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Journal of Pharma Research

Journal of Pharma Research
Available online through
www.jprinfo.com

Research Article
ISSN: 2319-5622

Structure Elucidation and Biological Activities of 1-(3-bromo-5-methylphenyl)-1*H*-indole from Marine *Streptomyces* sp LCJ85

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Received on: 14-11-2013; Revised and Accepted on: 26-11-2013

ABSTRACT

The marine Streptomyces sp LCJ85 isolated from sediments of Bay of Bengal produced biologically active compound 1-(3-bromo-5-methylphenyl)-1H-indole. The compound inhibited the human bacterial pathogens and with a effective mic value of 1.56 µg/mL against the Staphylococcus aureus. The DPPH assay showed good free radical scavenging by the compound with a IC_{50} value of 44 µg/mL. The HepG2 cells experienced a significant decrease in viability at very low oncentrations of 1-(3-bromo-5-methylphenyl)-1H-indole with an eventual decline at the high concentrations of indole compound in the cytotoxicity assay. The IC_{50} values of compound against the HepG2 cells was found to be 192 µg/mL. The results from these study proves that the marine actinomycete Streptomyces sp LCJ85 will be a effective source of biologically important secondary metabolites.

Key words: *Streptomyces, Resazurin, 1, 1-diphenyl-2-picryl hydrazyl, Free radical.*

INTRODUCTION

The actinomycetes are distributed widely in aquatic and terrestrial ecosystems. Actinomycetes are Gram-positive filamentous bacteria belonging to the phylum Actinobacteria. The actinomycetes play an excellent role in the recycling of biowaste by decomposing complex mixtures of polymers in to simpler ones [1]. There are 23,000 bioactive secondary metabolites have been reported from microorganisms and among these the actinomycetes produces over 10,000 compounds [2]. The pharmaceutical industry exploited the *Streptomyces* which forms the major primary antibiotic-producing organisms. The *Streptomyces* produces various useful secondary metabolites such as Aureolic acids (mithramycin), anthracyclins (doxorubicin), peptides (actinomycin D),antimetabolites (pentostatin), mitomycins and many others [3, 4].

The marine ecosystem is characterised by different unique parameters like high pressure, low temperature, absence of light etc. The actinomycetes in the marine environment have adapted themselves to survive in these environments. From various actinomycetes genera, the *Streptomyces, Amycolatopsis, Saccharopolyspora*, *Actinoplanes* and *Micromonospora* are the major producers of commercially important biomolecules [5]. The metabolites produced by these actinomycetes possess broad spectrum of bilogical activities such as antibacterial (Streptomycin, Chloramphenicol and tetracyclin), antiviral (tunicamycins), antiparasitic (avermectin), antifungal (nystatin), antitumor (mitomycin C, actinomycin and anthracyclines), enzyme inhibitory (Clavulanic acid) and diabetogenic (streptozotocin), etc. Since 1995, 12000 antibiotics are known and widely studied type of secondary metabolites. From these known antibiotics 55% are produced by filamentous bacteria of the genus *Streptomyces*, 12% from non filamentous bacteria, 11% from other actinomycetes and 22% from fungi. In the recent decades there has been tremendous increase in the discovery of novel metabolites with high bioactivity were isolated from marine *Streptomyces* [6-9].

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MATERIALS AND METHODS

Fermentation and purification of compound:

The fermentation was carried out using medium which consists of soy bean meal (20gr), mannitol (20gr) and yeast autolysate (10g) were dissolved in distilled water (1000ml), pH 7. The fully grown actinomycetes were transferred to the fermentation medium in 1 litre flasks (n=10) and incubated under shaking condition (150rpm) at 28°C for 7 days. The culture filterate was then separated and extracted with equal volume of ethylacetate and condensed under vacuum.

The purification was carried out by using column chromatography using silica gel (60-120 mesh). The hexane and chloroform were used as solvent system to elute the fractions. The column purification was repeated with silica gel of 230-450 mesh column to obtain pure compounds from the fractions obtained. The purified compounds were analyzed on TLC to check the homogenecity of the compounds.

Characterization of the purified compounds:

The purified compounds were subjected to Nuclear Magnetic Resonance studies, FT-IR, GC-MS spectroscopy and UV spectroscopy analysis.

NMR spectroscopy:

^{1}H NMR spectra of extracts and pure compounds as a purity check were recorded by Bruker spectrometer operating at 300 MHz. Spectra of pure compounds were processed using Bruker 1D WIN-NMR or 2D WIN-NMR software.

FT-IR analysis:

FT-IR spectra were recorded in FT-IR spectrometer SHIMADZU 8201 PC. 5 mg of the sample was mixed with KBr and made into pellets and placed in the instrument. FT-IR was recorded between 4000 and 400cm^{-1} for all the test samples under study.

GC-MS analysis:

Twenty five milligram of the sample was dissolved in 1 ml of spectroscopic grade methanol. About 2 µl of each of the dissolved sample was injected into the sample injection port of GC-MS (SHIMADZU QP 2000, Japan) The column (25mm x 0.25mm dia) was packed with ULBON HR-1; the temperature increased linearly from 100°C to 250°C and the carrier gas pressure fixed at 79.80 KPa for all the test samples studied. Mass spectra (EIMS) were recorded for each compound separated in succession by GC, the relative intensities corresponding to their retention time (Rt) of the molecular ion peak and the fragmented ion peaks were normalized with respect to the base peak.

UV spectrums for all the compounds were recorded in SHIMADZU UV- VIS-160 spectrometer. 1 mg of each sample was dissolved in 3 ml of HPLC grade methanol and the λ max was recorded between 200 nm and 500 nm where the samples exhibited absorption maxima.

Biological activity:

Minimum Inhibitory Concentration:

The minimum inhibitory concentration of the compound was carried out according to Sarkar *et al*. (2007) [10].

Preparation of Resazurin solution:

The Resazurin solution was prepared by dissolving a 270 mg tablet in 40 mL of sterile distilled water as described in previous experiment. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution.

Preparation of the plates:

Plates were prepared under aseptic conditions. A volume of 200µL of test material in 10% (v/v) DMSO was pipetted into the first row of the sterile 96 wells plate. To all other wells 100µL of nutrient broth was added. Serial dilutions were performed using a sterile pipette such that each well had 100µL of the test material in serially descending concentrations. To each well 10µL of resazurin indicator solution was added. Finally, 10µL of bacterial suspension (5×10^6 cells/mL) was added to each well to achieve a concentration of 5×10^5 cells/mL. The broad-spectrum antibiotic chloramphenicol was used as positive control. The plates were placed in an incubator set at 37 °C for 18-24 hrs. The colour change was then assessed visually. Any colour changes from purple to pink or colourless were recorded as reduction of dye by the viable bacteria. The lowest concentration at which no colour change occurred was taken as the MIC value.

Antioxidant Activity:

DPPH radical scavenging assay:

The antioxidant capacity of the samples was determined on the basis of their free radical scavenging activity and it was measured *in vitro* by using the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH). The DPPH is a stable free radical chemical containing an odd electron in its structure and it was usually utilized for the detection of the radical scavenging activity in chemical analysis. The DPPH solution (0.1 mM) in methanol was prepared and this DPPH was added to test tubes containing the test samples at different concentrations (5-60 µg/mL). all the tubes were incubated for 30 min, after 30 min; the absorbance was measured at 517 nm using Beckman spectrophotometer. The percentage of free radical scavenging at different concentrations of compounds was determined. The Ascorbic acid was used as a standard. The DPPH absorbs at 517 nm, and its concentration is reduced by the existence of an antioxidant. A dose response curve was plotted to determine the IC_{50} values. The following formula is used to calculate the percentage of radical scavenging.

$$\text{Radical scavenging (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

MTT assay: Cell Viability Assay:

A 5 mg per ml stock solution of MTT dye (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) was made in sterilized phosphated buffered saline (PBS). The lysis solution was made by mixing 0.6 ml of acetic acid and 99.4 ml of di-methyl sulphoxide (DMSO). The cells were incubated with the appropriate concentrations of samples for 24 and 48 hours. Then after the incubation the consumed medium was carefully removed from all the wells of the assay plate and was replaced with freshly prepared DMEM. The MTT dye (100µl per ml of stock solution) was added to each well and the culture plates were incubated for three hours in CO_2 chamber. The supernatant was aspirated carefully, taking care not to remove Formazan crystals formed with in the cells. Subsequently, lysis solution in amounts equal to that of the DMEM added before incubation was added and the cells were lysated over 5 minutes and mixed well. 200 µl of the lysate from each well of 24 well culture plate was transferred to the pre-marked 96 well plate and then the optical density (OD) of the lysate was measured at 595nm using ELISA reader. The percentage of OD value (OD for metal specimen/OD for control group x 100%) was calculated. A larger OD value represented higher cell viability and thus adhesion.

Molecular docking studies:

The two biologically active compounds isolated from marine *Streptomyces* sp LCJ85 were subjected for molecular docking [11] using Autodock (version 4) with Lamarckian genetic algorithm. This experiment was performed to analyse the antimicrobial mechanism of the two biological active marine compounds.

RESULTS

The purification was carried out by using column chromatography using silica gel (60-120 mesh). Two different compounds were obtained in the fractions with the hexane : chloroform (9 : 1) as solvent system. The column purification was repeated with silica gel of 230-450 mesh column to obtain pure compounds from the fractions obtained. The purified compounds were analyzed on TLC to check the homogenecity of the compounds and the R_f value was 0.71.

The structure of Compound can be detemined by the following data of ^1H and ^{13}C NMR spectra. Simalarly the functional group and molecular weight of the compound have been determined by the IR and Mass Spectra respectively. The ^1H NMR (300 MHz) spectrum of compound in $CDCl_3$ displayed peaks in the regions at 2.3 to 7.7, the major peaks at δ 7.619, δ 6.607, δ 7.594, δ 6.617, δ 7.147 and δ7.37 which probably indicates the protons of Indole. The substituent Methyl protons as a singlet at δ 2.426 and aromatic three protons also as a singlet at δ 7.221, δ 7.268 and δ 7.279 which is sharp peak due to the high deshielding around it.

In the ^{13}C NMR spectrum showed the presence of Methyl carbon at δ 21.66 and the aromatic skeleton of indole carbons shows at δ 1204.34, δ 104.15, δ 117.09, δ 122.30, δ 120.71,δ 110.61, δ 140.93 and δ 127.83.The substituent bromine attached carbon shows at δ 123.61 and methyl group attached carbon at δ 135.74. The peaks δ 130.10, δ 121.34, δ 129.49 and δ 120.70 shows the carbon atoms of aromatic ring.

The IR spectrum (KBr) showed an absorption band at 1334 cm^{-1} and aromatic skeleton gives band at 1465 cm^{-1}. The molecular formula was determined as $C_{15}H_{12}NBr$ by (-) EIMS and (+)-EIMS Which is showed the molecular ion peak at m/z 285 $[M-2]^+$ and m/z 287 $[M+2]^+$ respectively (**Table. 1**).

The UV spectra of the compound were measured using UV visible spectrophotometer. According to UV spectra the comound showed absorption maxima values at 300 λ_{max}.

The spectral analysis of the compound showed that the compound is 1-(3-bromo-5-methylphenyl)-1*H*-indole (**Fig. 1**)

Minimum Inhibitory Concentration:

The MIC of the compound 1-(3-bromo-5-methylphenyl)-1*H*-indole was performed by Resazurin reduction assay described by Sarkar *et al*. The change of blue color to pink color of the dye indicates that the cells are viable and the oxidoreductases present inside the bacterial cells converted the resazurin to resorufin which is pink in color. The compound 1-(3-bromo-5-methylphenyl)-1*H*-indole was active against the all tested human bacterial pathogens. This compound shows very effective mic value of 1.56 µg/mL against the bacteria *Staphylococcus aureus* when compared to the commercial antibiotic streptomycin. The mic values of this compound against other pathogens viz *Bacillus cereus, Escherichia coli* and *Proteus vulgaris* were less effective when compared to the streptomycin. The results are tabulated in **Table 2**.

Antioxidant activity:

The DPPH assay is a easy, rapid and sensitive method to screen the antioxidant potential of the given test compounds. Thus in the present study the antioxidant capacity of the compound1 was analysed by DPPH assay. The 1-(3-bromo-5-methylphenyl)-1*H*-indole showed significant free radical scavenging generated by DPPH. The free radical scavenging of compound1 was seen from the low concentration of 5µg/mL up to higher concentration of 60 µg/mL (**Fig. 2**). The svavenging activity increases with the increase in the compounds concentration. The compound 1-(3-bromo-5-methylphenyl)-1*H*-indole showed excellent free radical scavenging capacity in all concentrations with a IC_{50} value of 44 µg/mL and it is not more effective than the positive control α- tocopherol. The α-tocopherol was used as a standard to measure the free radical scavenging by DPPH assay. The α- tocopherol showed excellent antioxidant activity with a IC_{50} value of 25 µg/mL. The IC_{50} values of compound and standard were given **Table 3**.

Cytotoxic activity:

The cytotoxic activity of the 1-(3-bromo-5-methylphenyl)-1*H*-indole against HepG2 cells was assessed by MTT assay. The HepG2 cells showed exponential responses toward increasing concentration of compound. The HepG2 cells experienced a signficant decrease in viability at very low concentrations of compound with an eventual decline at the high concentrations of compound (**Fig. 3**). The IC_{50} values of compound 1-(3-bromo-5-methylphenyl)-1*H*-indole against the HepG2 cells was found to be 192 µg/mL.

The molecular docking of 1-(3-bromo-5-methylphenyl)-1*H*-indole was done with the *E. coli* Topoisomerase IV (PDB: 3FV5). This compound binds in the active site of the topoisomerase IV enzyme and shows good hydrophobic interactions (**Fig. 4**). This results substaniate with the wet lab work as this compound showed effective activity against the human bacterial pathogens. The binding of this compound in the active site may also be the mode of action of this compound.

Table No. 1: Physio-chemical properties of compound 1-(3-bromo-5-methylphenyl)-1*H*-indole

Properties	1-(3-bromo-5-methylphenyl)-1H-indole
R_f-Value	0.71
Molecular formulae	$C_{15}H_{12}BrN$
(+)-ESI-MS	287([M+2]$^+$, (bromide isotope - 81)
(-)-ESI-MS	285[M-2]$^+$, (bromide isotope - 79)
IR(KBr) υ cm-1	1334(m), 1465,

Table No. 2: Minimum Inhibitory Concentration of 1-(3-bromo-5-methylphenyl)-1*H*-indole against human bacterial pathogens (µg/mL)

Pathogens	Compound 2	Streptomycin
Staphylococcus aureus	1.56	3.12
Bacillus cereus	6.25	3.12
E.coli	6.25	1.56
Proteus vulgaris	12.5	6.25

Table No. 3: The free radical scavenging activities of compounds

Compounds	IC_{50} (µg/ml)
1-(3-bromo-5-methylphenyl)-1*H*-indole	44
α- tocopherol Compounds	25

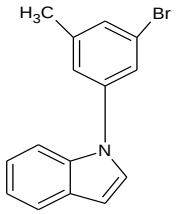

Fig.1: Structure of 1-(3-bromo-5-methylphenyl)-1*H*-indole

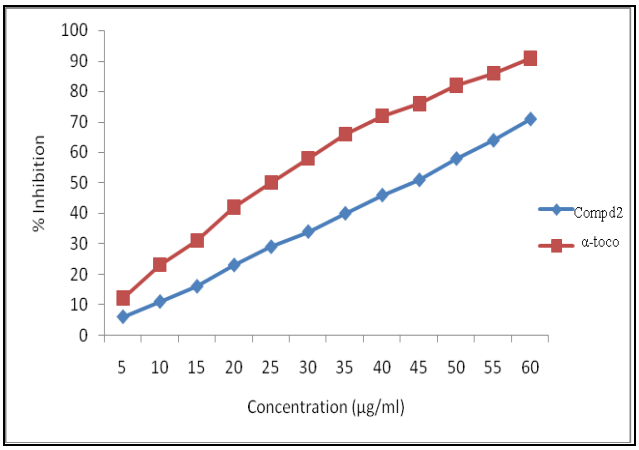

Fig. 2: *In vitro* DPPH radical scavenging activity of compounds

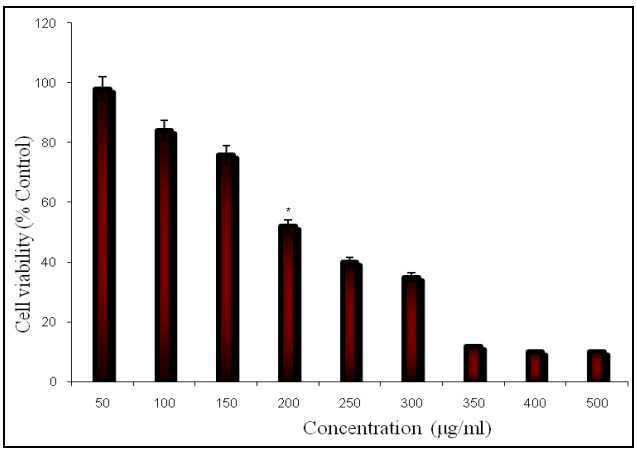

Fig. 3: Cytotoxic effects of Compound 2 against the HepG2 cell lines by MTT cell viability assay

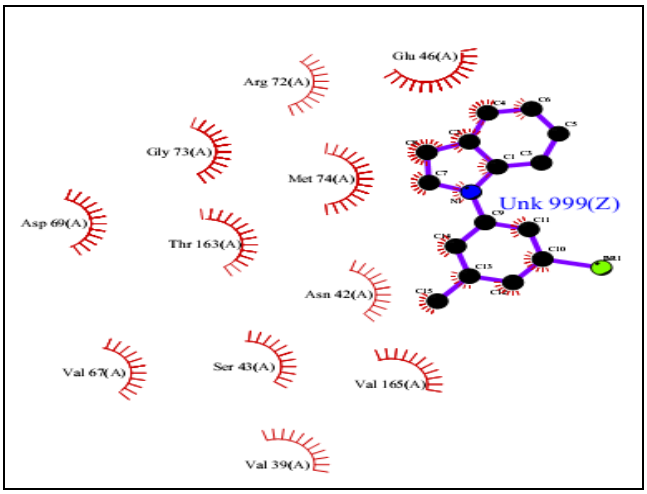

Fig. 4: Ligplot shows interactions of 1-(3-bromo-5-methylphenyl)-1*H*-indole with amino acid residues of Topoisomerase IV (PDB: 3FV5)

DISCUSSION

In the case of ^{1}H NMR Spectrum exhibit two multiplet signals at δ 7.17 range , Hence we can easily assume that should be an aromatic skeleton in addition to the two signals appeared at δ 7.18 range as a singlet. Two signals appeared at δ 2.42 and another one shows at δ 7.13 from these data the compound must be having heteroatom like nitrogen, oxygen etc. So, the compound must be having heterocyclic moiety as a indole. According to ^{13}C NMR spectra, sharp peak at δ 122.3 due to the high electronegative atom like halogens and from the isotopic abundance of bromine in 81% ($[M+2]^{+}$) by mass spectroscopy. A strong band of C=C Bond which is appeared at 740.61 cm^{-1} in the IR spectroscopy, confirms the aromatic skeleton of inodole. The UV spectra of the compound showed absorption maxima values at 300 λ_{max} measured using UV visible spectrophotometer. The compounds were found to be (1S,2S)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl dichloroacetate and 1-(3-bromo-5-methylphenyl)-1*H*-indole from the X-ray diffraction and spectral studies.

The compound 1-(3-bromo-5-methylphenyl)-1*H*-indole possessed good biological functions viz., antibacterial, anticancer and antioxidant activities. The minimum inhibitory concentration of this compound 2 against the human bacterial pathogens was excellent. This compound shows very effective mic value of 1.56 µg/mL against the bacteria *Staphylococcus aureus* when compared to the commercial antibiotic streptomycin. The two compounds with cytotoxicity activity against human liver cancer cell line 7402 and a human breast cancer cell line MDA-MB 435 were isolated from marine *Streptomyces carnosus* strain AZS17. Among these two compounds, compound 1 of *Streptomyces carnosus* strain AZS17 showed strong cytotoxicity with IC_{50} values of 0.6 µg/mL [12]. Similarly the compound 1-(3-bromo-5-methylphenyl)-1*H*-indole showed excellent cytotoxicity activity against HepG2 cell lines with a IC50 value of 192 µg/mL. The mode of action of cytotoxicity of the compound 1-(3-bromo-5-methylphenyl)-1*H*-indole needs to be further investigated. The compound Carbazomycin B showed both antibacterial and antioxidant activity. The Carbazomycin B was having much stronger antioxidant activity than α-tocopherol both in in vitro and in vivo studies [13]. Similarly the 1-(3-bromo-5-methylphenyl)-1*H*-indole showed good antioxidant activity in the invitro studies with a IC_{50} value of 44 µg/mL and less effective than the standard antioxidant agent α-tocopherol in the DPPH free radical scavenging assay. In the computational study, the compound 1-(3-bromo-5-methylphenyl)-1*H*-indole was docked with the topoisomerase enzyme

CONCLUSION

The study concludes that the compound 1-(3-bromo-5-methylphenyl)-1*H*-indole has effective biological activity and marine actinomycetes from the Bay of Bengal can be the potent source of biologically important novel compounds.

ACKNOWLEDGEMENTS

We thank Prof. R. Rengasamy, CAS in Botany, University of Madras for his constant help in this research. We thank National Institute of Ocean Technology, Pallikaranai, Chennai, for their help in arranging Cruises for sample collection and for other technical support.

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Conflict of interest: The authors have declared that no conflict of interest exists.

Source of support: Nil