive and fungal strains tested as illustrated in Figure 2c and d. At 10,000 µg/ml, *S. aureus* had the best anti-biofilm activity, followed by *B. subtilis*, *P. aeruginosa, E. coli*, *C. albicans,* and *A. niger* (Table 4). The result shows that Gram-positive bacteria were most affected, followed by Gram-negative bacteria, while fungi were least affected. The same study from other literature reported that Gram-positive strains are more sensitive than Gram-negative strains, where *S. aureus* was most sensitive (Lai *et al*., 2014). Our findings demonstrated that the plant extract had a greater impact on biofilm formation inhibition on Gram-positive bacteria than Gram-negative bacteria, but in comparison between bacteria and fungi, the findings from this study showed that the extract has greater anti-biofilm activity against bacteria than fungi. In other studies evaluating the anti-biofilm formation of methanolic pomegranate extract, the results revealed that *C. albicans* was less susceptible to the extract than *S. aureus* and *E. coli* (Bakkiyaraj *et al*., 2013). A high quantity of flavonoids, phenolics, tannins compounds, and other phytochemicals in *S. verticillata* extract may be the source and responsible for the activity observed. A previous study reported that flavonoids like quercetin, apigenin, and naringenin inhibit the formation of

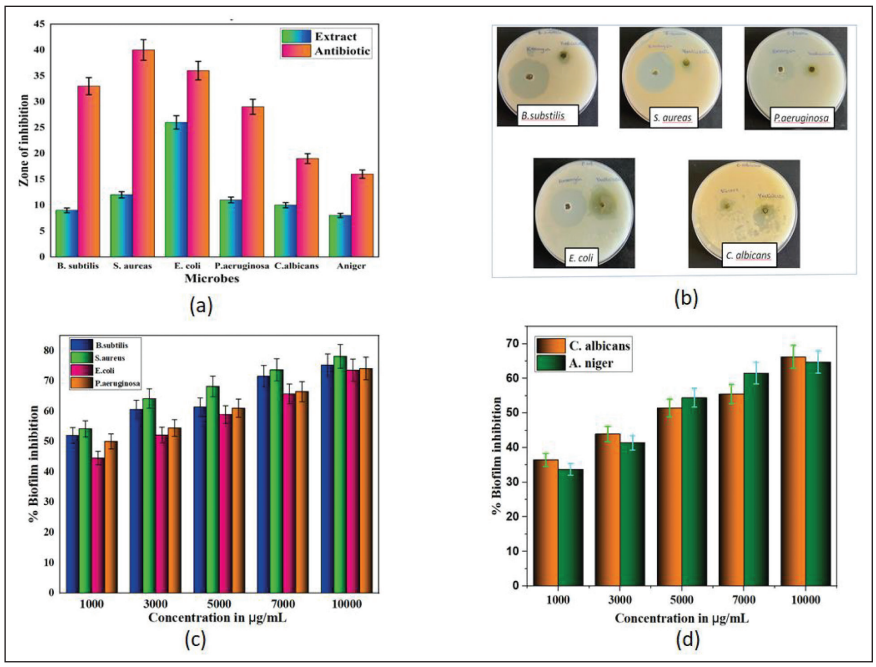

Figure 2. The effects of 80% ethanolic extract of *S. verticillata* on zone of inhibition and biofilm formation of microorganisms; a) Zone of inhibition shown by extract (100,000 µg/ml) in comparison with antibiotic against six microbes, b) plates showing effects of extract in terms of zone of inhibition for some microorganisms, c) biofilm inhibition effects shown by extract against four bacterial strains, d) effects of extract of biofilm formation against two fungal strains.

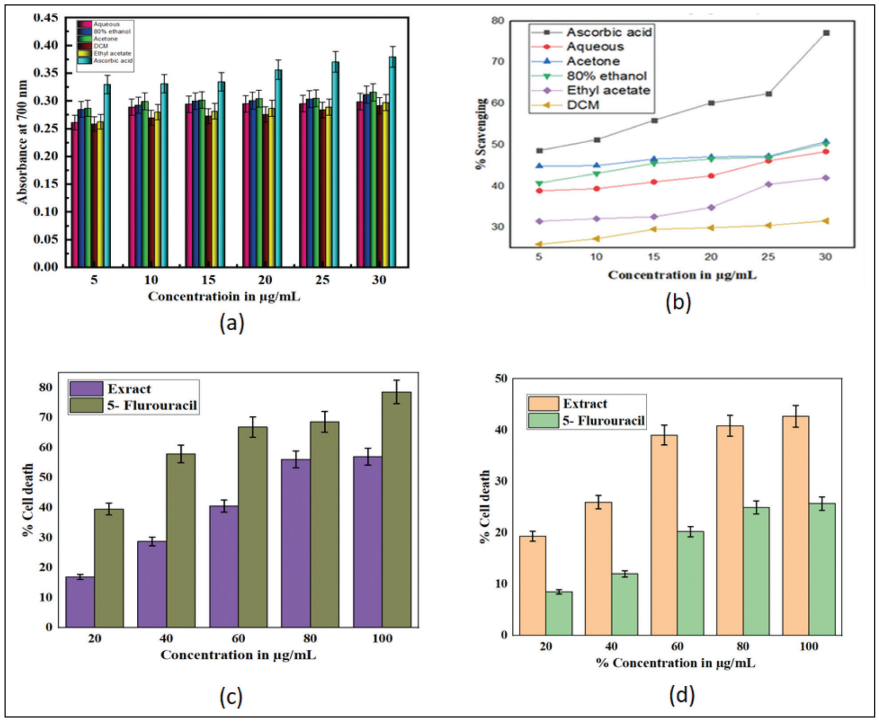

Figure 3. Antioxidant and cytotoxicity effects of *S. verticillata* extract; a) reducing power effect of different solvent extracts (aqueous, 80% ethanol, acetone, dichloromethane, ethyl acetate) in comparison with ascorbic acid, b) scavenging capacity of different solvent extracts (aqueous, 80% ethanol, acetone, dichloromethane, ethyl acetate) in comparison with ascorbic acid, c) effects of 80% ethanolic extract and standard (5- Flurouracil) on colon cancer cell lines, d) effects of 80% ethanolic extract and standard (5- Flurouracil) on normal kidney cancer cell lines.

Table 3. Zone of inhibition, MIC and MBC/MFC of *S. verticillata* on microorganisms.

Organisms	Inhibition zone of in mm		MIC (µg/ml)		MBC/MFC (µg/ml)	
	S. verticillata	Antibiotic	*S. verticillata*	Antibiotic	*S. verticillata*	Antibiotic
B. subtilis	9 ± 0.86	33 ± 0.79	8,000	500	16,000	1,000
S. aureus	12 ± 0.85	40 ± 0.55	4,000	500	16,000	1,000
E. coli	26 ± 0.75	36 ± 1.02	16,000	500	64,000	1,000
P. aeruginosa	11 ± 1.05	29 ± 1.08	8,000	500	32,000	1,000
C. albicans	10 ± 0.61	16 ± 0.13	8,000	1,000	32,000	2,000
A. niger	8 ± 0.45	13 ± 0.35	16,000	1,000	64,000	2,000

Inhibition zone diameters are expressed as Mean ± S. D (n = 3).

Table 4. Anti-biofilm activity of *S. verticillata* extracts against pathogenic bacterial and fungal strains.

Sample	Concentration (µg/ml)	% Biofilm formation inhibition					
		Gram + ve bacteria		Gram −ve bacteria		Fungi	
		B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. niger*
S. verticillata	1,000	52.09 ± 0.90	54.21 ± 0.56	44.62 ± 1.65	50.05 ± 1.35	36.41 ± 0.42	33.72 ± 0.84
	3,000	60.62 ± 1.02	64.24 ± 1.40	52.22 ± 1.25	54.57 ± 1.45	43.93 ± 1.02	41.42 ± 1.40
	5,000	61.44 ± 1.42	68.22 ± 1.50	58.94± 1.30	61.05 ± 1.60	51.44 ± 0.74	54.43 ± 0.95
	7,000	71.62 ± 1.08	73.73 ± 1.45	65.77± 1.9	66.51 ± 1.4	55.49 ± 0.58	61.53 ± 0.85
	10	75.26 ± 0.95	78.22 ± 0.80	73.59 ± 0.85	74.15 ± 1.7	66.27 ± 1.13	64.73 ± 1.01
Streptomycin	0.5	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	-	-
Micanozole (Standard)	0.5	-	-	-	-	100 ± 0.00	100 ± 0.00

The values in the table are given as Mean ± S. D (n = 3).

Table 5. Reducing power capacity of *S. verticillata.*

Concentration (µg/ml)	Absorbance at 700 nm of different solvent extracts					
	Aqueous	80% ethanol	Acetone	DCM	Ethyl acetate	Ascorbic acid
5	0.2608 ± 0.025	0.2847 ± 0.011	0.2865 ± 0.013	0.2586 ± 0.024	0.2622 ± 0.014	0.3295 ± 0.012
10	0.2886 ± 0.041	0.2920 ± 0.022	0.2990 ± 0.017	0.2696 ± 0.021	0.2798 ± 0.019	0.3309 ± 0.021
15	0.2941 ± 0.026	0.2996 ± 0.023	0.3015 ± 0.021	0.2726 ± 0.023	0.2813 ± 0.018	0.3344 ± 0.031
20	0.2947 ± 0.023	0.3006 ± 0.019	0.3040 ± 0.019	0.2758 ± 0.029	0.2866 ± 0.031	0.3560 ± 0.014
25	0.2951 ± 0.029	0.3031 ± 0.025	0.3044 ± 0.026	0.2837 ± 0.028	0.2887 ± 0.022	0.3703 ± 0.016
30	0.2984 ± 0.017	0.3113 ± 0.027	0.3153 ± 0.034	0.2915 ± 0.021	0.2970 ± 0.027	0.3793 ± 0.024

The values in the table are given as Mean ± S. D (n = 3).

biofilms by suppressing the communications between cells in the body (Kim *et al*., 2016). Polyphenols compounds play a crucial role as anti-biofilm agents; a previous study reported that these compounds inhibit biofilm production in *E. coli, S. aureus,* and *E. faecalis* (Slobodníková *et al*., 2016). Some literature suggested that tannins induce astringency, which could be involved in disabling the biofilm (Bakkiyaraj *et al*., 2013).

In cytotoxicity activity, the COLO205 colon Cancer cell lines and NRK52E normal kidney cell lines were used to assess 80% ethanolic extract of *S. verticillata* cytotoxicity effects by MTT assay, and their results are shown in Table 6. The extract showed a more significant inhibition effect on COLO205 cancer cell line a than NRK52E normal kidney cell line, as illustrated in Figure 3c and d, with IC_{50} values of 79.03 µg/ml for COLO205 colon cancer cell line and 112.58 µg/ml for NRK52E normal kidney cell line. Since human cell lines can more easily predict probable consequences and produce data that is more useful to humans, they were chosen for our investigation (Erhirhie *et al*., 2018). The effective comparison of cytotoxicity effects of chemicals and plant extracts can be done using the $IC_{50,}$ which assesses a substance's ability to stop the growth of 50% of cells. Chemicals and extracts are categorized as follows in accordance with the National Cancer Institute's and Geran Protocol's standards, $IC_{50} \leq$ 20 µg/ml = highly toxic to cells, IC_{50} 21–200 µg/ ml= moderately toxic to cells, IC_{50} 201–500 µg/ml = weakly toxic to cells, and IC_{50} > 501 µg/ml = no toxic to cells (Ogbole *et al*., 2017). The IC_{50} of 80% ethanolic extract of *S. verticillata* in NRK52E normal kidney cell line and COLO205 Colon Cancer cell line were 112.58 and 79.03 µg/ml, respectively, since the IC_{50} values for the two cell lines examined ranged from 21 to 200 g/ ml, the results clearly

show that the 80% ethanolic extract of *S. verticillata* displayed moderate cytotoxic action against them. Still, the extract had stronger activity against colon cancer and little activity in normal kidney cell lines. This cytotoxic potential may result from the plant extract's phytochemicals' capacity to trigger apoptosis and inhibit the cell cycle (Kamdem *et al*., 2022).

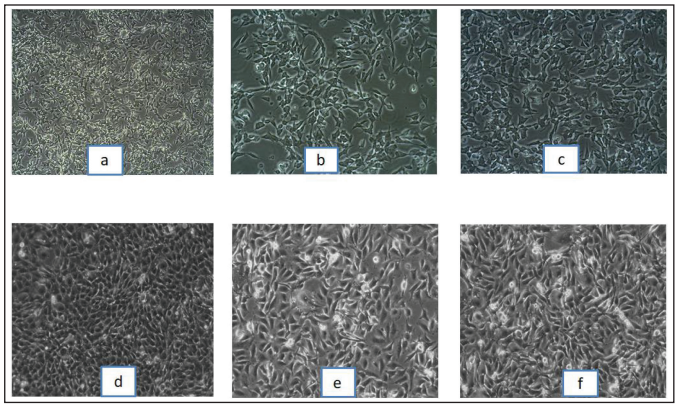

Figure 4. Microscopic images of colon cancer cell lines and normal kidney cell lines under treatment of 80% ethanolic extract of *S. verticillata*, control (lactic acid) and standard (5-Flurouracil); a) Colon cancer cell lines treated with control (lactic acid), b) colon cancer cell lines treated with standard (5-Flurouracil), c) colon cancer cell lines treated with *S. verticillata* extract, d) normal kidney cell lines treated with control (lactic acid), e) normal kidney cell lines treated with standard (5-Flurouracil), f) normal kidney cell lines treated with *S. verticillata* extract.

Bioactive compounds identification**,** the GC-MS chromatograms of the 80% ethanolic extract of *S. verticillata* revealed 5 compounds (Supplementary Material). The most abundant compounds in the 80% ethanolic extract included Butylated Hydroxytoluene (1), Hexadecanoic acid, ethyl ester (2), Linoleic acid ethyl ester (3), Ethyl 9,12,15-octadecatrienoate (4), Octadecanoic acid, ethyl ester (5) all compounds are represented in Figure 5. A thorough literature search in online databases such as PubChem, Scopus, Web of Science, and PubMed was conducted to assess whether any bioactivity has been reported regarding the identified compounds. The biological actions of certain identified compounds have been described in several studies; Butylated hydroxytoluene is reported to be a powerful antioxidant that is safe to use in the pharmaceutical and food industries (Yehye *et al*., 2015). Hexadecanoic acid-ethyl ester has antioxidant, antimicrobial, nematicide, hemolytic, hypocholesterolemic, and anti-androgenic properties (Aldakheel *et al*., 2020). The compound linoleic acid ethyl ester was reported to have the following properties: anti-arthritic, anti-inflammatory, nematicide, anti-coronary, hypocholesterolemic, hepatoprotective,

Table 6. Effect of *S. verticillata* extract against NRK52E normal kidney cell line and COLO205 colon cancer cell line.

Test sample	Concentration (µg/ml)	NRK52E normal kidney cell lines			COLO205 colon cancer cell lines		
		% cell alive	% cell death Mean ± SD	IC_{50}	% cell alive	% cell death Mean ± SD	IC_{50} µg/ml
Standard (5– Fluorouracil)	20	8.451	8.478 ± 0.37	194.48	60.449	39.551 ± 0.13	32.05
	40	12.179	11.986 ± 0.17		42.043	57.957 ± 0.25	
	60	20.298	20.217 ± 0.30		33.114	66.886 ± 0.24	
	80	25.021	24.940 ± 0.22		31.333	68.667 ± 0.13	
	100	26.264	25.682 ± 0.51		21.419	78.581 ± 0.21	
S. verticillata	20	19.635	19.332 ± 0.33	112.58	83.073	16.927 ± 0.16	79.03
	40	26.015	25.959 ± 0.17		71.241	28.759 ± 0.66	
	60	39.188	39.022 ± 0.21		59.463	40.537 ± 0.42	
	80	40.928	40.845 ± 0.29		43.906	56.094 ± 0.39	
	100	42.751	42.695 ± 0.17		43.002	56.998 ± 0.33	

The values in the table are given as Mean ± S. D (n = 3).

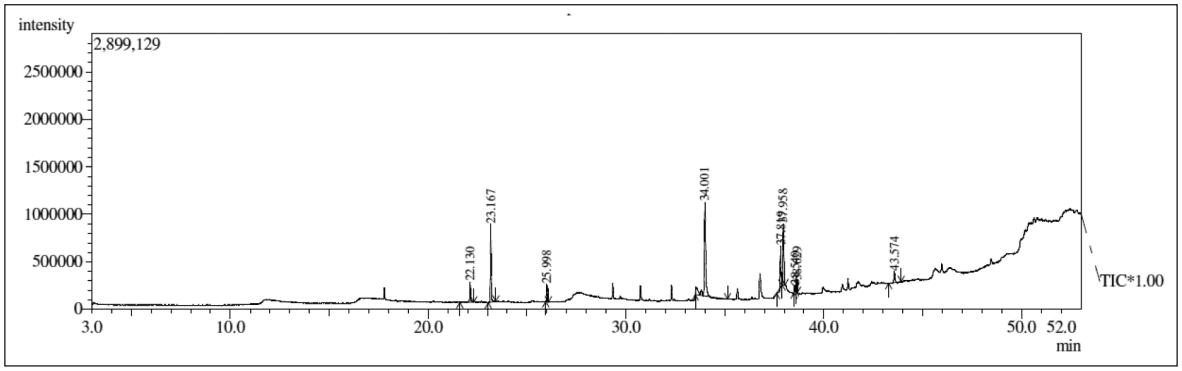

Figure 5. GC-MS chromatogram of 80% ethanolic extract of *S. verticillata*, five major bioactive compounds were identified.

anti-androgenic, antihistaminic, insectifuge, and anti- eczematic (Tyagi and Agarwal, 2017). Ethyl 9,12,15-octadecatrienoate was reported to possess antioxidant, antimicrobial, anti-inflammatory, and pesticide activity (Aldakheel *et al*., 2020). Since *S. verticillata* has been reported to be a sedative and treatment for unspecified medicinal infections/ disorders and associated symptoms (Thomas, 2020). With all the above justification, it can be concluded that *S. verticillata* is the potential for the treatment and management of a variety of non-infectious and infectious diseases and their symptoms.

CONCLUSION

The efficiency of the *S. verticillata* extracts as antimicrobials, anti-biofilm, antioxidants, and cytotoxicants was assessed. The plant extracts demonstrated good antimicrobial activity; the highest activity was observed against *S. aureus* with MIC value of 4000 µg/mL and MBC of 16000 µg/ml. The cytotoxicity activity was observed against COLO205 colon cancer cell lines with an IC_{50} of 79.03 µg/ml. Moreover, the plant extract exhibited strong antioxidant activity, and various bioactive substances with antimicrobial, anticancer, and antioxidant were identified. Based on those findings, this study points out that the plant species could be considered a new natural source of antimicrobial, antioxidant, and anticancer molecules with therapeutic potential. Therefore, isolation and characterization of bioactive compounds should be done; additionally, sub-acute and sub-chronic toxicity investigation of extracts and isolated bioactive compounds should be conducted to assess the side effects, biochemical, and hematological parameters.

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LIST OF ABBREVIATIONS

BSA, Bovine serum albumin; BSAE, Bovine serum albumin equivalents; CE, Catechin equivalents; CFU, colony forming unit; DCM, Dichloromethane; DMSO, Dimethyl sulphoxide; DNA, Deoxyribonucleic acid; DPPH, 2, 2- diphenyl picrylhydrazyl; DW, Dry weight; GAE, Gallic acid equivalents; GC, Gas chromatography; GC-MS, Gas chromatography mass spectrometer; HCl, Hydrochloric acid; IC_{50}, Inhibitory concentration of 50%; ICCR, Indian Council for Cultural Relations; MHB, Mueller Hinton broth; µg, microgram; MTT, 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide; MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration; MFC, Minimum fungicidal concentration; mg, milligram; NCCS, National Centre for Cell Science; OD, Optic density; PBS, Phosphate buffer solution; QE, Quercetin equivalent; rpm, revolution per minute; SD, Standard deviation; TSB, Trypticase soy broth; UV, Ultraviolet light; WHO, World Health Organization.

AUTHORSHIP CONTRIBUTIONS

Alfredi A. Moyo: Developed the idea, conducted the study and experimentation, gathered the information, and wrote the initial manuscript. Kishor S. Jagadhane: prepared all the figures. Sneha R. Bhosale: performed the data analysis and created all the tables. Sachin B. Shinde: Data analysis/interpretation. Vinod B. Shimpale: Identifying plants, editing, and reviewing manuscripts. Prashant V. Anbhule: Editing, reviewing, and finishing the manuscript.

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CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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SUPPLEMENTARY MATERIAL

Supplementary data can be downloaded from the link:[https://japsonline.com/admin/php/uploadss/4060_pdf.pdf]