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Purification and characterisation of bactericidal compound from *Actinobacterium dagang 5*

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Abstract

This research is concerned about isolation, purification, characterisation and screening of bactericidal metabolite from the culture filtrate of *Actinobacterium dagang 5*. The 2.58 g of crude extract was obtained from 10 L of culture filtrate of *Actinobacterium dagang 5* through ethyl acetate extraction. Later the concentrated crude extract was fractionated through silica gel column chromatography using hexane and ethyl acetate as eluting solvent system in gradient passion. The purified bactericidal compound having retention factor of 0.76 (8:2, hexane: ethyl acetate). The purified compound shows antibacterial activity against *Klebsiella pneumonia*, *Vibrio cholerae*, *Proteus vulgaris*. The compound was characterised and the structure was determined.

Keywords: *Actinobacterium dagang 5*, silica gel chromatography, FTIR, NMR, antibacterial activity

Introduction

For the last five decades antibiotics are playing major role in increase of life expectancy and aside reducing the morbidity and mortality of several infectious pathogens [1]. Recently antibiotic resistance is gradually increasing globally so the current urge on research is to develop the new drug for multiple drug resistant pathogens [2, 3, 4, 5]. Antibiotics are of synthesised by several pathways, which are interconnected and influenced by primary metabolites. The synthesis of antibiotic are of influenced by composition of culture medium, metabolic capacities, biosynthetic machinery [6, 7, 8]. Microbial natural product is the most important source for emerging drugs, of which actinomycetes are most eminent secondary metabolite producers [9]. It has enormous biosynthetic potential that remains to be competitor among other microbial family [10]. Bioactive compounds produced by the marine actinomycetes are of unique in nature with distinct chemical structure that could be used to combat resistant pathogens [11]. The aim of our current study is to isolate, purify and characterise the potent bactericidal bioactive compound.

Methods

Growth conditions of Isolate

The *Actinobacterium dagang-5* strain was isolated from the marine sediment sample from the Arichimunai (9.1794° N, 79.4183° E), Gulf of Mannar and their partial 16S rRNA gene sequence was deposited in gene bank and the accession number is KM925137. This strain was isolated by soil dilution technique using Starch Casein Agar (SCN) supplemented with nystain (50µg/ml), and nalidixic acid (50µg/ml) in order to avoid the fungal and bacterial contamination, respectively. The culture was maintained in yeast extract-malt extract agar (ISP 2) slants. The spores of *Actinobacterium dagang-5* was suspended in 15% glycerol and stored at -20°C.

Fermentation conditions

A loopful of pure culture of the strain was grown on ISP 2 Medium was transferred aseptically to the 250 ml of seed medium (glucose 10 g; malt extract 3.0 g; yeast extract 5.0 g; pH7.0 ± 0.2) in 1000 ml Erlenmeyer flask and incubated at 30°C for 7days on the rotary shaker at 180 rpm. The 10 L fermented culture medium(glucose 5; yeast extract 5; potassium nitrate 1; sodium chloride 1; di potassium hydrogen phosphate 0.5; magnesium sulphate 0.5; pH 7.2) was inoculated with 5 % (v/v) of preculture and incubated at 30°C for 7days [1, 12].

The Isolation and Purification of active compounds

To obtain the cell free supernatant the culture broth was filtered with a cheesecloth to separate the culture liquid and mycelium, the collected culture liquid was centrifuged at 10,000 rpm for 15 min. Later the cell-free supernatant was used for extraction of metabolites using organic solvent. Extraction of extracellular metabolites was done by adding ethyl acetate to the cell-free culture supernatant in the ratio of 1:1 (v/v) followed by vigorous shaking for 30 min. The solvent layer was separated and preserved, and extraction was repeated thrice with the aqueous layer. The ethyl acetate fractions were pooled and evaporated under vacuum using a rotoevaporator (BUCHI, Switzerland) [1, 13]. Later TLC was performed on a pre-coated silica gel TLC plates grade F₂₅₄ (E-Merck, Darmstadt, Germany) to determine the number of compounds present in the given sample. A total of 5 µL of sample was spotted at 1 cm from the bottom of silica gel plates using capillary tubes. Different solvents at various combinations and concentrations were used for metabolites profiling. Development of the chromatogram was done in closed tanks, in which the atmosphere has been saturated with eluent vapour by wetting a filter paper lining. The chromatogram was visualized under UV light (365 nm and 254 nm), iodine vapour and sulpho-vanillin reagent [6, 14, 15]. The concentrated crude metabolites were mixed with methanol-silica gel slurry and loaded into a silica gel 100–200 mesh (E-Merck, Darmstadt, Germany) column, packed in hexane: (the dimension of column was 450 × 30 mm). The column was eluted with stepwise gradient of (100:0; 90:10; 80:20; 70:30; 50:50; 30:70; 10:90, v/v) hexane/ethyl acetate solvents [1, 11]. Each fraction was concentrated and checked for its antibacterial activity against test pathogen. The fraction showing antimicrobial activity was further purified by silica gel 230–400 mesh (E-Merck, Darmstadt, Germany) column chromatography. The separation was done by gradient elution with low polar/high polar (gradient from 100% low polar/0% high polar to 0% low polar/100% high polar) using the flow rate of 2 mL/min. One hundred tubes of 10 mL each were collected and then analyzed by TLC. Fractions showing similar spots with same R_f values were pooled and concentrated by a speed-vac under low pressure with evaporating temperature of 40°C. All the fractions were tested for their antibacterial activity against the test pathogen. The active compounds were checked for their purity by Thin Layer Chromatography.

Characterization of purified molecules

The physical appearance of the purified compounds was determined visually. Solubility was checked with methanol, ethyl acetate, chloroform, hexane, DMSO and water. The purified compounds were dissolved separately in methanol at 2–10 µg/mL concentrations and their UV-Vis spectra were recorded using a UV-Vis spectrophotometer at 220nm.

Methanol was used as blank. IR spectra for the purified compounds were recorded on a Perkin-Elmer 1600 series. The GC-MS was recorded using the JEOL GCMATE II GC-MS with data system in a high resolution, double focussing instrument. Maximum resolution: 6000 maximum calibrated mass: 1500 Da. Source options: Electron impact (EI); Chemical ionization (CI) (JEOL, Akishima, Tokyo, Japan). ¹H NMR and ¹³C NMR of the purified compounds were recorded in CDCl₃ or deuterated DMSO with Tetramethylsilane (TMS) as internal standard solution using 400 MHz Bruker machine (Bruker, MA, USA).

Antibacterial studies of purified compound

The antibacterial activity of purified compound against bacterial pathogenic organisms such as *Klebsiella pneumoniae* (MTCC 1687), *Proteus vulgaris* (MTCC 3160), *Salmonella typhi* (MTCC 3231), *Shigella dysenteriae* (MTCC 3642), *Vibrio cholerae* (MTCC 3906) was investigated by well diffusion method. Pure cultures of the pathogenic bacteria were grown in Muller Hinton broth at 35°C for overnight on rotary shaker at 180rpm. Wells were made on MH agar plate at 6mm in diameter using a gel puncture. Plates were swabbed with the bacterial pathogens and purified compound at the various concentrations (25 µl, 50 µl, 75 µl, 100 µl) are loaded in each well, the positive and negative controls are also maintained. Plates are incubated at 37°C for 24hrs. After incubation the susceptibility of the test organism was recorded by measuring the diameter of the zone of inhibition.

Results and Discussion

Through ethyl acetate extraction, 2.58 g of crude extract was obtained from 10 L of culture filtrate of *Actinobacterium dagang* 5. The concentrated crude extract was fractionated through silica gel (100–200 mesh) column chromatography using hexane and ethyl acetate as eluting solvent system in gradient passion. Of the seven fractions (IMF1–IMF7) obtained, fraction IMF2 showed high inhibitory activity against bacterial pathogens and hence, it was further purified with silica gel (230–400 mesh) column chromatography. Three peaks were obtained, of which fractions 28–40 showed antibacterial activity against most of bacterial pathogens. As all these fractions were also showed single spot on TLC, pooled together and concentrated for further evaluation, spectral analysis and biological activities.

A white powder compound with no UV absorbing band on TLC with the R_f value of 0.76 (8:2, hexane: ethyl acetate) was purified from *Actinobacterium dagang* 5. This antibacterial compound was fully soluble in organic solvents such as methanol, ethanol and ethyl acetate. The UV absorption spectrum of the purified active compound dissolved in methanol (Fig. 1) exhibited absorption maximum at 220 nm. No absorption was found in the visible region.

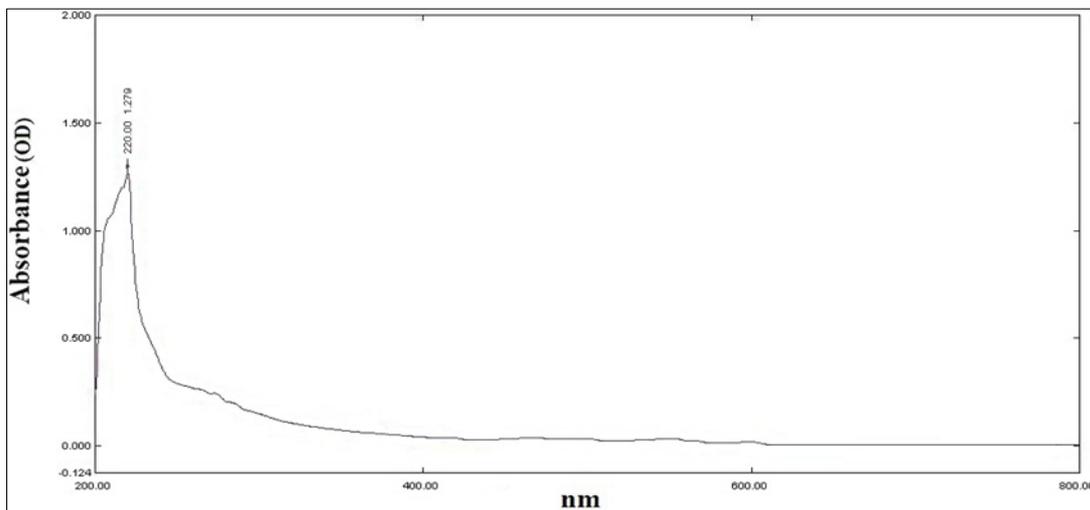


Fig 1: UV-Vis spectrum of the purified compound from *Actinobacterium dagang 5*

The IR spectrum (KBr) of purified compound displayed an absorption band at ν_{max} 3373 cm^{-1} (amine). A strong band at

ν_{max} 1673 cm^{-1} is indicative for carbonyl group. Strong bands between ν_{max} 1485–1321 cm^{-1} of methyl groups (Fig. 2).

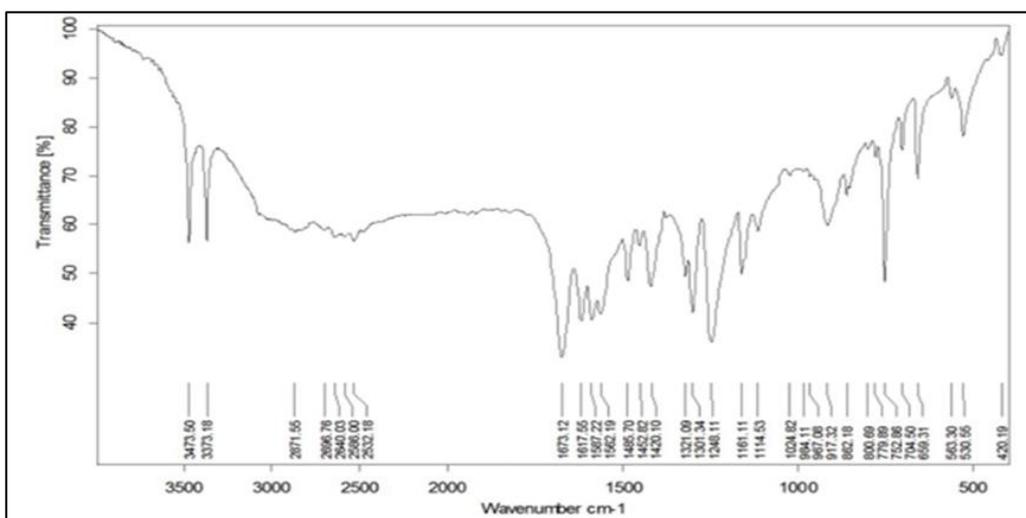


Fig 2: FTIR spectrum of the purified compound from *Actinobacterium dagang 5*

The GC-MS spectrum of the purified compound of *Actinobacterium dagang 5* showed a molecular ion peak at

m/z 174.80 (100, M^+) indicating the very characteristic fragmented peak of the purified compound (Fig. 3).

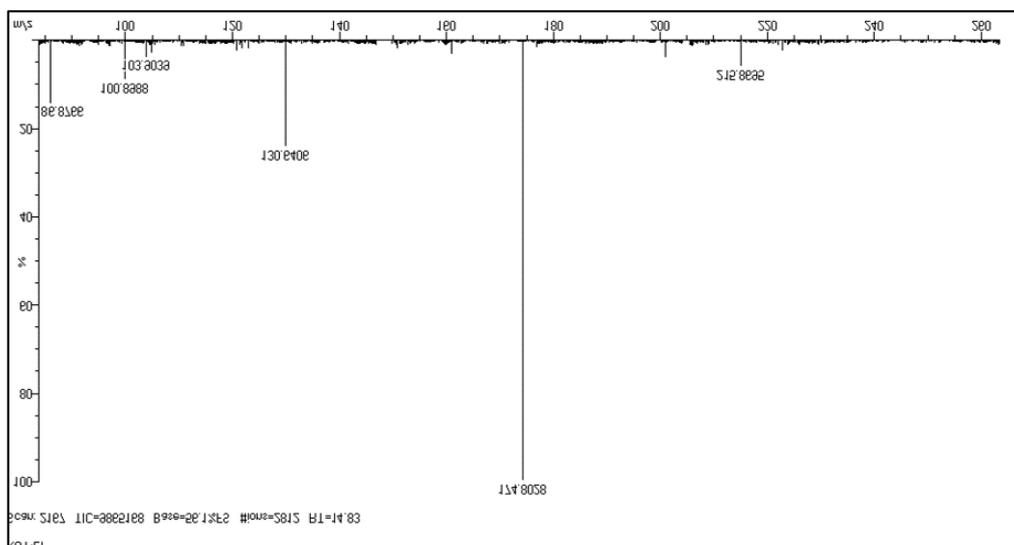


Fig 3: GC-MS spectrum of the purified compound from *Actinobacterium dagang 5*

The ^1H NMR shift of the purified compound showed the indole NH_2 proton appears at δ 10.15ppm and aromatic proton appears as multiples in range 7.28 to 7.59ppm. The $\text{N}-\text{CH}_2$ appears at triplet at 2.75ppm and indole ring attached CH_2

proton appears as triplet at δ 2.84ppm. The NH_2 proton appears as singlet at δ 2.00ppm in the purified compound (Fig. 4).

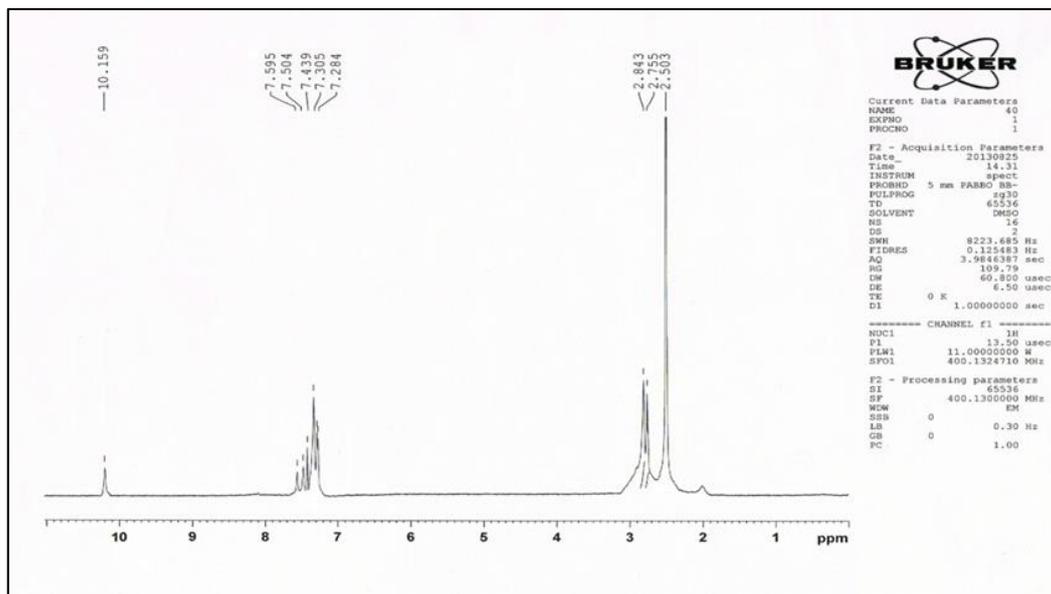


Fig 4: ^1H NMR spectrum of the purified compound from *Actinobacterium dagang* 5

The ^{13}C NMR spectrum showed a peak at δ 26.52 and δ 43.87 ppm for aliphatic methyl carbon. The aromatic carbon appeared

in the range of δ 112 to 123ppm and aromatic junction appears at δ 126.91 and 135.82 ppm (Fig. 5).

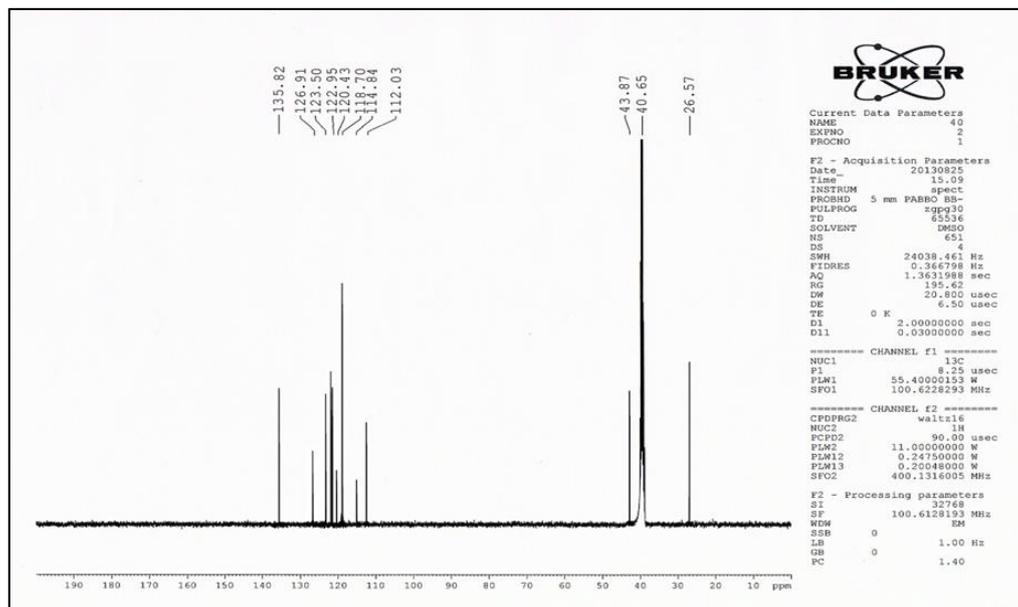


Fig 5: ^{13}C NMR spectrum of the purified compound from *Actinobacterium dagang* 5

The mass fragmentation search in the Mass Bank and molecular weight search between 150 and 175 in the Novel Antibiotic database, PubChem, NIST and SDBS databases revealed that a most similar compound with molecular mass of 160.22. Based on the physico-chemical properties, the purified compound was identified as 3-(2-aminoethyl) indole and its structure is depicted. Its molecular formula is $\text{C}_{10}\text{H}_{12}\text{N}_2$. Its calculated molecular weight is 138.22 g/mol and the exact mass is 138.10005 g/mol. The isolated compound is monoamine alkaloid group, which contain one amino group connected to an aromatic ring by a two carbon chain. The compound is synthesized by decarboxylation of tryptophan,

indole side chain amino acid. The tryptamine molecule constitutes a family of secondary metabolite with various biological activity such as antibacterial activity and neurotransmitter or neuromodulator [3]. The presence of indole groupings suggest antibacterial molecule.

The antibacterial activity of purified compound was investigated against various pathogenic organisms such as *Klebsiella pneumonia* (MTCC 1687), *Proteus vulgaris* (MTCC 3160), *Salmonella typhi* (MTCC 3231), *Shigella dysenteriae* (MTCC 3642), *Vibrio cholerae* (MTCC 3906) using agar well diffusion method. The diameter of zone of inhibition was measured and represented in (Table.1).

Table 1: Antibacterial activity of purified compound

Bacterial Pathogens	Antibacterial activity of purified compound and antibiotic ($\mu\text{g/ml}$) (Zone of Inhibition at mm)				Ampicillin 50 μl
	25 μl	50 μl	75 μl	100 μl	
<i>Klebsiella pneumonia</i> (MTCC 1687)	14	16	19	21	11
<i>Proteus vulgaris</i> (MTCC 3160)	5	6	9	11	15
<i>Salmonella typhi</i> (MTCC 3231)	8	12	13	14	12
<i>Shigella dysenteriae</i> (MTCC 3642)	11	12	13	16	13
<i>Vibrio cholera</i> (MTCC 3906)	12	13	17	18	10

The purified compound shows highest activity against *Klebsiella pneumonia* (14mm) and *Vibrio cholerae* (12mm), whereas the lesser activity against *Proteus vulgaris* (5mm) at the low concentration (25 μl) of purified compound. The diameter of inhibition was increased by increasing the amount of the concentration of compounds. The positive control used in these studies is ampicillin which shows least activity when compare to isolated compounds.

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