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Antioxidant and Cytotoxic Potentials of a Pure Alkaloid Isolated from (MDA-MB-231: A Triple Negative Breast Cancer Cell Line) from Marine *Actinomycetes*

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***Abstract*: Marine *actinomycetes* are immense source of novel compounds with many therapeutic applications due to their vast diversity. A novel *actinomycetes*, *Aciditerrimonas ferrireducens* isolated from marine sediments collected at Kakinada coast. Objective: To isolate and characterize biologically active novel compound (s) from crude methanolic extract and to analyze antioxidant activity and anti-proliferative potential against MDA-MB-231triple negative breast cancer cell line. Method: The homogeneity of potential fraction obtained gel from silica column chromatography was analyzed by HPLC. FTIR, NMR, and ESI-MS spectroscopy method were used to elucidate the chemical structure. Antioxidant activity was evaluated by DPPH, superoxide and hydroxyl radical scavenging activity. Anti-proliferative potential was assessed by MTT, BrdU and TUNEL assays. Result: The structure of active compound was elucidated as amide alkaloid, hexahydro-3-(2-methylpropyl) pyrrole [1,2 α] pyrazine-1,4-dione based on spectral data. The isolated amide alkaloid exhibited significant antioxidant activity and also anti-proliferative activity against MDA-MB 231 cells. Conclusion: A novel amide alkaloid from methanolic extract of marine *actinomycetes* exhibited significant antioxidant potential and anti-proliferative activity against TNBC cells.**

Keywords*: Amide alkaloid, Antioxidant, Anti-Proliferative and Marine *actinomycete

1. Introduction

Marine derived *actinomycetes* are efficacious source of novel compounds with various therapeutic applications. They are proven to produce novel bioactive compounds due to their vast diversity. Investigation on natural products produced by prokaryotic and eukaryotic organisms with vast pharmacological and medicinal possessions shows the way for clinical applications. Among more than 22,000 known microbial secondary metabolites, contribution made by different organisms are as follow *actinomycetes* 70%, fungi 20%, *Bacillus spp* 7% and 1–2% by other bacteria [1]. Various studies have proven that several new marine *actinomycetes* possess unique metabolic activity and can produce novel compounds with various biological activities including antibiotics, anti-parasitic and enzyme inhibitors and antiproliferative agents.

In recent years' new therapeutic agents have developed but unfortunately with some side effects and drug resistance. As *actinomycetes* are known to produce unique biological active compounds it is required to explore the available resources. We have isolated and characterize a novel *actinomycetes* strain. *Aciditerrimonas ferrireducens* which belong to family *Acidimicrobiaceae* from marine sediments collected at Kakinada coast [2]. In the present investigation an attempt has been made to isolate and characterize a novel bioactive compound from the crude methanolic extract of culture supernatant. Further, the isolated compound was evaluated for antioxidant and cytotoxic activity against MDA-MB-231 a triple negative breast cancer cell line.

2. Materials and methods

A. Chemicals, microbial culture and cell lines

Silica gel with mesh size 200-300, thin layer chromatography sheets, methanol, n- hexane, ethyl acetate, acetonitrile (HPLC grade) and all other solvents were purchased from Merck, India. Starch casein agar, rifampicin, ketoconazole, and nutrient agar from HiMedia, Mumbai. *Staphylococcus aureus* were obtained from MTCC, Chandigarh and maintained on nutrient agar at 27^{o}C in an incubator. MDA-MB-231 a triple negative breast cancer cell line was obtained from NCCS Pune, and maintained in advanced DMEM (Invitrogen), supplemented with 100 units of penicillin and streptomycin (Invitrogen) and 10% fetal bovine serum (Invitrogen) in 5% CO_2 incubator.

B. Fermentation

The isolated *actinomycetes* strain *A. ferrireducens* was used for isolation of alkaloids [3]. The starch casein broth was prepared by suspending starch casein (g) in 10lit of sterilized seawater and distilled water (1:1). The starch casein broth (pH 6) was supplemented with dextrose (0.5mg/100mL), leucine (0.5mg/100mL), rifampicin (10µg/mL) and ketocanozole

(75µg/mL). The spores of isolated *actinomycetes* was inoculated into the starch casein broth, pH 6 in 25mL conical flask and treated with UV light followed by EtBr. The treated spores were incubated for 7 days at 30°C. After the mycelium growth the supernatant was collected, filtrated and centrifuged at 5000 rpm for 10 min. The alkaloids from culture supernatant (10 L) was extracted by methanol by solvent extraction method [4]. The methanolic extract was subjected to evaporation at 60ºC using rotary evaporator. The semidry crude methanol extract was used for isolation of alkaloids.

C. Isolation and characterization of alkaloids

1) Silica gel column chromatography

Crude methanolic extract (20 g) was subjected to silica gel (Mesh size: 200-300) column chromatography (15 × 2.5 cm) using different solvent systems. The sample was eluted with a gradient solvent system beginning with n-hexane and ethyl acetate mixture in the ratio of 100:0, 75:25, 50:50, 25:75 and 0:100 followed by ethyl acetate and methanol in the ratio of 100:0, 75:25, 50:50, 25:75 and finally 100 % with methanol. 10 mL of each fraction was collected and the homogeneity was assessed by thin layer chromatography using appropriate solvent system. The spots were developed by spraying with 20% H_2SO_4 in methanol and subsequently heating for 5 min at 110ºC. Homogeneously, pure fractions with equal Rf values were pooled and crystalized. Purity of the sample was analyzed by reverse phase HPLC using C-18 column and linear gradient of acetonitrile: water of methanol: (25:35:40 v/v/v) at a flow rate of 1 mL/min. The absorbance was examined at 270nm for 10 min and the retention time was noted.

2) FT- IR spectroscopy

The functional group of isolated alkaloids was analyzed by Thermo Nicolet Nexus 670 spectrometer. Dry potassium bromide (100mg) was mixed with 1 mg of isolated alkaloid compound and the mixture was compressed into pellet. The KBr pellets were evaluated by means of FT-IR spectrophotometer at a wave number in the range of 4000 and 500cm-14cm-1 at room temperature.

3) NMR spectroscopy

Proton (^{1}H) and carbon (^{13}C) NMR spectroscopy was used to elucidate the structure of purified alkaloid compound. The isolated alkaloid (43mg) was dissolved in water and used for recording the spectra. ^{1}H NMR spectra was recorded from 1-10 ppm on AVANCE 300 spectrometer using deuterated water. ^{13}C NMR spectra was recorded from 1-200 ppm on AVANCE 300 spectrometer using deuterated water (D_2O). Tetramethyl silane was taken as inner standard. Chemical shifts were expressed in ppm and coupling constants (J) were demonstrated in hertz (Hz). The NMR instrument was operated at 500 MHz for 6 h at room temperature. Signals were referenced to tetramethyl silane to within ± 0.01 ppm [5].

4) Electrospray Ionization-Mass spectroscopy (ESI-MS)

Electrospray ionization is a soft ionization technique, characteristically used to define the molecular weights of isolated alkaloid. ESI-MS of isolated alkaloid was acquired in the positive ion mode on a positive ESI coupled with high resolution mass analyzer and Fourier transform mass spectrometer (FTMS) ranging between 100 and 750 m/z. The spectral analysis was done under the conditions: Flow rate - 0.5mL/min, Capillary voltage - 3kv, Fragmentor voltage -75v, Nebulizer pressure - 25 psi and drying gas temperature - 350oc.

D. Antioxidant activity

1) DPPH radical scavenging activity

DPPH radical scavenging activity of the isolated alkaloid (PPDH) was carried out by the procedure of Zakaria [6]. Concisely, 10µL of different concentrations of PPDH (2, 4, 6, 8 and 10 µg/mL) was added to 290 µL of DPPH (120 µM/L) and incubated at 37°C in dark for 30 min. The absorbance was noted at 517 nm by using spectrophotometer. Methanol was taken as control. The percent of DPPH radical scavenging activity was calculated using the formula.

$$\frac{A_{517}(\text{control})-A_{517}(\text{test})}{A_{517}(\text{control})} \text{ X100}$$

2) Scavenging of hydrogen peroxide (H_2O_2)

The H_2O_2 scavenging of PPDH was assessed by the method [7]. Concisely, 40 mmol/L hydrogen peroxide was prepared in PBS at (pH 7.4) and was incubated at different concentrations of PPDH (2, 4, 6, 8, and 10µg/mL) for 10 min. The amount of hydrogen peroxide turned into was determined by measuring the absorption at 230nm and molar coefficient of 81 L/ mol.cm. Ascorbic acid was used as positive control. The H_2O_2 radical scavenging activity was calculated.

$$\frac{A_{230}(\text{control})-A_{230}(\text{test})}{A_{230}(\text{control})} \text{ X100}$$

3) Nitric oxide radical scavenging activity

NO is generated from Na_2 [Fe (CN) 5NO] solution as per Griess reaction [8]. The reaction mixture (3mL) containing sodium nitroprusside (10mM) and PPDH at 2,4,6,8 and 10µg/mL was incubated at 25°C for 120 min. 0.5mL reaction mixture was added to 1/mL of 0.33% of $C_6H_7NO_3S$ in 20% frigid acidic corrosive and incubated at room temperature for 5 min. After incubation 1 mL of 0.1% N-1-naphthylethylenediamine dihydrochloride was added and incubated for 30 min at 25ºC. The absorbency of pink chromophore was measured at 540 nm. Ascorbic was used as positive control. The NO radical scavenging activity was calculated as,

$$\frac{A_{230}(\text{control})-A_{230}(\text{test})}{A_{230}(\text{control})} \text{ X100}$$

4) Hydroxyl radical (•OH) scavenging activity

The ability of sample to inhibit hydroxyl radical mediated peroxidation was measured by method [9] with some adaptations. The reaction mixture contained 100µL of PPDH (2,4,6,8 and 10µg/mL), (0.6mM) 100µL of deoxyribose (3mM) in phosphate buffer (20mM, pH 7.4), 500µL ferric chloride

(0.1mM) 500 µL EDTA (0.1mM), 500 µL of ascorbic acid (0.1mM) and 500 µL of H2O2 (1mM) and 800 µL of phosphate buffer so that the final volume is 3ml. After incubation for 1hr at 370C, 1.0 ml of TCA (2.8%) and 1.0ml of (thiobarbituric acid) TBA (1%) were added and incubated in water bath for 20 minutes at 100°C. The absorbance was measured at 532nm. Ascorbic acid was used as standard. The HO.- radical scavenging activity was calculated as,

$$\frac{A_{532}(\text{control})-A_{532}(\text{test})}{A_{532}(\text{control})} X100$$

5) Superoxide (O_2-) radical scavenging activity

The superoxide radical scavenging activity was measured by Seema kumari et al., [10] method. Superoxide anion were generated in non-enzymatic hydroxylamine (HA)-EDTA system through the reaction of HA, EDTA and oxygen. It was assayed by reduction of nitroblue tetrazolium (NBT). The reaction mixture containing 1.0ml of sodium carbonate (125mM), 0.4ml NBT (24mM) and 0.2 ml of EDTA (0.1mM). The reaction was initiated by adding 0.4ml of hydroxylamine (1mM) and 0.5ml of PPDH (2,4,6,8 and 10µg/mL). ascorbic acid was used as standard. After 5 minutes of incubation at room temperature, the absorbance was measured at 560nm. The superoxide radical scavenging activity was calculated as,

$$\frac{A_{532}(\text{control})-A_{532}(\text{test})}{A_{532}(\text{control})} X100$$

6) Total antioxidant activity

The total antioxidant activity of PPDH was determined by the process of [11]. The reaction mixture containing 0.3 µL of PPDH (2, 4, 6,8,10 µg/mL) 3.0 mL of reagent containing of 0.6 mol/L H_2SO_4, 28 mmol/L Na_3PO_4 and 4 mmol/L $(NH_4)_6MO_7O_{24}$. The mixture was incubated in a water bath at 95°C for 90 min. The absorbance was recorded at 695 nm. Ascorbic acid was used as positive control. The results were expressed in Ascorbic acid equivalents.

7) Total reducing power

Total reducing ability of PPDH was resolved as per the [12]. PPDH (2, 4, 6, 8 and 10 µg/mL) as mixed with 1% $C_6N_6FeK_3$ and incubated at 50 °C for 20 min. Then, 2.5 mL of 10% TCA was added to the reaction mixture and centrifuged at 5000 rpm for 10 min. The top layer of solution (2.5 mL) was collected and 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ was added. The absorbency was recorded at 700 nm. The highest absorption of the reaction mixture was considered as the diminishing power of PPDH. Total reducing ability was calculated as,

$$\frac{A_{700}(\text{control})-A_{700}(\text{test})}{A_{700}(\text{control})} X100$$

E. Cytotoxicity assays

1) MTT assay

MTT assay used to study the impact of PPDH on MDA-MB-231 cells. Monolayered cells (5×10^3/well) were treated with PPDH (2, 4, 6, 8, and 10µg/mL) for 24h. Untreated cells were used as a control. MTT reagent (5 mg/mL of PBS) was added to each well and incubated for 2h. DMSO was supplemented to solubilize formazan crystals. Absorbance at 595 nm was noted using ELISA plate reader. The percent reduction of cell viability was expressed by comparing with methanol treated cells, which set as 100%.

2) Sulphorhodamine B (SRB) assay

SRB assay was used to evaluate the effect of PPDH (2-10µg/mL) on survival of MDA-MB-231 cells [13]. Monolayer cells ($5x10^3$/well) were plated in 96-well plate were treated with PPDH for 48h. Cells treated with methanol was served as control. After 48h incubation, adherent cells were settled in situ by adding 50µl of cold 40% (w/v) TCA and incubated for 1h at 4°C. The cells were washed with MilliQ water. To every well, 50µl of Sulphorhodamine solution (0.4% w/v in 1% acidic corrosive) was added and incubated for 30min at room temperature. Unbound Sulphorhodamine was washed with 1% acidic corrosive. The plates were air-dried and 100µl of 10 mM Tris base, pH 10.5 was added to each well to solubilize the color. The plate was shaken for 20min on a plate shaker set at 200 rpm and the absorbance was read using ELISA reader at 570nm. Cell survival was computed as the percent absorbance contrasted with that of untreated control.

F. Apoptotic assays

1) Determination of cell death by propidium iodide staining method

The effect of PPDH on induction of cell death in *S.aureus* was evaluated using propidium iodide staining assay. Briefly, *S.aureus* cells were treated with PPDH at 10µg/mL, respectively for 24h. Untreated cells were served as controls. Then, 50µl of propidium iodide solution (15µM) was added to each well and incubated in the darkness for 10 min. The treated and untreated cells were filtered through black Millipore polycarbonate filters. The filters were then rinsed with water to remove the excess stain. The PI positive cells on filters were counted under the fluorescent microscope within 1h and expressed as percent control. Streptomycin was used as positive control.

2) TUNEL assay

S.aureus cells were treated with PPDH at 10µg/mL for 24h. The untreated well was served as control. The hydroxyl group of fragmented DNA in cells was labeled with (TdT) and (TUNEL) technique the use of an apoptosis in situ discovery equipment conferring to the company's instructions (Invitrogen USA). The cell nucleus become categorized through (DAPI) and the nick-ends were labeled. The treated and untreated bacterial cells were filtered through black Millipore polycarbonate filters. The positively fluorescein-labeled cells were visualized under fluorescence microscope and quantified. The percent of cell death was expressed.

3) BrdU proliferation assay

Bromodeoxyuridine assay used to study the impact of PPDH on cell expansion. Cells ($1x10^4$cells/well) were harvested in

triplicate into 96-well plates. The cells were treated with PPDH (2-10µg/mL) for 48h then, cells were labeled with BrdU (1:2000) for 12h. After incubation, cells were labelled with diluted BrdU (1:2000 in tissue culture medium) for12 h. Cells were fixed with fixative solution and incubated for 30 min at room temperature. After incubation, anti-BrdU antibody (1:100) added and incubated for 1h at room temperature. After washing, conjugate antibody (1:2000) added and incubated for 30 min at room temperature. After washing, tetra-methyl benzidine substrate added and incubated in dark for 15min. Ten stop solution was added and absorbance was read at dual wavelength of 450 and 540 nm within 30 min.

4) Detection of cytoplasmic membrane damage in S aureus

The cytoplasmic membrane damage was assessed by potassium leakage assay. The concentration of potassium was measured utilizing flame photometer at a wavelength of 766 and 480 nm. The instrument was aligned with standard potassium chloride solution at 0.05, 0.1, 0.5, 1.0 and 5.0 µg/mL in deionized water. *S aureus* (100µL) were treated with different concentration of potassium and the rise in the potassium level caused by PPDH (10µg/mL) or streptomycin (10µg/mL) combination became measured after separation of biological debris through centrifugation at 4000rpm for 30 min.

G. Statistical analysis

Statistical analysis and graphical exploration of the data were done using Microsoft Excel. All the experiments were carried out three times. Values are shown as means ±SD of at least three independent experiments. Significance set as $p<0.05$.

3. Results and discussion

A. Extraction of alkaloids

By using liquid-liquid fractionation, the alkaloids are extracted into methanol from 10L of culture broth, then methanol extract was evaporated to 20g of semi dry residue.

B. Separation of alkaloids

Silica gel column chromatography is commonly used for isolation of alkaloids from natural sources [14]. It offers isolation as well as purification of alkaloids. In the current study, methanolic extract of *actinomycetes* isolated from marine sediments was subjected to silica gel column chromatography with mesh size 200-300 for purification of alkaloid. Methanolic extract (20g) was mixed with the silica gel and carefully loaded on to column previously packed with silica gel. The column was initially equilibrated and eluted with n-hexane. 16 fractions (1-16) each with 5.0mL were collected. Further, fractions from 17-33 were eluded with combination of ethyl acetate and n-hexane in the ratio of 75:25. The fractions from 34-49 were eluted with ethyl acetate: n-hexane in the ratio of 50:50. The fraction, 50-65 were eluted with ethyl acetate: n-hexane in the ratio of 25: 75. Further, fractions 66-81 were eluted with ethyl acetate (100%). The fractions from 82-101 were eluted with ethyl acetate and methanol in the ratio of 75: 25. The fractions from 102 -121were eluded with a mixture of ethyl acetate and methanol in the ratio of 50:50. The fractions from 122 -139 were eluded with a mixture of ethyl acetate and methanol in the ratio of 25:75. The fraction 140-156 were eluted with 100% methanol. The fractions from 1 to 156 were negative for Wagner's test. In addition, these fractions were not showed absorbance at 240nm, a characteristic of alkaloids, indicating the absence of alkaloids. The fractions 157-175 were eluted with water: methanol (25:75). These fractions were positive for Wagner's test and also exhibited absorbance at 240nm, (0.884) indicating the presence of alkaloids (Table 1). The fractions were subjected to TLC investigation homogeneity. Among different fractions only eluents from fraction 157-175 showed single peak in TLC analysis (Table 2) which was further analyzed by HPLC.

C. Homogeneity of purified alkaloid

Fraction 157-175 were pooled for HPLC study using acetonitrile: water: methanol (25:35:40) as a mobile phase with flow rate of 1mL/min. HPLC is a simple and versatile technique used for both qualitative and quantitative analysis of secondary metabolites. C18 reverse phase column was used for analysis of alkaloid purity. The degree of purity of isolated compound is based on the stationary and mobile phases. The selection of stationary phase depends on nature of secondary metabolite. The resolution of mobile phase, such as acetonitrile, and methanol can be enhanced by reducing the polarity using aqueous mobile phase [15]. The chromatogram showed single peaks at retention time of 3.350, indicating purity of the sample (Fig 1). The pooled fraction was condensed using rotary evaporator and the yield was calculated as 6.4g. The structure of purified alkaloid was elucidated by various spectroscopic techniques.

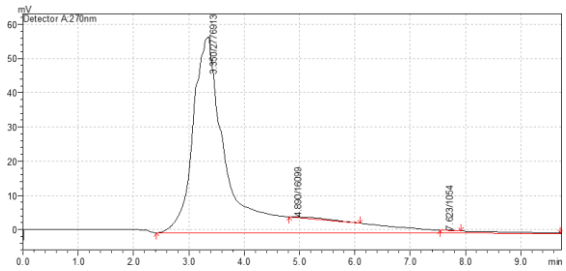


Fig. 1. HPLC Chromatogram of purified alkaloid.

- The HPLC analysis was carried out using methanol: acetonitrile: water (25:35:40v/v/v).
- C18 reverse phase column was used.
- Detection of eluted compound at 270 mn.
- Retention of compound was observed at 3.35min.

D. Elucidation of structure of alkaloid

Infrared (IR) spectroscopy is frequently used for functional group investigation. In the present study, the functional groups of isolated alkaloids were analyzed using FT-IR spectrometer (Thermo Nicolet Nexus 670) at a resolution of 4 cm-1 from wave numbers 500 cm-1 (Fig 2). The IR absorption of purified

Table 1
Silica gel column chromatography of methanolic extract of isolated *actinomycete*

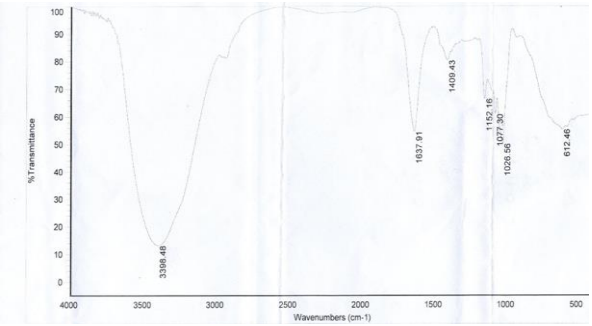
Eluent	Fraction number	OD at 240nm	Wagner's test
n-hexane: Ethyl acetate (100:0)	1-16	0.044	Negative
n- hexane: Ethyl acetate (75:25)	17-33	0.062	Negative
n- hexane: Ethyl acetate (50:50)	34-49	0.084	Negative
n- hexane: Ethyl acetate (25:75)	50-65	0.110	Negative
Ethyl acetate (100)	66-81	0.131	Negative
Ethyl acetate: Methanol (75:25)	82-101	0.059	Negative
Ethyl acetate: Methanol (50:50)	102-121	0.205	Negative
Ethyl acetate: Methanol (25:75)	122-139	0.167	Negative
Methanol (100)	140-156	0.427	Negative
Water: Methanol (75:25)	157-175	0.184	Positive

Table 2
Silica gel column chromatography of methanolic extract of isolated *actinomycete*

S. No.	TLC fractions	Solvent system	Rf value	No. of bands
1	1-16	n- hexane: Ethyl acetate (75:25)	0.3	6
2	17-33	n- hexane: Ethyl acetate (50:50)	0.3	6
3	34-49	n- hexane: Ethyl acetate (50:50)	0.3	5
4	50-65	n- hexane: Ethyl acetate (25:75)	0.4	5
5	66-81	Ethyl acetate (100)	0.4	4
6	82-101	Ethyl acetate: Methanol (75:25)	0.5	4
7	102-121	Ethyl acetate: Methanol (50:50)	0.7	4
8	122-139	Ethyl acetate: Methanol (25:75)	0.11	3
9	140-156	Methanol (100)	0.6	2
10	157-175	Water: Methanol (75:25)	0.8	1

spectrum of alkaloid exhibited major absorption peak at 3398 could be attributed to NH of amide [16] 1637 attributed to CONH of amide [17] and 1409 and 1077 indicating CH2 bending and out of plane vibration of CH_2 group [18]; [19]. The structure of compound ^{13}C and 1H NMR are two basic types, which are used to identify the resonance frequency of ^{13}C and ^{1}H nucleus of bioactive compounds. ^{1}H NMR spectrum of isolated alkaloid compound records chemical shifts and coupling constants and gives information about the relative number of hydrogen in compound. (Fig 3 a, b, c, d). ^{13}C NMR affords fundamental information on type of carbon groups in the compound. In the present study, purified alkaloid compound was subjected to ^{1}H and ^{13}C NMR using D_2O. In the NMR of spectrum of purified compound, the major peaks were observed at δ 5.286 ppm indicating CH_2 group. Since, it is closed to electronegative oxygen so went download field. The peak values from 3.87-3.71 ppm were due to CH_4 and CH_2 groups, however, δ 3.51 ppm peak is due to CH_2 proton. The peak at 3.49 ppm is due to CH proton. Based on the IR and ^{1}H and ^{13}C NMR spectral data, and also based on the available literature the possible structure of purified compound was proposed as hexa hydro pyrrole [1,2 α] pyrazine-1,4-dione. The mass spectrum of purified alkaloid compound exhibited major m/z peak at 157 (Fig 4) indicating its molecular weight, which is close to formula weight of purified alkaloid compound Mol.Wt 154.

- Major IR peak at 3398 cm-1 attributed to NH of amide.
- Major IR peak at 1637cm-1 attributed to $CONH_2$ of amide.
- Major IR peak at 1409 cm-1 indicating CH_2 bending.
- Major IR peak at 1077 cm-1 indicating out of plane vibration of CH_2 group.



- Fig. 2. IR absorption spectrum of purified alkaloid

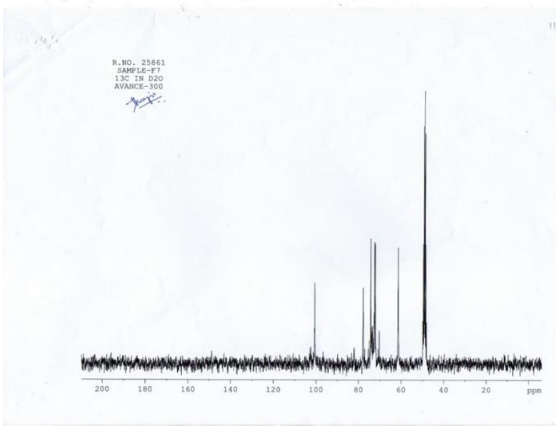


Fig. 3(a). ^{13}C NMR spectrum of purified alkaloid

- ^{13}C NMR spectrum of purified alkaloid recorded from 10-200 ppm

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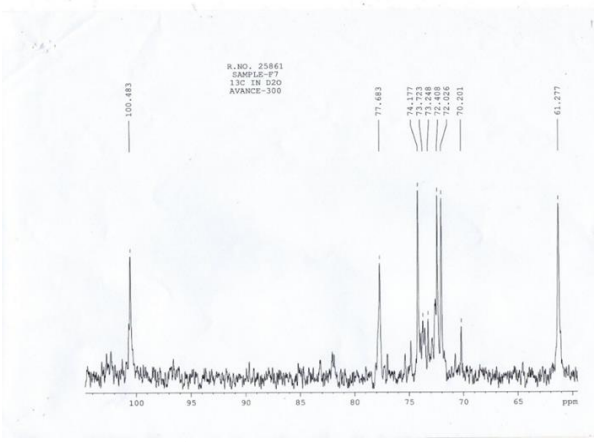

Fig. 3(b). ^{13}C NMR spectrum of purified alkaloid

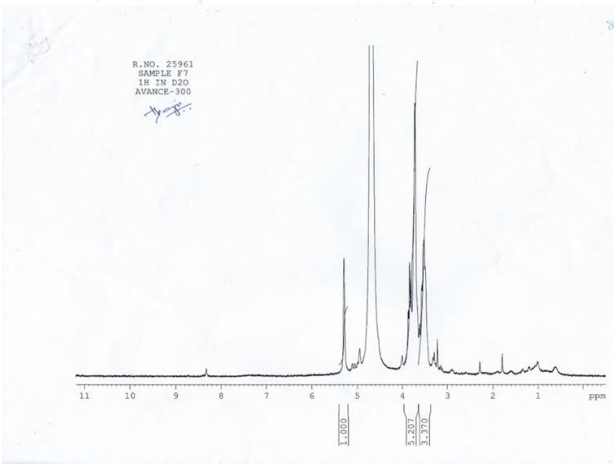

Fig. 3(c). ^{13}C NMR spectrum of purified alkaloid

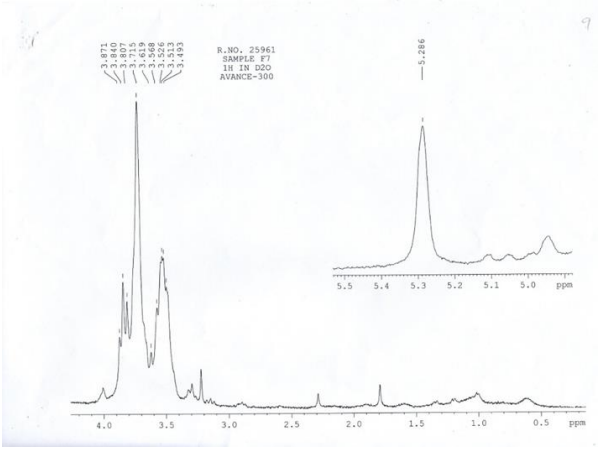

Fig. 3(d). ^{13}C NMR spectrum of purified alkaloid

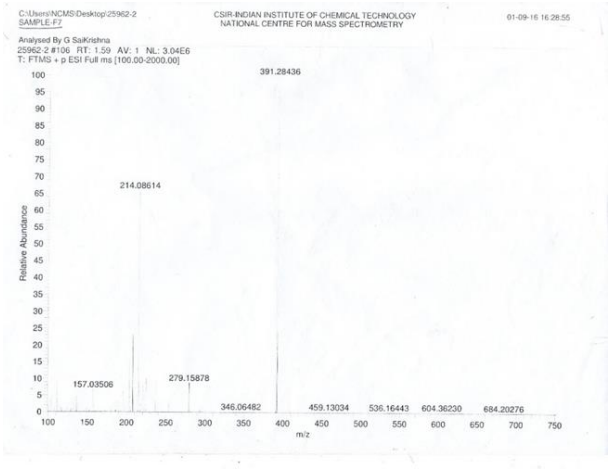

Fig. 4. ESI-MS spectrum of purified alkaloid

- ESI-MS of purified compound was acquired in the positive ion mode.
- ESI-MS coupled with mass analyzer.
- Fourier transform mass spectrum (FTMS) ranging from 100-750 m/z.
- Major peak with m/z was observed.

E. DPPH radical scavenging activity

The DPPH radical scavenging activity of PPDH at 2,4,6,8 and 10µg/mL was found to be 20.83±0.34, 42.66±0.36, 52.55 ±0.42, 63.45±0.37 and 85.43±0.38; ascorbic acid was 24.64±0.24, 46.32±0.42, 56.22±0.31, 68.54±0.34 and 89.45±0.42 respectively. IC_{50} of PPDH was 5.76 µg/mL and ascorbic acid was 4.3 µg/mL indicating potential radical scavenging activity. The capacity of PPDH to scavenge DPPH gives the antioxidant potential of PPDH. Dose dependent increase in antioxidant activity is observed compared to ascorbic acid (Fig 5).

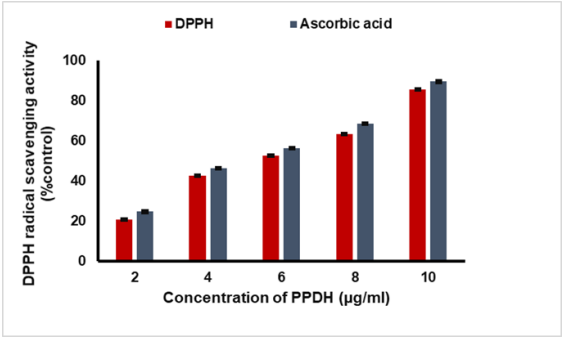

Fig. 5. DPPH radical scavenging activity

- DPPH without PPDH served as control
- DPPH radical scavenging activity of PPDH at concentration ranging from 2-10µg/ml
- The results were expressed in percent control.
- Ascorbic acid was used as positive control.

F. H_2O_2 scavenging activity of PPDH

The reducing capability of a compound from Fe_3+/ [Fe (CN) 6] 3− complex to the ferrous shape can also function as a giant indicator of its antioxidant capacity. The present result suggested that the compound PPDH isolated from *actinomycetes* could be useful to reduce to oxidative damage of cells induced by oxygen radicals, and may be utilized as probable antioxidant. PPDH was resolved and compared to the standard antioxidant, ascorbic acid (Fig 6). The results show that the activity of PPDH was 16.96±0.31, 34.64±0.23, 45.75±0.32, 56.23±0.46 and 72.14±0.36%, respectively and ascorbic acid was 21.66±0.51, 38.46±0.32, 55.45±0.51, 62.32±0.44 and 76.41±0.33%, respectively at 2,4,6,8 and 10µg/ mL, indicating that the PPDH show hydrogen peroxide scavenging activity similar to ascorbic acid.

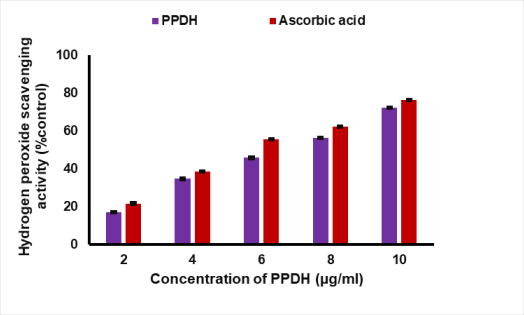

Fig. 6. Hydrogen peroxide scavenging activity of PPDH

- Hydrogen peroxide scavenging activity of PPDH at concentration ranging from 2-10µg/ml.
- The results were expressed in percent control.

G. NO radical scavenging activity of PPDH

At physiological pH, nitric oxides generate from Na_2[Fe $(CN)_5NO$] in aqueous solutions, which interact with O_2 and produce NO_3-. The isolated PPDH compound decrease nitrite generation by scavenging nitric oxide radical. The consequences of the existing study, reveals that the PPDH exhibited nitric oxide scavenging of 15.45±0.32, 25.33±0.12, 32.22±0.21, 45.12±0.24 and 68.45±0.42% respectively at 2,4,6,8 and 10µg/mL, which was lower than that of ascorbic acid as 22.42±39, 31.52±42, 41.32±44, 52.54±37 and 71.54±47 (Fig 7).

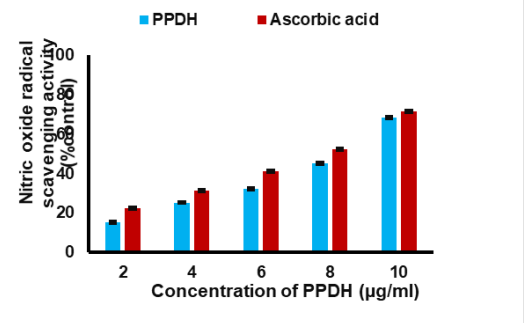

Fig. 7. Nitric oxide radical scavenging activity

- Nitric oxide radical scavenging activity of PPDH at concentration ranging from 2-10µg/ml
- The results were expressed in percent control.

H. Hydroxyl radical scavenging activity of PPDH

The •OH is highly sensitive radical released by various living structures and implicated in free radical mediated pathology. They are proficient for each molecule found in living cells. They mainly interact with nucleotides of DNA, causes strand breakage and accord to carcinogenesis [20]. The •OH scavenging action of PPDH was related to antioxidant ability. The results depicted as PPDH 19.20±38, 28.32±36, 41.46±39, 55.48±40, 81.56±42 and ascorbic acid as 21.44±34, 32.21±36, 48.46±39, 62.42±42 and 82.84±44 (Fig 8).

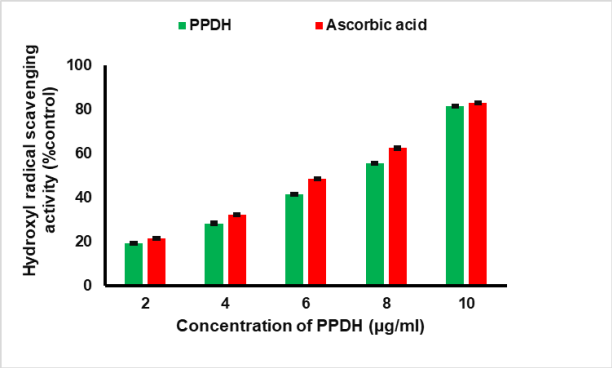

Fig. 8. Hydroxyl radical scavenging activity of PPDH

- Hydrogen radical scavenging activity of PPDH at concentration ranging from 2-10µg/ml
- The results were expressed in percent control.

I. Super oxide radical scavenging activity of PPDH

The O_2- ability of PPDH was resolved and associated to the standard antioxidant ascorbic acid (Fig 9). The results show that the hydrogen peroxide scavenging activity of PPDH was 17.82±0.31, 32.62±0.22, 41.72±0.31, 54.21±0.41 and 70.12±0.34% respectively and ascorbic acid was 21.68±0.53, 39.44±0.31, 52.42±0.49, 59.31±0.41 and 75.39±0.29% respectively at 2,4,6,8 and 10µg/ mL. The IC_{50} value of PPDH and ascorbic acid was 7.4 µg/ mL and 5.7 µg/ mL respectively. These outcomes designate that the PPDH was capable of super oxide scavenging activity in a concentration dependent manner.

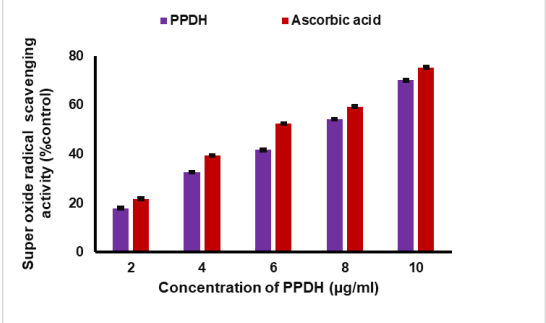

Fig. 9. Super oxide radical scavenging activity of PPDH

- Super oxide radical scavenging activity of PPDH at concentration ranging from 2-10µg/ml
- The results were expressed in percent control.

J. Total antioxidant activity

Total antioxidant activity of the PPDH at 2, 4, 6, 8 and 10 µg/mL was 0.60±0.04, 1.01±0.02, 1.60±0.01, 2.06±0.02 and 2.62±0.01 AEAS in µg/L. However total antioxidant activity of ascorbic acid was 0.80±0.02, 1.20±0.02, 1.80±0.03, 2.40±0.01 and 2.81±0.02 µg/mL (Fig 10, 11). The total reducing activity of PPDH was 48.80±0.03, 52.20±0.02,68.62±0.01,85.20±0.02 and 89.80±0.03 The results show that total antioxidant activity of PPDH was improved in concentration dependent manner, similar to ascorbic acid in terms of total antioxidant and reducing capacity. The production of secondary metabolites by marine bacteria are economically feasible. The marine bacteria produce long-lasting and some potential antioxidant. The optimized media for serious biomass can expand the generation of the sought metabolite. The present study shows that ascorbic acid at 2, 4, 6, 8 and 10 µg/mL and antioxidant activity of PPDH. Previously it was reported that bioactive compound on free radicals was comparable to that of hydrogen donors [21].

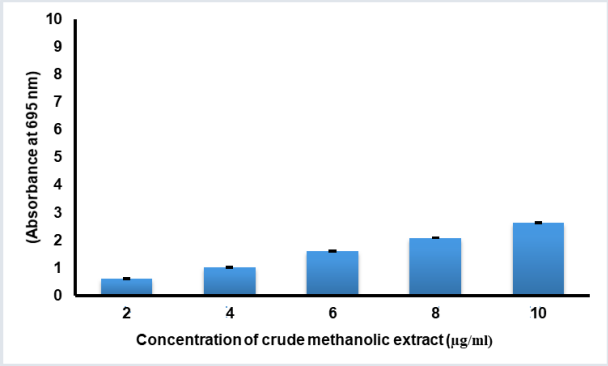

Fig. 10. Total antioxidant activity of PPDH

- The total antioxidant activity of PPDH is increased.
- The total antioxidant activity of PPDH expressed in ascorbic acid equivalent of µg/mL.
- The absorbance was measured at 695 nm.

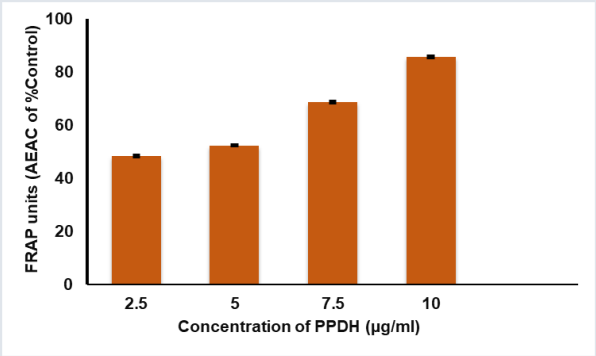

Fig. 11. Total reducing activity of PPDH

- Total antioxidant activity of at concentration ranging from 2.5-10µg/ml
- Total antioxidant activity expressed in FRAP units of antioxidant activity in ascorbic acid equivalents (AEAC)
- The AEAC units of PPDH were expressed in percent control

K. Cytotoxic and antiproliferative activity of PPDH

The PPDH (2-10 µg/mL) from isolated *actinomycetes* exhibited significantly high cytotoxic activity against MDA-MB 231 cell. Further, in protein based Sulforhodamine B assay, the cytotoxic activity of PPDH was increased and percent of survival was decreased with increasing concentration. The IC_{50} value of standard anticancer drug, paclitaxel exhibited IC_{50} value 6.2 and 5.5ng/mL as assessed by MTT and SRB methods, respectively against triple negative breast cancer cells MDA-MB 231. However, the IC_{50} value of PPDH was 6.2µg/mL ($p<0.05$) in MTT assay and 5.82µg/mL in SRB assay. Propidium iodide (PI) staining is widely used to indicate dead cells in either eukaryotes or prokaryotes. In the current study, propidium iodide staining was employed to evaluate the consequence of PPDH on induction of bacterial cell death. The results indicate that the propidium iodide positive *S. aureus* cells with PPDH and streptomycin was 82 and 86%, respectively whereas untreated was 16%. Further, to determine the effect of PPDH on DNA fragmentation in S. aureus, TUNEL assay was performed (Fig 12, 13,14 and 15). The results show that the TUNEL positive *S. aureus* cells with PPDH and streptomycin was 79 and 81%, respectively however, the tunnel positive cells in untreated control was 11%. To study the effect of PPDH on cytoplasmic membrane damage, potassium leakage was used as an indicative of membrane damage that potassium leakage with PPDH was 445 ppm and streptomycin was 48.2ppm. The BrdU assay is a reliable in vitro non-radioactive method, which is very oftenly, used to quantify cell proliferation [22]. BrdU incorporation into DNA was measured in MDA-MB 231 cells treated with extract for 48 h exposure. As seen in Figure the crude methanolic extract decreased BrdU incorporation in concentration reliant way, when compared to untreated cells with IC_{50}value 58.9µg/mL.

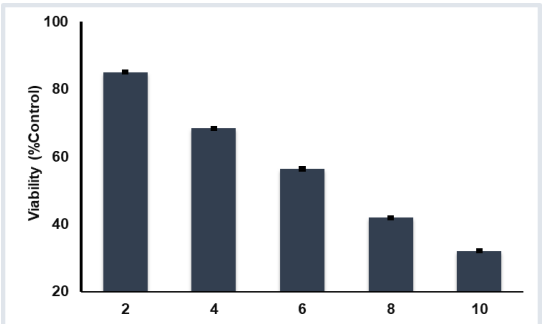

Fig. 12. Cytotoxic effect of concentration of amide alkaloid (µg/mL) of PPDH on viability of MDA MB-231 breast cancer cells

- The viability of MDA-MB-231 at concentration ranging from 2-10µg/ml as determined by MTT assay.
- The viability was expressed in percent control.

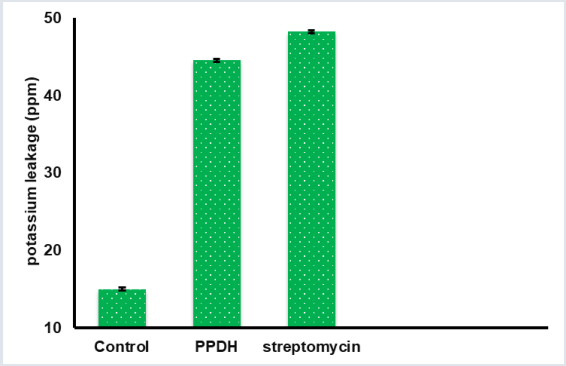

Fig. 13. Cell death by propidium iodide

- Results indicate that the propidium iodide positive *S. aureus* cells with PPDH and streptomycin
- Potassium leakage (ppm).

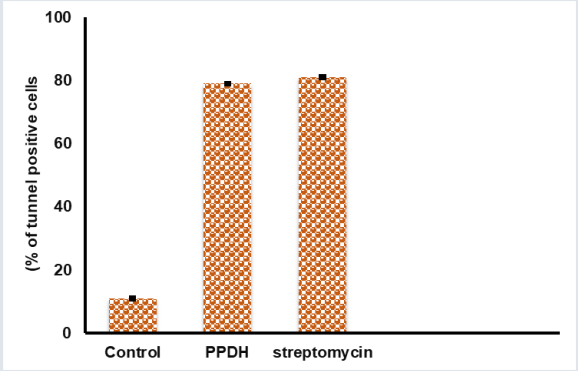

Fig. 14. Tunnel assay for PPDH

- Results show that the tunnel positive for *S. aureus* cells with PPDH

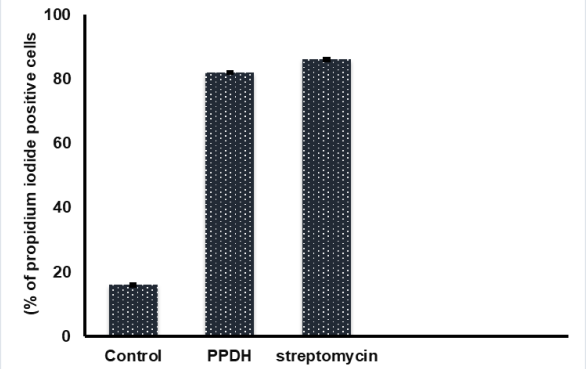

Fig. 15. Effect of PPDH on membrane damage of *S.aureus*

- % of propidium iodide positive cells.

4. Conclusion

In the current study, methanolic extract was imperiled to silica gel column chromatography for additional purification of alkaloids. Based on spectral studies the structure of purified compound was proposed as hexa hydro pyrrole [1,2α] pyrazine-1,4-dione ($C_7H_{10}N_2O_2$). It exhibited significant antioxidant activity which is comparable to ascorbic acid. PPDH antiproliferative activity against MDA-MB-231 a triple negative breast cancer cell line. Thus, PPDH can be further explored for its anticancer properties.

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