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# Isolation, structure elucidation and bioactivity of secondary metabolites produced by marine derived *Streptomonospora arabica* VSM-25

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The strain VSM-25 with an exhilarating bioactive potential isolated during our systematic screening of marine actinomycetes was identified as *Streptomonospora arabica* based on polyphasic taxonomy. The ethyl acetate extract of culture filtrate was purified by silica gel column chromatography. The chemical structure of active compounds was determined by NMR, FTIR, and ESIMS and were established as Indole-3-carboxaldehyde (C1), 2, 3-dihydroxy benzoic acid (C2), Vanillic acid (C3), Daidzein (C4), and 3, 4-Dihydroxy benzaldehyde (C5). The antimicrobial activities of the compounds were tested against medicinally and agriculturally significant bacteria and fungi. C1 displayed a high inhibitory effect against bacteria and fungi to that of the other compounds tested. C5 exerted the strongest scavenging activity of free radicals such as DPPH and NO at a concentration of 400 µg/mL. C1 inhibited alpha-amylase effectively at 400 µg/mL although it was less potent than acarbose. C3 and C4 exerted significant anti-inflammatory and anti-arthritic activities at 400 µg/mL. The anti-inflammatory activity of compound C3 was found to be more potent than Diclofenac sodium, the reference drug. MTT assays of five compounds against MDA-MB-231 and MCF-7 cell lines using taxol as standard documented cytotoxicity. C4 showed highest activity of 67.81% and 54.33% ($IC_{50}$ -1 µg/mL) against MDA-MB-231 and MCF-7. The cytotoxicity of five compounds was also evaluated by soft agar colony forming assay to determine the ability of MDA-MB-231 cells to proliferate while cell cycle arrest at sub G1 and induction of apoptosis was documented with MDA-MB-231 cells after treatment with C1, C2, C3, C4, and C5.

**Keywords**: Anti-inflammatory activity, Antimicrobial activity, Antioxidant activity, Cell cycle arrest, Cytotoxicity, *Streptomonospora arabica*

Marine microbes have unfolded the greatest genetic and metabolic diversity to acclimate themselves to utmost environmental conditions distinguished by low temperature, lack of light, high atmospheric pressures, variable salinity, and low levels of oxygen[1-2]. Among such microbes, marine actinobacteria are the propitious source of structurally diverse and versatile bioactive secondary metabolites with many therapeutic applications[3]. Soil actinobacteria have been widely exploited, but marine actinobacteria are inadequately investigated to that of terrestrial counterparts[4]. In recent years marine actinomycetes have received considerable attention since they have developed unique biochemical and physiological capabilities for easy accustom to extreme habitats and also afford to produce compounds with antibacterial, antifungal, antimitotic, antiviral, cytotoxic, and antineoplastic activities[5].

Screening of phylogenetically distinct and uncommon organisms from extreme ecosystems is a thoughtful approach to unearth novel chemical structures with medicinally pertinent biological activities[6]. The evolution of multidrug-resistant pathogens and the increased prevalence of cancer inspired interest in the exploration and isolation of potent bioactive metabolites of natural origin for potential pharmaceutical and industrial applications[7]. Studies related to the biopotentiality of anticancer metabolites based drug discovery from marine actinobacteria is too limited[8]. In the recent literature, the deep sea has emerged as a new frontier in natural products chemistry, when there is an indispensable requirement for novel drug templates to encounter the emerging drug resistance and cancer[1].

The genus *Streptomyces* alone produces a large number of bioactive molecules, raising the alluring

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probability that additional chemically prolific taxa anticipate their discovery[9]. This ambition may be reached by inspecting new microbial sources for the production of bioactive compounds, such as non-*Streptomyces* actinomycetes. Rare actinomycetes produce unique, unprecedented compounds exhibiting excellent bioactive potency[10]. It is difficult to isolate rare actinomycetes genera by using conventional isolation approaches. Novel genera can be isolated taking into account several factors during the isolation procedures, such as the selection of untouched niches for sample collection, unique enrichment procedures, selective isolation methods, use of specific selective isolation media, phylogenetic analysis, culture-independent methods, and digital image analyses[11]. Subsequently, employing pre-treatment of sediment samples by drying and heating accelerated the isolation of rare actinomycetes[12].

The marine habitat of the Bay of Bengal, coastal Andhra Pradesh, India is less explored for actinobacterial diversity and bioactive metabolites. Hence there is a probability to identify novel actinobacteria in this habitat. Accordingly, the present study was designed to investigate the marine sediment samples of the Bay of Bengal with the ultimate objective of discovering rare actinomycetes. In the systematic screening program, the morphologically distinct actinobacterial strain VSM-25 was selected from 57 actinobacterial strains isolated from marine sediment samples which exhibited high antimicrobial potential. Therefore in the present study, attempts have been made to study the taxonomic position of the strain, as well as purification, structure elucidation, and biological evaluation of isolated compounds responsible for its bioactive potential.

## Materials and Methods

### Sampling

Marine sediment samples were collected at different depths of the Bay of Bengal of north coastal Andhra Pradesh, India, and blended to achieve a composite sample for further investigation. Sediment samples were collected using clean, dry, sterilized ziploc bags and transferred to the laboratory. The collected sediment samples were dehydrated in a laminar air flow cabinet for a certain period of time and sieved to exclude large particles. Samples were preserved at 4°C until pre-treatment for restraining undesirable microorganisms.

### Isolation

The dried sediment sample was pre-treated with sodium dodecyl sulphate (0.05%) and yeast extract (5%)[13]. The pre-treated sediment sample (1 g) was suspended in 10 mL of sterile distilled water, homogenized by vortexing. Serial dilutions were prepared down to $10^{-4}$ dilution and 10 µL of $10^{-4}$ dilution was plated on Marine Agar medium[14] supplemented with filter-sterilized antimicrobial agents such as nalidixic acid (50 µg $mL^{-1}$) and nystatin (50 µg $mL^{-1}$) to retard the growth of Gram-negative bacteria and fungi, respectively. The inoculated plates were incubated at 30°C for 2 to 4 weeks to isolate slow growing actinomycetes. After incubation tough and leathery colonies partially embedded into the agar, identified as actinobacterium were selected and subcultured on YMD (ISP-2) agar slants for further characterization.

### Identification

A total of 57 actinobacterial isolated strains, were screened for their bioactive potential and 22 strains exhibited antimicrobial activity. Among 22 strains, 10 strains were active against all the test micro-organisms. The strain VSM-25 with significant antimicrobial activity was selected and maintained by subculturing on yeast extract malt extract dextrose (YMD) agar medium at 4°C for further characterization. The isolate was preliminarily characterized as described in the International *Streptomyces* Project (ISP). The cultural characteristics of the strain were studied on different ISP and non-ISP media such as ISP-1, ISP-2, ISP-3, ISP-4, ISP-5, ISP-6, ISP-7, Starch-casein agar, Czapek-Dox agar, and nutrient agar media[15]. The micro morphology of the strain was examined under microscopy (Olympus) by the slide culture method[16]. Physiological and biochemical characterization of strain was evaluated using standard protocols[17].

### Identification by Molecular Approach

The strain grown in YMD broth at 30°C for 2-3 days was centrifuged at 10,000 rpm, 4°C for 20 min, and the pellet was used for the extraction of genomic DNA[18]. The 16S rRNA gene was amplified in thermo cycler (PCR) with a reaction mixture containing 1 X PCR buffer, *Taq* polymerase (2 U), dNTP (200 µM), template DNA (50 ng), forward primer (20 µM) (5'-GAGTTTGATCCTGGC TCA-3') and reverse primer (20 µM) (5'-ACGGCTA CCTTGTTACGACTT-3'). The final volume of the

PCR was made up to 100 µL by adding distilled water and the reaction mixture. PCR amplification was carried out with initial denaturation at 94°C (3min) and 30 cycles of denaturation at 94°C (1 min), annealing at 65°C (1 min) and extension at 72°C (1 min) and further 5 min extension at 72°C. The amplified product was tested on 1% agarose gel and then purified with a DNA purification kit (Helini Bio molecules) according to manufacturer instructions. The bands were analyzed under UV light and documented using Gel Doc. The amplified product was sequenced by a dideoxy chain termination approach using 3100-Avant Genetic Analyzer (Applied Bio systems, USA). The obtained sequences were analyzed for homology using the Basic Local Alignment Search Tool (BLAST) (NCBI). The phylogenetic tree was constructed using the Neighbor-joining method[19] and employing Molecular Evolutionary Genetic Analysis (MEGA7) software[20].

**Fermentation and Extraction**

A seed culture of *S. arabica* VSM-25 was grown in 100 mL YMD broth and incubated on a rotary shaker (250 rpm) at 30°C for 48 h. Seed culture at a concentration of 10% (100 mL of the seed culture in 1000 mL of production medium) was transferred to an optimized fermentation medium consisting of 2 % Galactose, 1% Peptone, 1% Soy bean fluor, 0.05% $K_2HPO_4$, 3% NaCl with pH adjusted to 8. After cultivation of strain for 11 days, culture filtrates (40 L) were extracted twice with ethyl acetate. The extract was concentrated by rotary evaporation to yield 3.7 g dark brown crude extract and then freeze-dried.

**Purification and Structure Elucidation of Bioactive Compounds**

Isolation and purification of bioactive compounds were carried out by applying the crude extract to a silica gel G column (80 × 2.5 cm, Silica gel, Merck, Mumbai, India). The separation of crude extract was conducted via gradient elution with Chloroform: Methanol. The eluent was run over the column and small volumes of eluent collected in test tubes were analyzed via thin-layer chromatography (TLC) using silica gel plates (Silica gel, Merck, Mumbai, India) with Chloroform: Methanol solvent system. Compounds with identical retention factors ($R_f$) were combined and assayed for antimicrobial activities. Among the 10 main fractions eluted, 8 were polar residues and two were non-polar residues. Based on $^1$HNMR spectral data and bioactive screening, four polar fractions were selected for further studies. Four fractions were collected at different eluent conditions ($CHCl_3$: MeOH) (First at 80-20 *v/v*; second at 70-30 *v/v*; third at 60-40 *v/v* and fourth at 40-60 *v/v*), respectively. Further purification of fractions through silica gel column using different gradient eluent systems for final elucidation of compounds yielded one compound from a first fraction (C1), two from second (C2, C3), one from the third fraction (C4), and another one compound (C5) from fourth of the polar residue. The structures of active compounds were analyzed based on Nuclear Magnetic Resonance ($^1$H NMR and $^{13}$ C NMR) model: Varian Gemini 200 (Samples were made in $CDCl_3$ with Trimethyl Saline as standard), Electron Spray Ionization Mass spectrophotometry (ESIMS/ESIMS-QTOF); model: Micromass VG-7070H, 70 eV spectrophotometer; Agilent and Fourier Transform Infrared (FTIR), model: Thermo Nicolet Nexus 670 spectrophotometer with NaCl optics.

**Test Microorganisms**

Gram-positive bacteria: *Bacillus cereus* (MTCC 430), *Streptococcus mutans* (MTCC 497), *Staphylococcus aureus* (MTCC 3160), *Staphylococcus epidermis* (MTCC 120), *Bacillus subtilis* (ATCC 6633), and *Bacillus megaterium* (NCIM 2187). Gram-negative bacteria: *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus vulgaris* (MTCC 7299), *Serratia marcescens* (MTCC 118), *Xanthomonas campestris* (MTCC 2286), *Xanthomonas malvacearum* (NCIM 2954), and *Salmonella typhi* (ATCC 14028). Medically important dermatophytes: *Candida albicans* (ATCC 10231) and *Epidermophyton floccosum* (MTCC 145). Medically and agriculturally important filamentous fungi: *Aspergillus niger* (ATCC 1015), *Aspergillus flavus* (ATCC 9643), *Fusarium oxysporum* (MTCC 3075), *Fusarium solani* (MTCC 4634), *Penicillium citrinum* (MTCC 6489), *Verticillium alboatrum,* and *Alternaria alternata* (MTCC 6572). The test micro-organisms used in the study were procured from ATCC, University Boulevard, Manassas, USA, MTCC, and NCIM, India, and preserved at 4°C.

**Minimum Inhibitory Concentration (MIC) Assay**

The antimicrobial spectra of bioactive compounds of strain were determined in terms of MIC against a wide variety of Gram-positive and Gram-negative bacteria and fungi by using agar plate diffusion

assay[21]. Triplicate sets of plates were maintained for each concentration of the test sample. Nutrient agar and Czapek-Dox agar media were prepared to grow bacteria and fungi, respectively. The purified compounds were dissolved in dimethyl sulfoxide at concentrations ranging from 0 to 1000 µg/mL and used to assay against test bacteria and fungi. The inoculated plates were examined after 24-48 h of incubation at 37°C for bacteria and 48-72 h at 28°C for fungi. The lowest concentration of bioactive metabolites exhibiting significant antimicrobial activity against test microorganisms was taken as the MIC of the compound.

**DPPH Radical Scavenging Assay**

The DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging activity of compounds isolated from *S.arabica* was executed according to the method described by Myagmar and Aniya[22] with slight modification. The reaction solution contains 1.0 mL of 0.1 mM DPPH-methanol solution, 0.95 mL of 0.05 M Tris-HCl buffer (pH 7.4), and 50 µL of different concentrations of five test samples and reference compound (200 µg/mL and 400 µg/mL). The reaction mixture was incubated for 30 min in dark at ambient temperature and the optical density of each sample was measured at 517 nm corresponding to blank using a UV-Vis spectrophotometer. All tests were run in triplicate and averaged. Ascorbic acid was used as a reference compound. The methanolic solution of DPPH served as a control. The DPPH assay method was reported as radical scavenging activity (RSA %) using the following equation:

$RSA\% = [A_{control}-A_{sample}]/[A_{control}]\times 100$

$A_{control}$ =absorption of blank sample;

$A_{sample}$= absorption of test compounds

0.5 mL of the C1 (Indole-3-Carboxaldehyde), C2 (2, 3-Dihydroxybenzoic acid), C3 (Vanillic acid), C4 (Diadzein), and C5 (3, 4-Dihydroxybenzaldehyde) purified from strain were added to DPPH at concentrations 200 µg/mL and 400 µg/mL and calculated as above

**Determination of Nitric Oxide (NO) Radical Scavenging Efficacy**

The reaction mixture in this assay contained 0.5 mL of sodium nitroprusside (5 mM) in phosphate-buffered saline at pH 7.4 was mixed with five test samples (C1, C2, C3, C4, and C5) separately, and the standard ascorbic acid with a concentration of 200 µg/mL and 400 µg/mL. The reaction mixture was incubated for 60 min in light at 25°C. After incubation, 1.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% of napthylene diaminedihydrochloride)[23] was added and incubated for 30 min at 25°C in dark. The absorbance of pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with napthyl ethylene diamine dihydrochloride (NED) was read at 546 nm. All the tests were executed in triplicates. The inhibition percentage of free radicals was calculated using the following formula

$$\%\,\text{of free radical scavenging} = \frac{(\text{Abs of control} - \text{Abs of test})}{\text{Abs of control}} \times 100$$

**α-Amylase Inhibitory Assay**

The α-amylase inhibitory assay was performed as per the standard protocol[24]. 500 µL of five test samples *viz*., C1, C2, C3, C4 and C5 at concentrations of 200 µg/mL and 400 µg/mL added to 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.5 mg/mL) were pre-incubated for 10 min at 25°C. After pre-incubation, 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer (adjust pH 6.9 with 0.006 M sodium chloride) was added to each tube. The reaction mixtures were subjected to incubation for 10 min at 25°C after which 1 mL of 3, 5-dinitrosalicylic acid color reagent was added to terminate the reaction. The tubes were incubated in a boiling water bath for 5 min and cooled at room temperature. Finally, the reaction mixture was diluted with the addition of 10 mL of Milli Q water. Absorbance was recorded at 540 nm. Acarbose, a known α-amylase inhibitor was used as a standard drug and the percentage inhibition of α-amylase enzyme was calculated using the formula

$$\%\,\text{inhibition of } \alpha - \text{amylase} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Suitable reagent blank and inhibitor controls were simultaneously carried out.

***In vitro* Evaluation of Anti-inflammatory Activity**

An *in vitro* anti-inflammatory activity of isolated compounds was performed as per the modified protocol[25]. The reaction mixture (5 mL) contained 0.2 mL of bovine serum albumin (5% aqueous solution), 2.8 mL of the phosphate-buffered saline (pH 6.3), and 2 mL of five test compounds at

concentrations of 200 µg/mL and 400 µg/mL. The same volume of double distilled water served as control. The reaction mixtures were incubated at 37°C ± 2°C in a BOD incubator for 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm by using the vehicle as blank. Diclofenac sodium (200 µg/mL and 400 µg/mL) was used as a reference drug and treated similarly for determination of absorbance. Percentage inhibition of protein denaturation was calculated by using the following formula.

$$\text{Percentage Inhibition} = \frac{\text{Absorbance of control}}{\text{Absorbance of Test/Absorbance of control}} \times 100$$

**Evaluation of *in vitro* Anti-arthritic Activity**

Inhibition of protein denaturation method was used for evaluation of *in vitro* anti-arthritic activities of five compounds (C1, C2, C3, C4, and C5)[26]. The standard drug taken for the present study is diclofenac sodium. The test solution (0.5 mL) contains 0.45 mL of bovine serum albumin (5% *w/v* aqueous solution) and 0.05 mL of test compounds of various concentrations. The standard solution (0.5 mL) consists of 0.45 mL of bovine serum albumin and 0.05 mL of Diclofenac sodium and control contains 0.45 mL of bovine serum albumin and 0.05 mL of distilled water while product control lacked BSA. 0.05 mL of two concentrations (200 µg/mL and 400 µg/mL) of test compounds and standard drug diclofenac sodium (200 µg/mL and 400 µg/mL) were taken and 0.45 mL of BSA (0.5% *w/v*) was mixed. Each solution was adjusted to pH 6.3 using 1 N HCl. The samples were incubated at 37°C for 20 min followed by 57°C for 30 min. After cooling, 2.5 mL of phosphate buffer was added and absorbance was measured at 660 nm. The results were compared with diclofenac sodium. The percentage inhibition of protein denaturation can be calculated as:

$$\%\ \text{inhibition of protein denaturation} = \frac{(\text{Absorbance of Test Solution} - \text{Absorbance of Product Control})}{\text{Absorbance of Test Control}} \times 100$$

**Cell Lines and Culture Conditions**

The cell lines used in the present study were obtained from National Centre for Cell Science, Pune, India. Cell lines MDA-MB-231 were cultured on Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10% (*v/v*)), L-glutamine (2 mM), penicillin (10 units/mL) and streptomycin (10 µg/mL), while breast cancer cell line MCF-7 was cultured on Roswell Park Memorial Institute medium 1640 supplemented with fetal bovine serum (10%; (*v/v*)), L-glutamine (2 mM), penicillin (10 units/mL) and streptomycin (10 µg/mL) in a humidified atmosphere (95%) with 5% of $CO_2$ at 37°C.

***In vitro* Cytotoxicity by MTT Assay**

The cytotoxicity of compounds was assessed based on measurement of *in vitro* growth in 96-well plates by cell-mediated reduction of tetrazolium salt to water-insoluble formazan crystals, as per the micro-culture MTT assay[27]. Cells were seeded in 96-well microtiter plates at a density of $5\times10^3$ per well (100 µL) containing 0.1 mL of medium. After overnight incubation, the cells were treated with different test concentrations of bioactive compounds (0.01, 0.1, 1, 10, 100, 400 µg/mL) at identical conditions with three replicates of each concentration. After 24 h of incubation, cell viability was assessed by adding 20 µL of MTT (5 mg/mL in PBS) per well and the plates were incubated at 37°C for 4 h. The formazan crystals formed in cells were dissolved with 100 µL of 0.1% acidified isopropanol, and the rate of color development was measured at 570 nm using a micro plate reader. The sample treatment for the present study was taken as follows Negative Control: MDA-MB-231 and MCF-7 Cells and DMSO, positive control: MDA-MB-231 and MCF-7 cells, Taxol and DMSO and test samples include MDA-MB-231 and MCF-7 cells and different concentrations of the five test samples (0.01, 0.1, 1, 10, 100, 400 µg/mL) and DMSO. All the experiments were carried out in triplicates.

**Soft Agar Colony Formation Assay (SACFA)**

SACFA is a widely used method to evaluate the transformation of the cells *in vitro. T*he cells are dispersed in the culture plate and cell growth is supported by a conditioned medium that provides necessary growth factors. Unlike normal cells that require substrate for growth, the transformed cells do not require substrate for their growth and are anchorage independent. The common application of the SACFA can be used to test specific compounds for their potency to inhibit tumor growth *in vitro*. The colony count and size are the quantitative read-outs of assay and can be compared with control. The method also executes the results with reference to drug concentration.

The experiment was executed in 24 well tissue culture plates. 1000 µL base layer of agarose (1%) is

prepared by autoclaving the agarose solution. 2.1 mL of agarose solution (1%) is added to 7.9 mL of DMEM medium supplemented with 10% of FBS. MDA-MB-231 that has attained 90% of confluency were carefully washed twice in pre-warmed phosphate-buffered saline and then trypsinized by adding 5 mL of trypsin/EDTA solution and incubated for 5 min at 37°C. The cells were centrifuged for 10 min at 500 g and the resultant cells were suspended in 10 mL of pre-warmed DMEM medium with 10% FBS and the number of cells was counted by hemocytometer. The cell suspension was incubated for cells to attain a density of $2.5\times10^6$ cells/mL with a pre-warmed DMEM medium. Cell suspension with $2.5\times10^4$ cell/mL is prepared in a 1:1 mixture of 0.3% agarose solution and 2X DMEM medium supplemented with 10% FBS. 1000 µL of cell suspension was added to the top of the base layer and allowed to solidify and the plates were incubated overnight at 37°C with 5% $CO_2$. Based on the $IC_{50}$ values of MTT assay, C1 (100 µg), C2 (10 µg), C3 (1 µg), C4 (2 µg), and C5 (400 µg) were added in an appropriate well. The culture pates were incubated for 3 weeks and the medium was changed every 3 days. The cells were stained with 0.05% of crystal violet for 1 h. The plates were scanned for colonies using an Olympus fluorescence micro plate cytometer.

#### Analysis of MDA-MB-231 Cell Cycle Arrest by Flow cytometer

MDA-MB-231 cells cultured in 60 mm dishes were treated with the respective compounds C1, C2, C3, C4, and C5 at 16 µg/mL and incubated for 24 h. After trypsinization followed by thorough washing, cells were treated with 1% paraformaldehyde for 20 min at room temperature, centrifuged, and washed twice with PBS. The cell pellet was collected and 70% ethanol was added and incubated overnight at −20°C. After incubation, the cells were subjected to washing twice with PBS. The cells were then treated with 0.1% Tween 20 for permeabilization for 20 min at room temperature and again washed with PBS twice. The cells were treated with Propidium Iodide (PI) (5 µg/mL) and 250 µg/mL RNAse and incubated for 60 min to provide the nuclear signal for fluorescence-activated cells. The prepared samples were analyzed with a FACS analyzer for different stages of the cell cycle.

#### Statistical analysis

All experiments were executed in triplicates and the data obtained were expressed as mean ± standard error and the *P*-values < 0.05 were considered as statistically significant.

## Results

Screening of marine sediment samples of the Bay of Bengal for actinobacteria led to the isolation of morphologically distinct and potent actinobacterium isolate VSM-25. The taxonomic position of strain was described based on conventional and molecular approaches. Cultural characteristics of strain were studied by growing isolate on ten selective media (Table 1). The strain exhibited good growth on ISP-2 (Yeast extract malt extract dextrose agar), ISP-3 (Oat meal agar medium), ISP-4 (Inorganic salts starch agar), ISP-5 (Glycerol asparagine agar), ISP-6 (Peptone yeast extract iron agar), Czapek-Dox agar medium and Nutrient agar medium. Moderate "growth was observed on ISP-1 (Tryptone yeast extract agar) and ISP-7 (Tyrosine agar) while it was poor on starch casein agar medium. The color of aerial mycelium was white while substrate mycelium varied from yellow to light orange. No pigment was observed on any of the media tested including melanin pigmentation on ISP-7. Micro morphology studies revealed single spores on aerial mycelia (Fig. 1).

The physiological and biochemical characteristics of the isolate are presented in (Table 2). Growth of the strain occurred in the pH range of 6-10 with optimum growth at pH 8. The temperature range for growth was 20-40°C with an optimum temperature being 30°C. The strain exhibited salt tolerance up to 10% with optimum growth at 5% NaCl. The salt concentration has a profound effect on the production of antibiotic from microbes due to its effect on the osmotic pressure to medium[28]. VSM-25 exhibited a positive response to catalase production, casein hydrolysis, Voges-Proskauer test, citrate utilization but negative for nitrate reduction, starch hydrolysis, gelatin liquefaction, $H_2S$ production, methyl red test, and indole production. It could produce industrially important enzymes such as cellulase, asparaginase, protease, pectinase, and chitinase. The strain efficiently utilized carbon sources such as glucose, galactose, fructose, and mannitol (Table 2). Carbohydrate utilization plays a distinguished role in the taxonomic characterization of actinomycetes strains[29]. The strain exhibited sensitivity to different antibiotics like ampicillin, amoxicillin, rifampicin, tetracycline, gentamicin, and chloramphenicol but showed resistance to streptomycin, kanamycin, vancomycin, and trimethoprim (Table 2).

Table 1 — Cultural characteristics of strain VSM-25 on various ISP & Non-ISP media

| S No | Name of the Medium | Response |
|---|---|---|
| 1 | Tryptone yeast extract agar (ISP-1) | |
| | Growth | Moderate |
| | Aerial mycelium | White |
| | Substrate mycelium | Yellow |
| | Pigmentation | Nil |
| 2 | Yeast extract malt extract dextrose agar (ISP-2) | |
| | Growth | Good |
| | Aerial mycelium | White |
| | Substrate mycelium | Pale yellow |
| | Pigmentation | Nil |
| 3 | Oat-meal agar (ISP-3) | |
| | Growth | Good |
| | Aerial mycelium | Creamy white |
| | Substrate mycelium | Light orange |
| | Pigmentation | Nil |
| 4 | Inorganic salts starch agar (ISP-4) | |
| | Growth | Good |
| | Aerial mycelium | White |
| | Substrate mycelium | Pale yellow |
| | Pigmentation | Nil |
| 5 | Glycerol asparagine agar (ISP-5) | |
| | Growth | Good |
| | Aerial mycelium | White |
| | Substrate mycelium | Brown |
| | Pigmentation | Nil |
| 6 | Peptone yeast extract iron agar (ISP-6) | |
| | Growth | Good |
| | Aerial mycelium | Creamy white |
| | Substrate mycelium | Yellow |
| | Pigmentation | Nil |
| 7 | Tyrosine agar (ISP-7) | |
| | Growth | Moderate |
| | Aerial mycelium | White |
| | Substrate mycelium | Light orange |
| | Pigmentation | Nil |
| 8 | Starch casein agar | |
| | Growth | Poor |
| | Aerial mycelium | - |
| | Substrate mycelium | - |
| | Pigmentation | Nil |
| 9 | Nutrient agar | |
| | Growth | Good |
| | Aerial mycelium | White |
| | Substrate mycelium | Pale yellow |
| | Pigmentation | Nil |
| 10 | Czapek-Dox agar | |
| | Growth | Good |
| | Aerial mycelium | White |
| | Substrate mycelium | Yellow |
| | Pigmentation | Nil |

Gene sequence of 16S rRNA of strain was blasted against nucleotide database of the NCBI. The library search reported matching strains and the sequences had been aligned with the set of published sequences based on conserved primary sequence and additionally with aid of nucleotide blast similarity search evaluation. The 16S rDNA gene sequence of isolate VSM-25 showed a close relation with *Streptomonospora arabica* (Fig. 2). The rDNA sequence was deposited in the GenBank database of NCBI with an accession number KU507598. Molecular identification of the strain was carried out through 16s rRNA gene fragment analysis, a dynamic tool to recognize microorganisms up to the genus level[30].

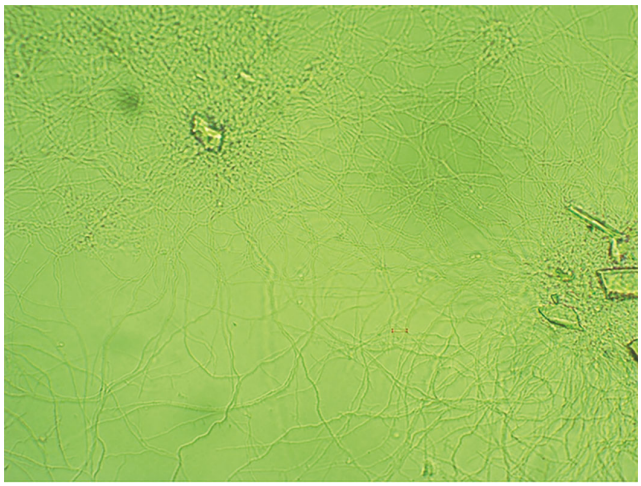

Fig. 1 — Micro morphology of the marine actinobacterium VSM-25 grown on ISP-2 media

The evolutionary history was inferred using the Neighbor-Joining method[19]. The optimal tree with the sum of branch length = 128.14474640 is shown. The tree is drawn to scale, with branch lengths in the same units as those of evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method[31] and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. Codon positions included were $1^{st}$ + $2^{nd}$ + $3^{rd}$ + Non-coding. All positions containing gaps and missing data were eliminated. There were a total of 876 positions in the final data set. Evolutionary analyses were conducted in MEGA 7[20].

**Purification and Structure elucidation of Bioactive compounds**

Compound C1 was obtained as a white crystalline powder, thoroughly soluble in dimethyl sulfoxide, methanol, and chloroform. The $^{1}$H-NMR spectrum of compound C1 showed signals at $^{1}$H NMR (300 MHz,

Table 2 — Morphological, physiological and biochemical characteristics of strain VSM-16

| S No | Characteristics | Response |
|---|---|---|
| | Morphological characters | |
| 1 | Cell shape | Mycelial |
| 2 | Sporophore morphology | Straight, Non fragmented |
| 3 | Colour of aerial mycelium (ISP-2) | White |
| 4 | Colour of substrate mycelium (ISP-2) | Pale yellow |
| Physiological characters | | |
| 5 | Gram reaction | P |
| 6 | Acid-fast reaction | N |
| 7 | Production of melanin pigment | N |
| 8 | Range of temperature for growth | 20-40°C |
| 9 | Optimum temperature for growth | 30°C |
| 10 | Range of pH for growth | 6-10 |
| 11 | Optimum pH for growth | 8 |
| 12 | NaCl tolerance | 10% |
| 13 | Optimum NaCl for growth | 5% |
| Biochemical characters | | |
| 13 | Catalase production | P |
| 14 | Nitrate reduction | N |
| 15 | Hydrogen sulphide production | N |
| 16 | Starch hydrolysis | N |
| 17 | Gelatin liquefaction | N |
| 18 | Methyl red test | N |
| 19 | Vogues-Proskauer test | P |
| 20 | Indole production | N |
| 21 | Citrate utilization | P |
| 22 | Casein hydrolysis | P |
| 23 | Urease | N |
| 24 | Cellulase | P |
| 25 | Asparaginase | P |
| 26 | Protease | P |
| 27 | Amylase | N |
| 28 | Pectinase | P |
| 29 | Chitinase | P |
| Utilization of the carbon sources | | |
| 30 | Glucose | G |
| 31 | Galactose | G |
| 32 | Fructose | G |
| 33 | Mannitol | G |
| 34 | Inositol | M |
| 35 | Maltose | M |
| 36 | Lactose | M |
| 37 | Xylose | W |
| 38 | Arabinose | W |
| 39 | Raffinose | W |
| 40 | Rhamnose | W |
| Antibiotic susceptibility (µg/mL) | | |
| 41 | Ampicillin (10) | S |
| 42 | Amoxicillin (10) | S |
| 43 | Streptomycin 10) | R |
| 44 | Tetracycline (30) | S |
| 45 | Kanamycin (30) | R |
| 46 | Vancomycin (30) | R |
| 47 | Rifampicin (5) | S |
| 48 | Chloramphenicol (10) | S |
| 49 | Trimethoprim (5) | R |
| 50 | Gentamicin (10) | S |

P = Positive; N = Negative; G=Good growth; M = Moderate growth; W=Weak growth; S=Sensitive; R=Resistant

DMSO-*d6*, 298 K): $\delta$ 11.63 (s, 1H, NH), 9.90 (s, 1H, CHO), 8.16-8.10 9m, 1H, Ar-H), 7.83 (s, 1H, Ar-H), 7.43-7.38 (m, 1H, Ar-H), 7.22-7.12 (m, 2H, Ar-H) (Suppl. Fig. 1); while $^{13}$C exhibited signals at (125 MHz, DMSO-*d6*+$CDCl_3$, 298 K): $\delta$ 184.2, 136.6, 136.3, 123.7, 122.9, 121.6, 120.6, 118.0, 111.5 (Suppl. Fig. 2). ESIMS/Q-TOF analysis of the compound gave a molecular ion m/z at 144 (M-H) (Suppl. Fig. 3). The IR spectrum exhibited absorption bands at $V_{max}$ IR (KBr): 3168, 3042, 2977, 2929, 2817, 1928, 1634, 1572, 1519, 1442, 1388, 1333, 1294, 1241, 1125, 1080, 1003, 884, 789, 761 $cm^{-1}$ (Suppl. Fig. 4). Based on the above spectral data, bioactive compound C1 was identified as Indole-3-carboxaldehyde with molecular formula $C_9H_7NO$ (Fig. 3 (C1))

Compound C2 was obtained as a colorless amorphous solid, completely soluble in methanol and water. The $^1$H-NMR spectrum of compound C2 showed signals at $^1$H NMR (300 MHz, DMSO-*d6*, 298 K): $\delta$ 7.25 (dd, 1H, $J$ = 1.4, 8.0 Hz, Ar-H), 6.95 (dd, 1H, $J$ = 1.7, 8.0 Hz, Ar-H), 6.61 (t, 1H, $J$ = 8.0 Hz, Ar-H), 4.59 (brs, 1H, OH) (Suppl. Fig. 5); while $^{13}$C exhibited signals at δ (125 MHz, $CDCl_3$, 298 K): $\delta$ 172.7, 149.7, 145.0, 120.8, 120.0, 118.0, 113 (Suppl. Fig. 6). ESIMS/Q-TOF analysis of the compound gave a molecular ion m/z at 154 (M-H) (Suppl. Fig. 7). The IR spectrum exhibited absorption bands at $V_{max}$ IR (KBr): 3372, 3047, 2578, 1678, 1640, 1598, 1475, 1434, 1353, 1302, 1258, 1233, 1158, 1070, 944, 903, 836, 797, 745 $cm^{-1}$ (Suppl. Fig. 8). Based on the above spectral data, bioactive compound C2 was identified as 2, 3-Dihydroxy benzoic acid with molecular formula $C_7H_6O_4$ (Fig. 3 (C2)).

Compound C3 was obtained as a colorless amorphous powder, totally soluble in ethanol, acetone, and water. The $^1$H-NMR spectrum of compound C3 showed signals at $^1$H NMR (300 MHz, DMSO-*d6*, 298 K): $\delta$ 9.0 (brs, 1H, OH), 7.53-7.41 (m, 2H, Ar-H), 6.79 (d, 1H, $J$ = 8.3 Hz, Ar-H), 3.81 (s, 3H, OCH3) (Suppl. Fig. 9); while $^{13}$C exhibited signals at δ (125 MHz, DMSO-*d6*+$CDCl_3$, 298 K): $\delta$ 167.7, 150.3, 146.4, 12.3, 121.5, 114.1, 112.0, 52.2 (Suppl. Fig. 10). ESIMS analysis of the compound gave a molecular ion m/z at 167 (M-H) (Suppl. Fig. 11). The IR spectrum exhibited absorption bands at $V_{max}$ IR (KBr): 3483, 2956, 2649, 1865, 1680, 1597, 1522, 1472, 1433, 1456, 1382, 1282, 1237, 1205, 1111, 1027, 917, 883, 804, 761, 722 $cm^{-1}$ (Suppl. Fig. 12). Based on the above spectral data, bioactive compound

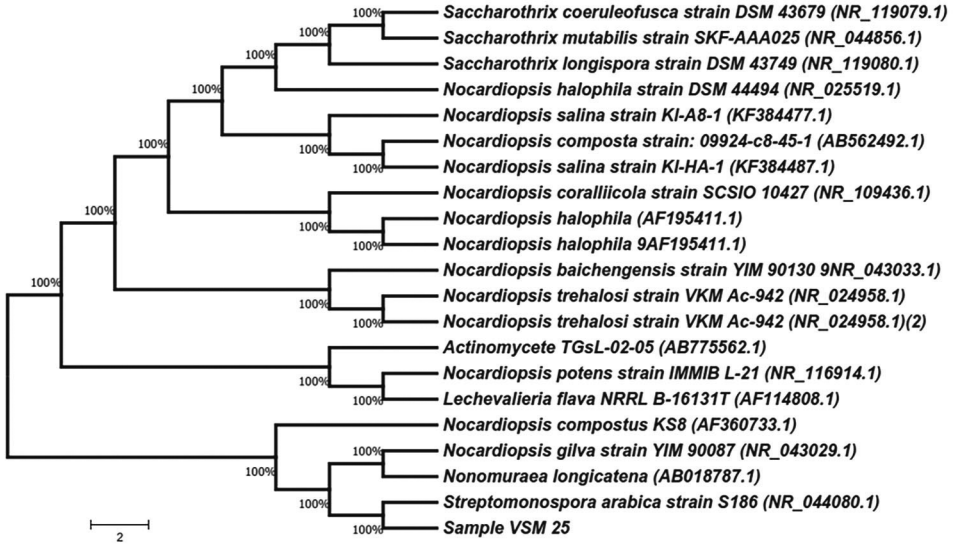


Fig. 2 — Evolutionary relationships of taxa

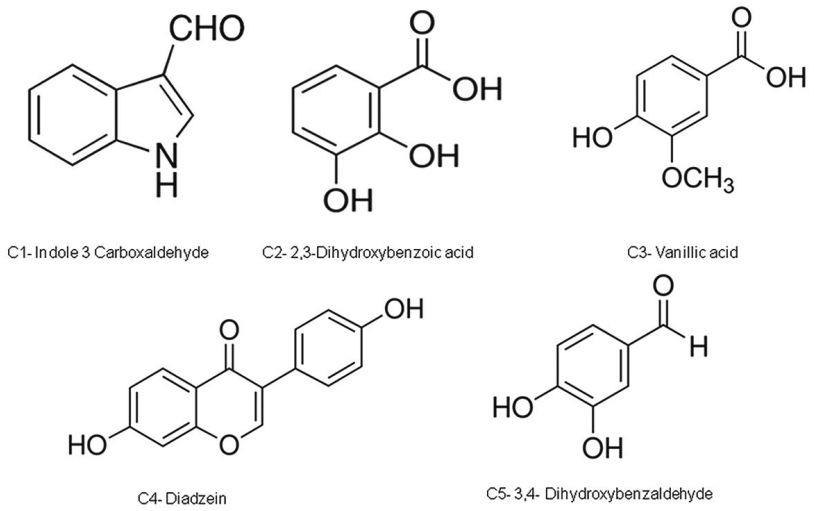


Fig. 3 — (A, B, C, D, E) Structures of compounds (C1-C5) produced by *S.arabica*

C3 was identified as 4-Hydroxy-3-methoxy benzoic acid (Vanillic acid) with molecular formula $C_8H_8O_4$ (Fig. 3 (C3)).

Compound C4 was obtained as white amorphous powder, wholly soluble in dimethylsulfoxide, ethanol, and DMF. The $^1$H-NMR spectrum of compound C4 showed signals at $^1$H NMR (300 MHz, DMSO-*d6*+$CDCl_3$, 298 K): $\delta$ 8.94 (s, 1H, OH), 7.98 (d, 1H, $J$ = 8.8 Hz, Ar-H), 7.81 (s, 1H, Olefinic), 7.27 (d, 2H, $J$ = 8.5 Hz, Ar-H), 6.86-6.74 (m, 5H, Ar-H and OH) (Suppl. Fig. 13); while $^{13}$C exhibited signals at (125 MHz, DMSO-*d6*+$CDCl_3$, 298 K): $\delta$ 175.3, 162.0, 157.4, 156.7, 151.3, 129.5, 127.0, 124.0, 122.4, 116.7, 114.9, 114.6, 101.9 (Suppl. Fig. 14). ESIMS analysis of the compound gave a molecular ion m/z at 277 (M+Na) (Suppl. Fig. 15). The IR spectrum exhibited absorption bands at $V_{max}$ IR (KBr): 3221, 1632, 1599, 1516, 1459, 1388, 1279, 1241, 1190, 1095, 1043, 953, 890, 843, 84, 733 cm$^{-1}$ (Suppl. Fig. 16). Based on the above spectral data, bioactive compound C4 was identified as Daidzien with molecular formula $C_{15}H_{10}O_4$ (Fig. 3 (C4)).

Compound C5 was obtained as a pale-yellow solid, slightly soluble in methanol and water. The $^1$H-NMR spectrum of compound C5 showed signals at $^1$H NMR (300 MHz, DMSO-d6, 298 K): $\delta$ 9.64 (s, 1H, CHO),

9.21 (brs, 1H, OH), 8.75 (brs, 1H, OH), 7.27 (d, 1H, *J* = 1.9 Hz, Ar-H), 7.17 (dd, 1H, *J* = 1.9, 8.0 Hz, Ar-H), 6.86 (d, 1H, *J* = 8.3 Hz, Ar-H) (Suppl. Fig. 17); while $^{13}C$ exhibited signals at δ (125 MHz, DMSO-*d6*+$CDCl_3$, 298 K): *δ* 190.4, 151.3, 145.1, 128.8, 124.2, 114.9, 114.3 (Suppl. Fig. 18). ESIMS/Q-TOF analysis of the compound gave a molecular ion *m/z* at 137 (M-H) (Suppl. Fig. 19). The IR spectrum exhibited absorption bands at $V_{max}$ IR (KBr): 3325, 2873, 1651, 1596, 1534, 1444, 1297, 1192, 1166, 1118, 876, 812, 777, 753 $cm^{-1}$ (Suppl. Fig. 20). Based on the above spectral data, bioactive compound C5 was identified as 3,4-dihydroxy benzaldehyde with the molecular formula $C_7H_6O_3$ (Fig. 3 (C5)).

**Minimum inhibitory concentration (MIC) assay**

Antibacterial activities of bioactive compounds along with crude extract in terms of MIC are represented in (Table 3). The bioactive compounds exhibited antibacterial activity against a panel of Gram-positive and Gram-negative bacteria, for which MIC values showed wide variation ranging from 8 to 512 µg/mL. The best activity of compound C1 was recorded against *Staphylococcus aureus* (8 µg/mL) followed by *Xanthomonas campestris*, *Bacillus megaterium,* and *Streptococcus mutans* (16 µg/mL). The compounds C2 and C3 exhibited mild bioactivity with MIC values ranging from 256-512 µg/mL, while the compound C4 displayed mild to moderate bioactivity with MIC values varying from 128-512 µg/mL. In this study, no bioactivity was defined as MIC>512 µg/mL (Table 3). The micro-organisms with high sensitivity towards compound C5 were *Xanthomonas malvacearum*, *Pseudomonas aeruginosa*, *Bacillus subtilis,* and *Staphylococcus epidermis* (32 µg/mL). Compared with standard drug tetracycline, the compound C1 displayed high sensitivity against *Staphylococcus aureus* and *Streptococcus mutans* and in other cases the positive control exhibited good antibacterial activity over the metabolites of strain.

Antifungal activity against yeasts and fungi and corresponding MIC values are recorded in (Table 4). Compound C1 exhibited significant MIC value against *Candida albicans, Aspergillus niger, and Fusarium solani* (16 µg/mL) whereas compounds C3 and C5 exhibited weak to moderate bioactivity. The compound C2 did not exhibit any antifungal activity while the compound C4 displayed very weak activity only against *Candida albicans*. The results of MIC assay (Tables 3 & 4) showed that compound C1 was able to prevent the growth of all organisms tested including *Salmonella typhi*, fungi, Gram-positive and Gram-negative bacteria, within the concentration range of 8 to 128 µg/mL. Other compounds displayed selective activity, against test organisms. Only a few reports were documented on the antimicrobial

Table 3 — MIC of bioactive compounds isolated from *Streptomonospora arabica* VSM-25 against test bacteria

| Test organism | MIC (µg/mL) | | | | | |
|---|---|---|---|---|---|---|
| Bacteria | C1 | C2 | C3 | C4 | C5 | Antibiotic |
| *Bacillus cereus* | 32 | 512 | 512 | 128 | 64 | 8 |
| *Streptococcus mutans* | 16 | - | 512 | 512 | 64 | 32 |
| *Staphylococcus aureus* | 8 | - | - | 256 | 128 | 32 |
| *Staphylococcus epidermis* | 32 | 512 | - | 128 | 32 | 16 |
| *Bacillus subtilis* | 32 | 512 | 512 | 256 | 32 | 32 |
| *Bacillus megaterium* | 16 | 256 | 512 | 256 | 64 | 16 |
| *Escherichia coli* | 32 | 512 | 256 | 512 | 64 | 8 |
| *Pseudomonas aeruginosa* | 32 | - | 256 | 512 | 32 | 8 |
| *Proteus vulgaris* | 32 | - | - | - | 128 | 16 |
| *Serratia marcescens* | 64 | - | - | 512 | 128 | 32 |
| *Xanthomonas campestris* | 16 | 512 | 512 | 256 | 64 | 16 |
| *X. malvacearum* | 32 | - | 512 | 256 | 32 | 8 |
| *Salmonella typhi* | 64 | - | - | - | 256 | 8 |

Data were statistically analysed by one-way analysis of variance (ANOVA) and found to be significant at 0.05% (n=3)
C 1-Indole-3-carboxaldehyde
C 2-2, 3-dihydroxy benzoic acid (Pyrocatechuic acid)
C 3-Vanillic acid
C 4-Daidzein
C 5-3,4-Dihydroxy benzaldehyde (Protocatechualdehyde)
(-)=MIC>512
Antibiotic -Tetracycline against bacteria

Table 4 — MIC of bioactive compounds isolated from *Streptomonospora arabica* VSM-25 against test fungi

| Test organism | MIC (µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| Yeasts | C1 | C2 | C3 | C4 | C5 | Antibiotic |
| *Candida albicans* | 16 | - | - | 512 | 64 | 16 |
| *Epidermophyton floccosum* | 32 | - | - | - | - | 16 |
| Fungi | | | | | | |
| *Aspergillus niger* | 16 | - | 512 | - | 128 | 16 |
| *Aspergillus flavus* | 32 | - | 256 | - | 128 | 8 |
| *Fusariumm oxysporum* | 32 | - | - | - | - | 16 |
| *Fusarium solani* | 16 | - | 512 | - | 512 | 32 |
| *Penicillium citrinum* | 32 | - | 512 | - | 256 | 8 |
| *Verticillium alboatrum* | 256 | - | - | - | - | 64 |
| *Alternaria alternata* | 128 | - | - | - | - | 32 |

Data were statistically analysed by one-way analysis of variance (ANOVA) and found to be significant at 0.05% (n=3)
C 1-Indole-3-carboxaldehyde
C 2-2, 3-dihydroxy benzoic acid (Pyrocatechuic acid)
C 3-Vanillic acid
C 4-Daidzein
C 5-3,4-Dihydroxy benzaldehyde (Protocatechualdehyde)
(-)=MIC>512
Antibiotic -Griseofulvin against yeast and Amphotericin-B against fungi

activities of Indole-3-carboxaldehyde (C1), 2, 3-dihydroxy benzoic acid (C2), Vanillic acid (C3), Diadzien (C4), and 3,4-Dihydroxy benzaldehyde (C5)[32-37]. This study, therefore, provides significant baseline information for antimicrobial activities of the above compounds.

**DPPH radical scavenging assay**

The DPPH radical scavenging assay is a basic screening method widely used for testing of anti-radical activity of a variety of compounds[38]. The principle of this assay is based on the measurement of the ability of antioxidants to scavenge the stable DPPH radical. The change of color of DPPH radical from violet to yellow upon reduction is made evident by a decline in absorbance at 517 nm. DPPH free radical is reduced to hydrazine on reaction with donor hydrogen. The method is being authoritative and uncomplicated and has been endorsed to measure antioxidant activity of samples of divergent origin[39].

The DPPH radical scavenging activity of five compounds in comparison with ascorbic acid as standard reference is shown in (Fig. 4A). Dose-dependent relationship response was found in DPPH radicals scavenging capacity. The test samples significantly scavenged DPPH with increasing concentrations. Compound C5 (3,4-dihydroxy benzaldehyde) exerted the strongest scavenging activity with a maximum inhibition of 64.562, which was significantly greater than that of other compounds at 400 µg/mL. The other four compounds *viz*., C1, C2, C3, and C4 displayed a maximum inhibition of 59.87%, 61.625%, 52.153%, and 34.217%, respectively at 400 µg/mL, while ascorbic acid showed 72.50% of inhibition at the same concentration. Significant ($P< 0.001$) DPPH radical scavenging activity was evident for all the tested concentrations of C1, C2, and C3 while the compound C4 showed moderate activity (200 and 400 µg/mL). It can be concluded that all the compounds possessed antioxidant and free radical scavenging activities. This data confirmed the observation of earlier reports on these compounds[40-42].

**Nitric oxide (NO) radical scavenging efficacy**

NO is formed from L-arginine by NO synthase and is a free radical with a single unpaired electron[43]. NO is not a very reactive free radical but over-production of NO leads to health complications such as neurodegenerative and chronic inflammatory diseases such as rheumatoid arthritis and ischemia reperfusion. NO scavengers compete with oxygen in a lower production of NO[44].

From Fig. 4B, it is elucidated that four compounds C1, C2, C3, and C5 among the five tested showed a significant NO radical scavenging activity ($P< 0.01$) in a dose-dependent manner. The results showed Indole 3 Carboxaldehyde, 2,3-Dihydroxybenzoic acid, Vanillic acid, and 3,4-dihydroxy benzaldehyde showed high scavenging activities of 66.22, 62.12, 58.16, and 70.66, respectively, but not as potent as ascorbic acid (79.22) at 400 µg/mL. Almost all the compounds tested possessed strong scavenging activity against NO. Compound C5 revealed strong

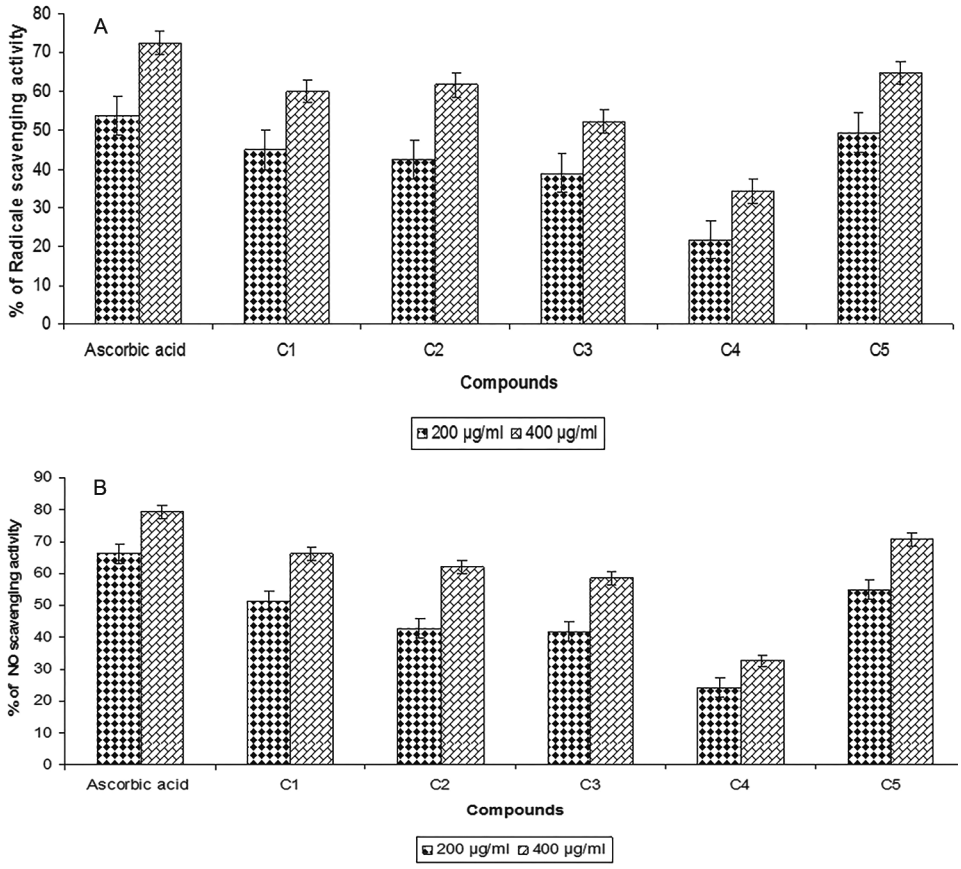


Fig. 4 — (A) % of DPPH; and (B) % of NO radical scavenging activity. C1 (Indole 3 Carboxaldehyde), C2 (2,3-Dihydroxybenzoic acid), C3 (Vanillic acid), C4 (Daidzein) and C5 (3,4-Dihydroxybenzaldehyde isolated from strain VSM-25. Data are expressed as mean ±standard deviation (n =3) and is found to be significant ($P \leq 0.05$)

inhibitory activity at concentration 400 µg/mL while the rest of the compounds showed good to weak inhibition. This study makes a valuable addition to the existing information. Several biomolecules such as proteins, lipids, and DNA are damaged due to the actions of ROS. Antioxidants play a crucial role in the inactivation of reactive oxygen species (ROS) generated in the body. Though human cells protect themselves against oxidative damage, sometimes these are not sufficient to prevent ROS-induced cellular damage[45]. Plants and microbes have an innate ability to biosynthesize a wide range of antioxidants capable of attenuating ROS-induced damage in humans[46].

**α-Amylase Inhibitory Assay**

Diabetes mellitus is a major endocrine disorder in nearly all countries due to lifestyle leading to reduced physical activity and increased hyperlipemia[47]. Pancreatic α-amylase, a key enzyme in the small intestine plays a major role in the digestion of starch yielding glucose and maltose[48]. Inhibition of α-amylase at the brush borders of the intestine and carbohydrate absorption after food intake is one therapeutic approach[49]. Thus amylase inhibitors received considerable attention and are helpful to control diabetes. The treatment of diabetes and its complications has been targeted using many natural products without any adverse effects.

The ability of five compounds to inhibit α-amylase activity *in vitro* was executed and the results are presented in (Fig. 5). The percentage of α-amylase activity inhibition is directly proportional to the increasing concentration of the five compounds from VSM-25 include C1 (78.04%), C2 (65.85%), C3 (75.60%), C4 (71.10), and C5 (56.09%). Acarbose is taken as the standard drug that showed 82.92% of inhibition of α-amylase activity. The percentage of α-amylase activity inhibition of five compounds was plotted in comparison to Acarbose. Of all the compounds, Indole 3 Carboxaldehyde exhibited the highest percentage of α-amylase inhibition activity at

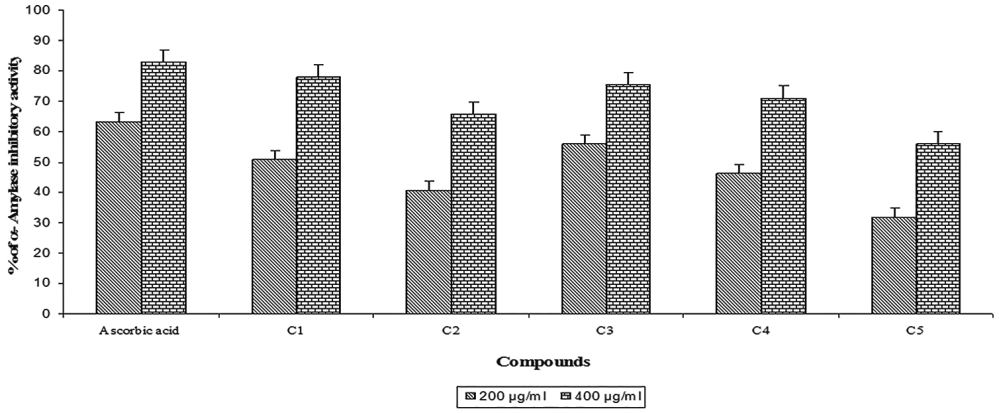


Fig. 5 — α-Amylase inhibitory activity of the C1 (Indole -3 -Carboxaldehyde), C2 (2,3-Dihydroxybenzoic acid), C3 (Vanillic acid), C4 (Daidzein) and C5 (3,4-Dihydroxybenzaldehyde) isolated from strain VSM-25. Data are expressed as mean ± standard deviation (n =3) and is found to be significant ($P \leq 0.05$)

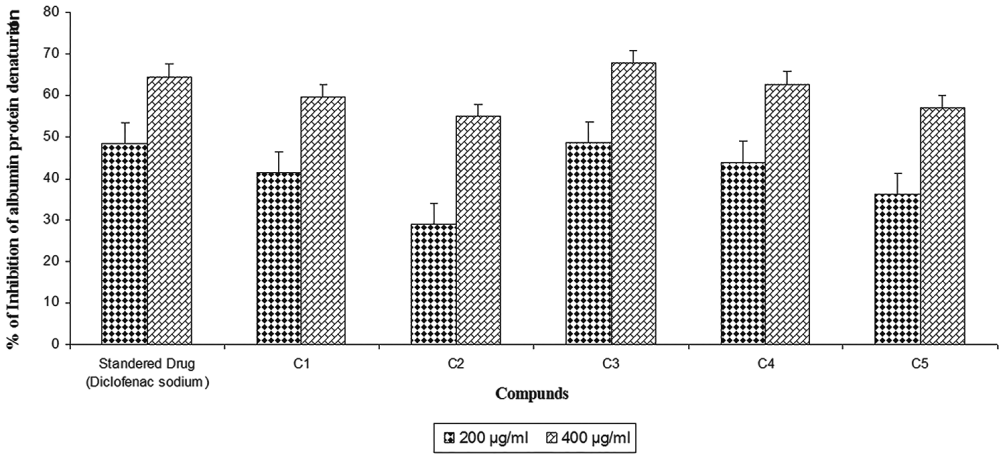


Fig. 6 — (Anti-Inflammatory activity) % of Inhibition of albumin protein denaturation by C1 (Indole 3 Carboxaldehyde), C2 (2,3-Dihydroxybenzoic acid), C3 (Vanillic acid), C4 (Diadzein) and C5 (3,4-Dihydroxybenzaldehyde) isolated from the strain VSM-25. Data are expressed as mean ±standard deviation (n =3) and is found to be significant ($P \leq 0.05$)

400 µg/mL but lower than that of Acarbose at the same concentration. From the results, it can be suggested that Indole 3 Carboxaldehyde may reduce the rate of absorption of carbohydrates and there by effectively contribute to the management of diabetes by decreasing post-prandial hyperglycemia.

**Anti-inflammatory activity**

*In vitro* anti-inflammatory effect of five compounds was evaluated against denaturation of the bovine serum albumin. The present findings suggest concentration-dependent inhibition of albumin denaturation. The percentage of albumin inhibition of five compounds at concentration 400 µg/mL was C1 (59.67%), C2 (54.83%), C3 (67.74%), C4 (62.67%) and C5 (57.03%). Diclofenac sodium is taken as the standard drug that showed 64.51% of protein denaturation which is also concentration-dependent. However, the effect of diclofenac sodium was found to be less when compared with vanillic acid (67.74%) (Fig. 6).

Using animals for experiments related to pharmacological research is associated with problems such as ethical issues and a lack of rationale for their use when other suitable methods are available. Hence *in vitro* assay of anti-inflammatory activity was selected. Agents that offer protection[50] and that can prevent denaturation of protein are worthwhile for anti-inflammatory drug development[51]. In course of our efforts in identifying anti-inflammatory agents from *S. arabica* VSM-25, we have isolated 5 compounds from the crude ethyl acetate extract, among which compounds C3 and C4 displayed significant anti-inflammatory activities.

**Anti-arthritic activity**

The effect of the five compounds C1 to C5 on inhibition of protein (Bovine serum albumin) denaturation is shown in (Fig. 7). No significant effect against the inhibition of protein denaturation was observed for compounds C1, C2, C3, and C5. In the case of compound C4, the inhibition of protein denaturation is concentration-dependent and maximum inhibition was observed at 400 µg/mL concentration. Compound C4 and the standard drug Diclofenac sodium recorded protein inhibition of 60.23% and 75.19%, respectively, at the same concentration. Most of the clinical investigators reported that protein denaturation is one of the causes of rheumatoid arthritis and for the generation of auto antigens in certain rheumatic diseases[52]. The denaturation of proteins is probably due to alteration in electrostatic, hydrogen, hydrophobic, and disulfide bonding. Daidzein showed anti-arthritic activity in a dose-dependent manner and therefore provides important baseline information for the anti-arthritic activity of the compound and may be proposed for controlling the production of auto-antigens.

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**MTT cell viability assay for cytotoxicity testing against MDA-MB-231 and MCF-7 Cell lines**

*In vitro* cytotoxicity of five compounds isolated was evaluated by MTT 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay on MDA-MB-231 and MCF-7 cells. $IC_{50}$ values were determined by exposing MDA-MB-231 and MCF-7 cell lines to various concentrations (0.01, 0.1, 1, 10, 100, 400 µg/mL) of five compounds at which 50% of MDA-MB-231 and MCF-7 cell growth was inhibited compared to control cells. Results indicated that five compounds had significant cytotoxic activity against MDA-MB-231 which showed $IC_{50}$ values of C1(69.69%) at 400 µg/mL, C2 (53.42%) at 10 µg/mL, C3 (57.72%) at 1 µg/mL, C4 (67.81%) at 1 µg/mL and C5 (60.52) at 400 µg/mL (Fig. 8 A-E) and for MCF -7 C1 (53.75%) at 400 µg/mL, C2 (53.15%) at 100 µg/mL, C3 (51.26%) at 10 µg/mL, C4 (54.33%) at 1 µg/mL and C5 (50.84) at 100 µg/mL (Fig. 8 A-E). The negative control showed 100 % cell viability and the effect of the positive control (Standard drug Taxol) of all the experiments is illustrated in (Figs 5 & 6).

**Inhibition of anchorage independent growth of MDA-MB-231 cells**

Anchorage independent assay is the most well-known stringent *in vitro* assay to determine the inhibition potency of compounds to inhibit tumor cell growth[53]. Cytotoxicity of five compounds was determined by anchorage independent growth of MDA-MB-231 cells. The concentration of the compounds C1 (400 µg), C2 (10 µg), C3 (1 µg), C4 (1 µg), and C5 (400 µg) selected for the present study was determined based on the results obtained from the MTT assay that showed $IC_{50}$ value which inhibited 50% of the cells. As shown in Figure 9, incubation of MDA-MB-231 cells after the addition of compounds in soft agar for 21 days significantly inhibited the anchorage independent growth with compounds C1, C4, and C5 (Fig. 10). They showed a reduction in colony formation and colony size when compared with DMSO control. The size of the colony for C1, C4, and C5 was within the range of 10 to

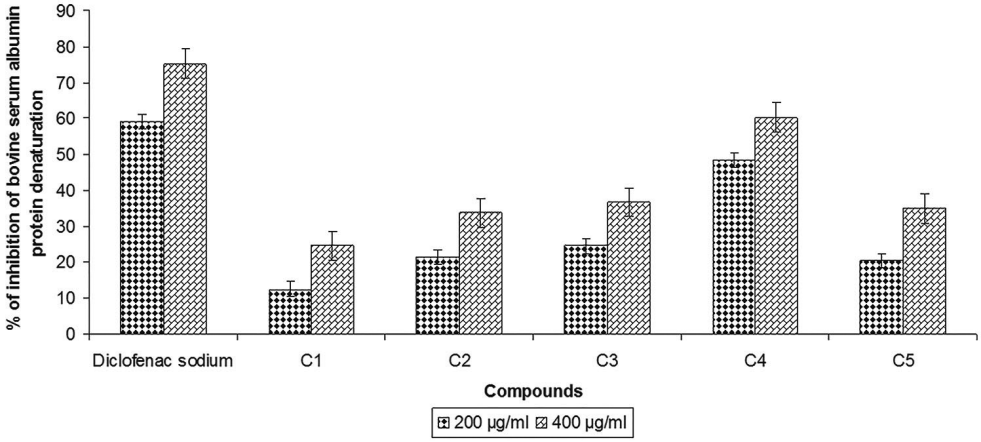


Fig. 7 — % of Bovine serum albumin protein denaturation by C1 (Indole 3 Carboxaldehyde), C2 (2, 3-Dihydroxybenzoic acid), C3 (Vanillic acid), C4 (Diadzein) and C5 (3, 4-Dihydroxybenzaldehyde) isolated from the strain VSM-25. Data are expressed as mean ± standard deviation (n =3) and is found to be significant ($P \leq 0.05$)

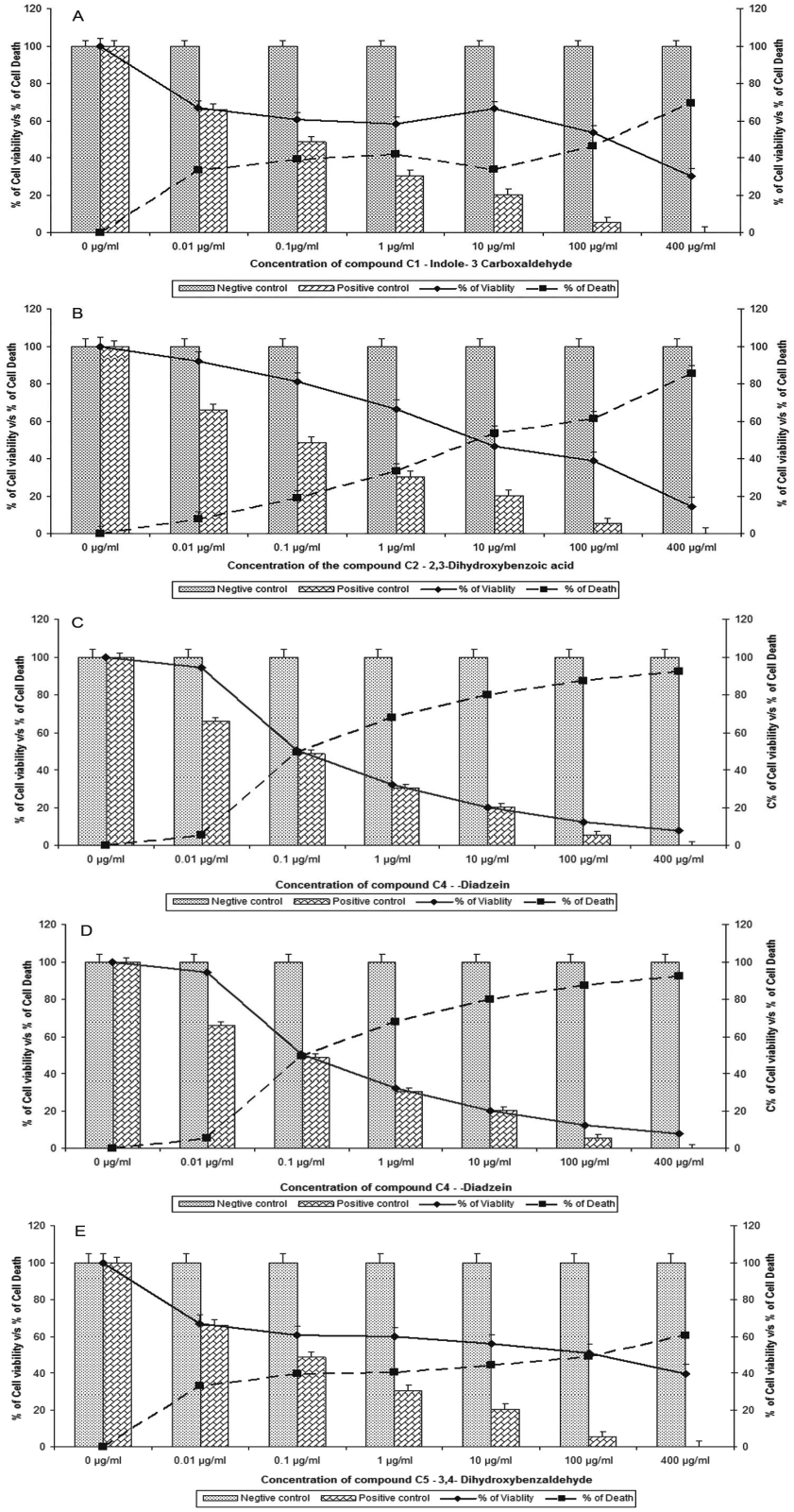


Fig. 8 — A-E MTT Assay for cytotoxicity of the compounds C1-Indole 3 Carboxaldehyde, C2-2,3-Dihydroxybenzoic acid, C3-Vanillic acid, C4-Diadzein and C5-3,4-Dihydroxybenzaldehyde at varying concentrations (0.01 µg/mL, 0.1 µg/mL,1 µg/mL,10 µg/mL,100 µg/mL and 400 µg/mL) against MDA-MB-231. Data are expressed as mean ± standard deviation (n =3) and is found to be significant ($P \leq 0.05$)

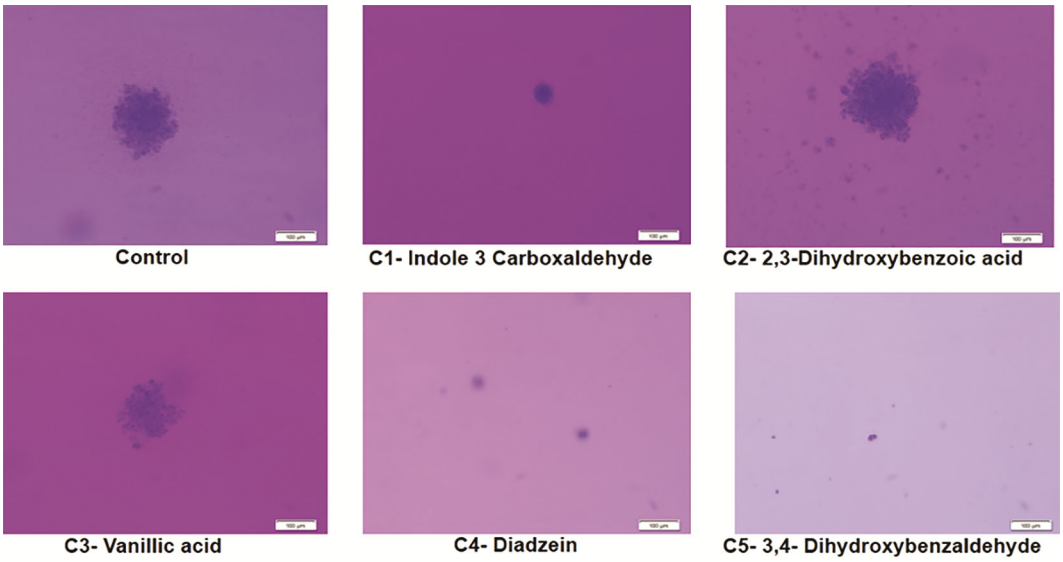


Fig. 9 — Image showing the formation of the MDA-MB-231 cells treated with compounds at concentration C1 (400 µg), C2 (10 µg), C3 (1 µg), C4 (1 µg) and C5 (400 µg). The treated cells were grown on soft agar. After 3 weeks colonies were imaged using inverted microscope

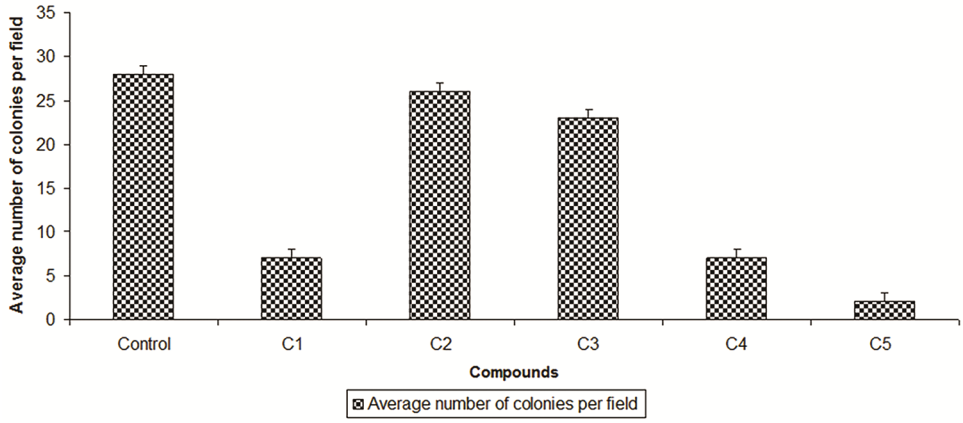


Fig. 10 — MDA-MB-231 Colonies per field grown on soft agar after treatment with compounds C1 (400 µg), C2 (10 µg), C3 (1 µg), C4 (1 µg) and C5 (400 µg) (DMSO) served as control. After 3 weeks individual colonies per field were found to be 28 for control, C1 (7), C2 (26), C3 (23), C4 (7) and C5 (2). Experiments were repeated 3 times (n = 3). The statistical significance of total cell count per field difference between the control and treatment samples was determined by two-sample Student's t-test (*$P \leq 0.005$)

30µm while C2 and C3 were in the size range of 110 µm to 140 µm when compared with DMSO standard that exhibited colony size of 180 µm. The average number of colonies per field in DMSO control was found to be 28. For cultures treated with C2 and C3 showed 26 and 23 colonies per field. But cultures treated with C1, C4, and C5 exhibited 7, 7, and 2 colonies per field (Fig. 11). This shows that 90% inhibition of the MDA-MB-231 cell lines by C1, C4, and C5 compounds.

**Arrest of MDA-MB-231 cell cycle**

After analyzing the cytotoxic activity of the compounds C1, C2, C3, C4, and C5, we aimed to evaluate the effect through induction of cell cycle arrest by Flow cytometry. The MDA-MB-231 cell line treated with compounds C1, C2, C3, C4, and C5 were analyzed by flow-cytometry to determine the cell cycle phases after 24 h of incubation. Analysis of the treated cells in the G1 phase showed evidence of apoptosis induction after 24 h of incubation. Accumulations in the cell cycle of the MDA-MB-231 sub G1 peak (apoptotic cells) was 8.91% for the control cells. Analysis of the G1 peak of the cell cycle in the treated MDA-MB-231 cell line showed an increase in apoptosis. The results indicate that the treated cells compared with the control group showed the arrest at the G1. An increase in the G1 phase


Fig. 11 — Quantification of MDA-MB-231 Colony Number of C1-Indole 3 Carboxaldehyde, C2-2,3-Dihydroxybenzoic acid, C3-Vanillic acid, C4-Diadzein and C5-3,4-Dihydroxybenzaldehyde. Treatment of MDA-MB-231 cells were grown in soft agar at concentrations C1 (400 µg), C2 (10 µg), C3 (1 µg), C4 (1 µg) and C5 (400 µg) of compounds (0 µM (DMSO). After 3 weeks individual colonies larger than 40 µm were counted. Experiments were repeated 3 times (n = 3). The statistical significance of total cell count difference between the control and treatment samples was determined by two-sample Student's t-test (*$P \leq 0.005$)

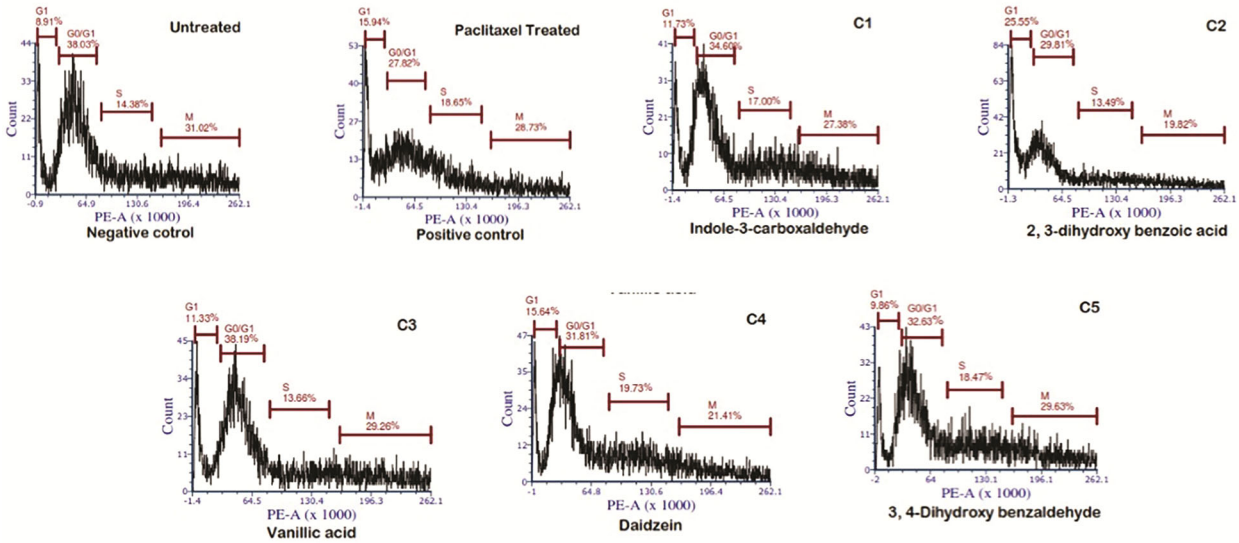


Fig. 12 — Cell Cycle analysis by flow cytometry of cells MDA-MB-231 treated with Paclitaxel, C1-Indole 3 Carboxaldehyde, C2-2,3-Dihydroxybenzoic acid, C3-Vanillic acid, C4-Diadzein, and C5-3,4-Dihydroxybenzaldehyde after 24 h of incubation

population was observed after 24 h of incubation for the cells treated with compounds C1, C2, C3, C4, and C5. This was followed by a slight decrease in the proportion of the S phase and a significant decrease in the G2/M phase percentage. As per the data obtained from the experiments, percentage of the untreated cells and standard drug (Paclitaxel) treated cells in the subG1, G1, S and G2/M phases were calculated as 9.67%, 39.73%, 18.60%, 32.04% and 16.85%, 30.46%, 23.46%, 29.55% and proportion of treated cells with compound C1 (11.49%, 39.74%, 20.40% and 28.67%), C2 (27.79%, 35.55%, 16.18% and 20.90%), C3 (9.29%, 37.63%, 22.47% and 30.88%), C4 (14.61%, 40.55%, 23.38% and 22.63%) and C5 (11.40%, 40.57%, 17.64% and 30.43%) (Fig. 12).

## Discussion

The genus *Streptomonospora* belongs to the suborder *Streptosporangiales* and family *Nocardiopsaceae*[54], is much unnoticed for its biosynthetic capacity and had been uncharacterized genetically and biosynthetically compared to other strains of the order *Actinomycetales.* Mikhail *et al*.[55] isolated an abundant bioactive compound,

streptomonomycin, from the culture broth of halophilic *Streptomonospora alba*, the first reported natural product from the genus *Streptomonospora*. Mikhail *et al*.[55] also reported the first genome sequence of *Streptomonospora.* Streptomonomycin, an uncommon hydrophobic lasso peptide with a unique Ser1-Asp 9 linkage exhibits antibiotic activity against several Gram-positive organisms, most notably *Bacillus anthracis*, a causative agent of anthrax with MIC value of 4 µg/ mL. Thus the discovery of streptomonomycin elucidates that poorly studied cultivable bacteria continue to be assets of readily discoverable natural products.

In the present study, compounds isolated from *Streptomonospora arabica* VSM-25 were indoles (C1), two phenolic acids (C2, C3), isoflavonoids (C4), and phenolic aldehydes (C5). Indole derivatives are very crucial heterocyclic compounds in drug discovery studies. There has been an increasing awareness in the use of indole derivatives as bioactive molecules against microbes and cancer cells[56]. Our results emphasize that compound C1 is an exceptionally potential inhibitor with the inhibitory effect of 100% against test microbes. It is also inhibited alpha-amylase effectively but not potent as acarbose. The phenolic compound pyrocatechuic acid (C2) is reported to have diverse pharmacological activities such as an antioxidant, a radical scavenger and a siderophore whereas vanillic acid (C3) has been reported for antisickling and anthelminthic activities and also could suppress hepatic fibrosis in chronic liver injury[57]. Anti microbial activities of compounds C2 and C3 were much less explored; however, the present study recorded weak antimicrobial activity against test microbes with the inhibitory effect of 27% (C2) and 54% (C3), respectively. Compound C3 exerted high anti-inflammatory activity by 67.74% at the concentration of 400 µg/mL and was stronger than the standard drug Diclofenac sodium, suggesting that it can be used for further studies.

Compound C2 showed significant DPPH and nitric oxide scavenging activities of 61.62% and 62.12%, which proved that it also has good antioxidant capacity. Compound C4 was previously reported as a natural product from *Streptomyces* sp. isolate[58]. It has been reported to exhibit moderate antibacterial activity against multi-drug resistant Gram-negative bacteria[35]. In similarity with previously documented data, Diadzein showed moderate to weak activity against pathogenic bacteria while it exhibited very weak activity against *Candida* and absolutely no activity against filamentous fungi. From the present preliminary findings, it can be concluded that Daidzein possessed marked anti-inflammatory and anti-arthritic activities of 62.67% and 60.23% although it was less potent than diclofenac sodium. 3,4-Dihydroxy benzaldehyde is widely used in medicine as an anti-aging, antioxidant, and antiinflammatory agent, and only a few reports were documented for its antimicrobial activity against *Ralstonia solanacearum* (20 µg/mL) and *Plesiomonas shigelloides* (≤ 60 µg/mL)[36, 33]. This study has revealed the antimicrobial activity of 3, 4-Dihydroxy benzaldehyde against a panel of pathogens with an inhibitory effect of 81%. Compound C5 showed significant and concentration-dependent antioxidant and free radical scavenging activities of DPPH and nitric oxide free radicals by 64.56% and 70.66% as compared to other compounds tested. Based on the results obtained, it can be concluded that 3, 4-Dihydroxy benzaldehyde exhibited potent antioxidant and free radical scavenging activities.

Anticancer activity of Indole-3-carboxaldehyde, 2, 3-Dihydroxybenzoic acid, vanillic acid, and 3, 4-Dihydroxybenzaldehyde was previously documented against various cancer cell lines[59-60]. In our present study, the potential of compounds C1 to C5 was investigated to inhibit cancer cell growth in MDA-MB-231 and MCF-7 cell lines. Similar to previously documented data, our results showed compounds C1, C2, C3, C4, and C5 exerted significant cytotoxicity with variation in the percentage of cell line inhibition against MDA-MB-231 and MCF-7 cell lines. Compounds C3 and C4 displayed $IC_{50}$ values of 1 µg/mL (57.72%; 67.81%) against MDA-MB-231, whereas C4 exhibited $IC_{50}$ value of 1 µg/mL against MCF-7 cell lines. The data reported in the present paper indicate that isolated compounds exhibit significant anticancer activities against the above-said cell lines at impressively low concentrations. Soft agar assay is employed for routine screening of specific compounds with cytotoxic potency that suppresses tumor growth *in vitro*. Colony count and colony size are the quantitative estimates of the assay that compares between treated cells and control to determine the difference of cell tumorigenicity. Based on colony size and count, conclusions could be drawn that the drug is an effective inhibitor of the tumor[61]. Michael *et al*.[62] reported cytotoxicity of pulmonary carcinoid tumor cell lines using soft agar assay. In addition, the anti-proliferative activity of a natural phenyl propionate derivative from *Mirabilis*

*himalaica* against HepG2 cell lines was reported using a soft agar assay[63]. Deoxyelephantopin (DOE) is a natural bioactive sesquiterpene lactone from *Elephantopus scaber*, a traditional herb in Chinese and Indian medicine whose anti-cancer activity is determined by soft agar assay[64]. The data reported in the paper indicate that cultures treated with compounds C1, C4, and C5 exhibited a very a smaller number of colonies per field. This identifies that compounds C1, C4, and C5 showed 90% inhibition of MDA-MB-231 cell lines in anchorage independent assay.

Human diseases such as cancer are considered to be cell cycle-related diseases due to recent conformation of the ability of the natural products to act as apoptotic, cell cycle inhibitors, and as cell cycle modulators[65-66]. The regulation of the cell cycle coordinates mainly at the G1/S, G2/M, and M phases by a series of check points. During the process of tumorigenesis[67], many compounds that act as anti-tumor agents can arrest the cell cycle and induce apoptosis which eliminates the damaged and unregulated cells[68]. Flow cytometric investigation showed that compounds C1, C2, C3, C4, and C5 induced early apoptosis in MDA-MB-231 cell lines. The increased proportions of the cell in the subG1 or G1 phase justify that compounds C1, C2, C3, C4, and C5 induced apoptosis in MDA-MB-231 cells resulting in the degradation of the DNA. Percentage of cells at the G2/M phase decreased, which corresponds to the increase in the percentage of cells at the G0 phase that indicates the arrest of the cell cycles at the G0/G1 phase by the compounds C1, C2, C3, C4, and C5 after 24 h. The obtained results indicate that compounds C1, C2, C3, C4, and C5 showed inhibitory activity on the cellular growth more by apoptosis rather by necrosis in the MDA-MB-231 cells.

The reported bioactive compounds of the present work, extracted from the strain VSM-25 showed significant bioactivities. To the best of our knowledge, this is the first report of isolation, characterization, and detailed biological evaluation of compounds Indole-3-carboxaldehyde (C1), 2, 3-dihydroxy benzoic acid (C2), Vanillic acid (C3), Daidzein (C4), and 3,4-Dihydroxy benzaldehyde (C5) from the genus *Streptomonospora*.

## Conclusion

The reported bioactive compounds of the present work, extracted from the strain VSM-25 showed significant bioactivities. To the best of our knowledge, this is the first report of isolation, characterization, and detailed biological evaluation of compounds Indole-3-carboxaldehyde (C1), 2, 3-dihydroxy benzoic acid (C2), Vanillic acid (C3), Daidzein (C4), and 3,4-Dihydroxy benzaldehyde (C5) from the genus *Streptomonospora*. The documented results reported that C1 showed the 100% inhibitory activity against test microbes. inhibited alpha-amylase effectively but not potent as acarbose. But compound C2 reported the diverse pharmacological activities such as an antioxidant, a radical scavenger and a siderophore. The compound C3 exerted high anti-inflammatory activity by 67.74% at the concentration of 400 µg/mL and was stronger than the standard drug Diclofenac sodium, suggesting that it can be used for further studies. The study also reported that compounds C1 to C5 inhibit cancer cell growth in MDA-MB-231 and MCF-7 cell lines. In addition C2, C3 and C4 exerted significant cytotoxicity with variation in the percentage of cell line inhibition against MDA-MB-231 and MCF-7 cell lines. The result of the soft agar assay is employed to test cytotoxic potency that suppresses tumor growth *in vitro*. The data reported in the paper indicate that cultures treated with compounds C1, C4, and C5 exhibited a very a smaller number of colonies per field. This identifies that compounds C1, C4, and C5 showed 90% inhibition of MDA-MB-231 cell lines in anchorage independent assay. In addition the compounds C1, C2, C3, C4, and C5 showed inhibitory activity on the cellular growth more by apoptosis rather by necrosis in the MDA-MB-231 cells.

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## Conflict of interest

All authors declare no conflict of interest.

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