



Cytotoxicity and anti-cancer activity of ancistrocline: A naphthyl iso-quinoline alkaloid extracted from the stem of *Ancistrocladus heyneanus*

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ABSTRACT

Mixture of Naphthyl Isoquinoline alkaloids (NIQAs) were extracted in methanol from the stem of *Ancistrocladus heyneanus*. We report our initial attempt to study the cytotoxicity and anti-cancer activity of these NIQAs using Vero cell lines and HeLa cell lines. NIQAs were found to be non toxic to Vero cells (i.e. normal cell lines), as percentage viability was found to be more than 50% at all tried concentrations of Ancistrocline. Whereas, the percentage viability of HeLa cells at all concentrations of alkaloids extract was less than the Vero cells. Moreover, with increase in concentration of Alkaloids there was a decrease in viability of HeLa cells suggesting that NIQAs extracted from the only Indian species of *Ancistrocladus heyneanus*, has anti cancer property. Hence, NIQAs were separated using TLC and one of the TLC band was found to be consisting of molecular weight 421 by LC/MS, which was further identified as Ancistrocline by NMR. In previous studies, Ancistrocline is proven to have anticancer activity. So, we can infer that anticancer activity was due to presence of Ancistrocline as one of the alkaloid.

Keywords: *Ancistrocladus heyneanus*, Ancistrocline, Vero Cells, HeLa Cells, Anti-cancer, Cytotoxicity

INTRODUCTION

Ancistrocladus heyneanus is a liana known to contain Naphthyl Isoquinoline alkaloids (NIQAs). The structurally unique NIQAs are characterized by a biaryl system consisting of naphthalene and an iso-quinoline moiety. Many of these alkaloids display atropisomerism; since biaryl axis usually is rotationally hindered due to the presence of bulky ortho-substituents. These natural biaryl alkaloids were discovered in *Ancistrocladus* by Govindachary and Parthasarathy (1970) [1]. NIQAs, at their biaryl axis connecting naphthalene portion with iso-quinoline show restricted rotation leading to stable atropisomers (rotational isomers). Bringmann and Pokorny (1995) have shown its unusual molecular structure of achiral biaryl axis is produced by an unparalleled biosynthesis of iso-quinoline alkaloids from acetic acid units [2]. The structurally unique NIQAs produced by *Ancistrocladaceae* are different from all other 2500 iso-quinoline alkaloids isolated from plants. This is because of their unusual structure, mostly rotationally hindered biaryl axis between the two molecular parts and also because of their unprecedented biosynthetic origin.

Most of the species of this genus *Ancistrocladus* are found in Africa, where it is used in tribal medicine for treating Chagas disease, African sleeping sickness & Leishmaniasis (Bringmann et al 2004) [3]. There have been reports of NIQAs from *Ancistrocladus* having anti-HIV [4] and antimalarial property also [5].

In the quest of possibility of having any anti-cancer alkaloid in *Ancistrocladus* have found that Ancistrocline extracted from *A. tectorius* (an African species) exhibited anticancer activity [6]. From India only one species of *Ancistrocladus* i.e. *Ancistrocladus heyneanus* has been recorded. Therefore, interest was aroused to see whether NIQAs present in the stem of this Indian species has anti-cancer property or not. Our NMR analysis results have revealed that the one

of the isolated NIQAs from *Ancistrocladus heyneanus* is Ancistroline that has been reported to have anticancer activity. Hence, anticancer activity observed in the stem extract of NIQA is possibly due to the presence of Ancistroline as one of the alkaloid.

However, prior to testing anti-cancer activity of any molecule it is imperative that its cytotoxicity should be assayed. Hence, we have studied both cytotoxicity and anti-cancer activity of mixture of NIQAs from stem extract.

MATERIALS AND METHODS

2.1. Extraction of NIQAs

500 ml Dichloromethane (Merck, USA) was added to 500g dried stem powder and refluxed using Soxhlet apparatus for 6 hr. Remaining Dichloromethane was discarded (this step was repeated once more) and the residual powder was dissolved in 250ml Dichloromethane and 250ml Methanol (Emplura, USA). This extract was concentrated to dryness under reduced pressure to get crude extract. Crude extract was mixed with 90% Methanol + n-hexane (Emplura, USA) and shaken thoroughly. This resulted into two layers of Methanol and Hexane. Hexane layer was discarded and the Methanol layer that contained alkaloids was used for testing cytotoxicity and anticancer activity. Methanol layer was also used for separation of alkaloids from mixture of NIQAs by TLC.

2.2. Cell Lines used for Cytotoxicity and Anticancer activity

The cell lines used for studying anti-cancer activity and cytotoxicity of NIQAs extracted in methanol from stem of *A. heyneanus* were:

- i. The Vero cell lines (normal cell lines)
- ii. HeLa cell lines (Cancer cell line)

2.3 Cytotoxicity and Antitumor Activity Assay

Cytotoxicity is done to rapidly assess whether the compound is harmful for normal cell line or not, by studying the impact of compound in question on cellular damage. The procedure followed in this paper is to measure metabolic function of the cellular ATP levels or mitochondrial activity by a yellow tetrazolium dye MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. Mitochondria of dead cells cannot metabolize dyes. The enzymes (succinate dehydrogenase) in the mitochondria of the living cells reduce MTT to formazan dyes, giving a purple color. Thus it can also be used to determine cytotoxicity of potential medicinal agents and toxic materials.

Both the Cell lines were separately trypsinized and re-suspended in Minimum essential medium (MEM) without phenol red. Cells were then seeded into 96 tissue culture well plate. Cell concentration was adjusted to 5×10^5 cells/ml and incubated overnight at 37°C in humidified incubator having 5% CO₂ for 24h. After incubation, culture medium was replaced with test solutions (1×10^4 , 3×10^4 , 5×10^4 , 8×10^4 , 1×10^5 ppm of NIQAs in methanol) and incubated further for 48 h at 37°C in CO₂ incubator. These solutions were later replaced with MTT (200 µg/ml) and cells were incubated for 2.5 h at 28±2°C in dark to initiate formation of formazan. Medium was then replaced with 150µl of DMSO (Sigma, USA). This complex was mixed gently for 15 minutes to dissolve formazan crystals. Finally, the dissolved formazan in DMSO was transferred to fresh 96 well plates and read on microplate reader (Thermo, USA) at 570nm. A blank containing all other solutions except test solutions and control containing cells and DMSO was also added. Based on the absorbance obtained at 570nm, % cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = 100 - \% \text{ cell survival}$$

$$\% \text{ Cell survival} = \frac{AT - AB}{AC - AB} \times 100$$

Where, AT – Sample reading
 AB – Blank cells (cells + DMSO)
 AC – cell control (only cells without any test compound)

2.4. Separation of alkaloid extracted in Methanol by TLC

Methanol layer of above mentioned extract was used for separation of alkaloids by TLC. Mobile phase used for TLC was Methanol: Ethyl acetate: 17% Ammonia (1:8:1). The TLC developing chamber was saturated with the mobile phase solvent and sealed with High Vacuum Silica grease. Methanol extract was loaded onto the readymade TLC sheets (Silica 60F254, MERCK) of dimensions 20x20 cm and with the help of a micropipette single long band of Methanol extract was loaded. The plate was then dried and placed in the saturated chamber of TLC to run the chromatogram. After the solvent reached to desired front, the plate was removed and air dried again. Dragendorff

reagent was sprayed to find out location of separated alkaloid bands and Retardation factor (Rf) values were calculated.

2.5. Elution of alkaloid from each band obtained by TLC

It was done separately in Methanol. Elute was dried at 50⁰C in an oven to remove the solvent.

2.6. Liquid Chromatography & Mass Spectrometry (LC-MS) analysis

The LC-MS analysis was performed using Shimadzu LC 2010 with mass detector API-2000, HPLC grade water (Make-Merck) and HPLC grade acetonitrile (Make-Merck) were mixed in the ratio of (1:1) and degassed by vacuum degasser. This degassed mixture of water and Acetonitrile was used as the mobile phase. Quaternary gradient pump was used to carry the mobile phase from the mobile phase reservoir to the detector via injector port and HPLC column flow rate (mobile phase) was set as 1.0 ml/minute.

HPLC column used was Zorbax SBC18, where SB stands for stable bond and C18, stands for Octadecyl carbon. The dimension of HPLC column was 250mm×4.6mm, 5µm i.e., the length of the column 250mm, internal diameter of the column was 4.6mm and the particle size of the Silica material was 5µm. The temperature of HPLC column was 25°C. Injection volume of the dissolved sample was set to 100µl to inject the Alkaloid sample from sample vial.

Prior to use HPLC column was washed with a mixture of water and acetonitrile (80:20) for 30 minutes followed by another wash using a mixture of water and acetonitrile (30:70) for another 30 minutes. HPLC column was saturated with mobile phase (50:50, water and acetonitrile) for one hour. After that the stable base line alkaloid samples were injected in the chromatograph Mass detector used was API-2000. Mass spectra obtained were analyzed.

2.7. NMR Analysis of alkaloids

BRUKER NMR model No.400MHz was used for the analysis of NIQ alkaloids. NMR tube was dried and placed in a 10 mL graduated cylinder. To remove any insoluble impurity from the alkaloid sample, a small piece of a Kimwipe was stuffed into a small Pasteur pipette which was placed on top of the NMR tube. To stuff the Kimwipe properly it was pushed in using a larger pipette. 5 mg of dried sample was dissolved in 0.5mL pure dry deuterated chloroform and was carefully transferred to the NMR tube (using a pipette) through the Kimwipe filter. Then NMR tube was capped and NMR analysis was accomplished.

RESULTS AND DISCUSSIONS

As mentioned above crude extract was mixed with 90% methanol + n-hexane and shaken thoroughly. This resulted into formation of two layers i.e. of Methanol (that contained NIQAs) and n-hexane (that contained fats and lipids) was discarded. Only methanol extract containing mixture of alkaloid was used for cytotoxicity assay.

3.1. The MTT Cell Proliferation and Viability Assay

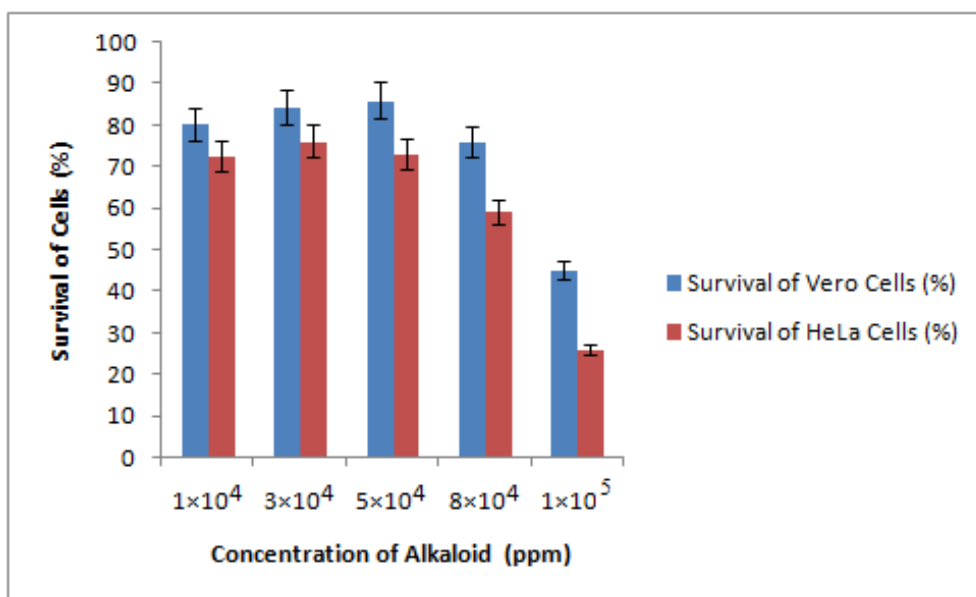
Methanol layer was envisaged for Cytotoxicity and anticancer activity assays. As mentioned above the succinate dehydrogenase reduced yellow MTT to insoluble purple formazan dye crystals, which was dissolved in DMSO and the absorbance was read at 570nm directly in the wells. The data was analyzed by plotting cell number versus absorbance, allowing quantisation of changes in cell proliferation. The rate of tetrazolium reduction is proportional to the rate of cell proliferation.

During assay, various concentrations of Alkaloid were added to estimate its cytotoxicity to normal and cancer cells. Comparatively less work has been done on the anti-tumor activity of NIQAs from *Ancistrocladus*. Previously, Betulinic acid (a naturally occurring pentacyclic triterpenoid) that has been isolated by Bringmann *et al* (1997) from *A. heyneanus*, is known to have antiretroviral, antimalarial, and anti-inflammatory properties, has recently been discovered potential as an anticancer agent, by inhibition of topoisomerase [7, 8]. In 2008, Bringmann *et al* have isolated 7 natural naphthoquinones from **callus culture** of *A. abbreviatus* and some of them strongly induced apoptosis in human tumor cells derived from two different B cell malignancies, B cell lymphoma and multiple myeloma, without any significant toxicity towards normal peripheral mononuclear blood cells [9]. So far direct proof of any NIQAs having anti-cancer property has not been published.

Table 1: Effect of NIQAs from stem extract of *A. heyneanus* on the viability of Vero cell lines and HeLa cell lines

Alkaloid Concentration (ppm)	Survival of Vero Cells (%)	Survival of HeLa Cells (%)
1 X 10 ⁴	80.25	72.72
3 X 10 ⁴	84.22	76.22
5 X 10 ⁴	85.95	72.95
8 X 10 ⁴	76.09	59.09
1 X 10 ⁵	45.13	26.13

In our work, the NIQs extracted from stem extract of *A. heyneanus* was found to be non toxic to Vero cells, as percentage viability was higher than HeLa cells at all concentrations of alkaloid extract. Fig 1 represent that as concentration of NIQAs increases, percentage viability of HeLa cells decreases as compared to Vero cells. The IC₅₀ value of Vero cells and HeLa cells can approximately be stated as 10x10⁴ ppm and 8x10⁴ ppm respectively. It can be clearly observed that IC₅₀ value of Vero cells is more than that of HeLa cells. This means lower concentration of NIQAs is enough to kill HeLa cells. Hence, it can be interpreted that Alkaloid (Ancistrocline) is cytotoxic to HeLa cells and comparatively biocompatible to Vero cells.

**Fig 1:** Histogram depicting impact of NIQAs extract from stem of *A. heyneanus* on cell growth of Vero and HeLa cell lines

3.2. Separation of alkaloids from mixture of NIQAs by TLC

TLC of alkaloids after spraying with Dragendorff reagent exhibited three orange colored spots, that is indicator of alkaloids (Fig 2). The R_f values of these three alkaloid bands were found to be 0.86, 0.71 and 0.64. This is the first report where R_f values to separated NIQAs are assigned. Since there is no information available about R_f values yet, separated bands were subjected to LC/MS and NMR for molecular weight and structure identification of alkaloid respectively. LC/MS and NMR analysis of only alkaloid – 2, is presented in this paper (Fig 3 and 4 and in Table 1); because that was found to be anticancer alkaloid Ancistrocline [6].

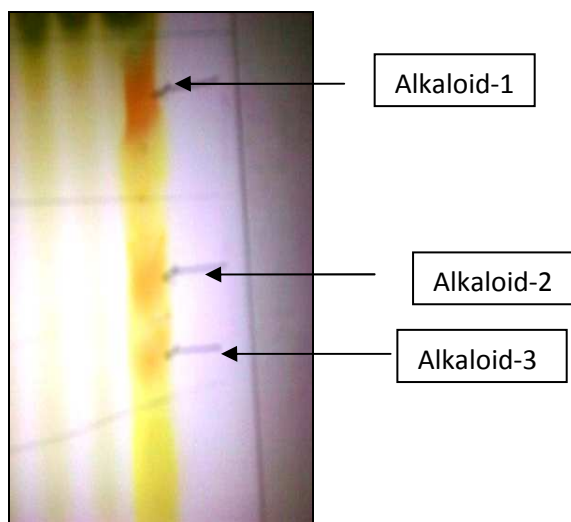
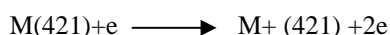


Fig 2: TLC of leaf extract of *A. heyneanus* carried out using Methanol: Ethyl Acetate: Ammonia solution (1:8:1) as mobile phase, showing three orange colored spots of alkaloids when sprayed with Dragendorff's reagent

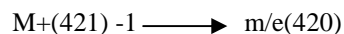
3.3. Mass Spectrum of Alkaloid separated as Band 2 by TLC

In the mass spectrum of alkaloid -2 from band-2 (Fig 3) the m/e value of the molecular ion peak is 421. Hence, we can say that the molecular mass of alkaloid from band-2 is 421. Also there are some peaks in the mass spectrum indicating that the molecule undergoes fragmentation. The m/e values of the peaks are 420, 407 and 406. The m/e values of the fragmented peaks may be explained as below:-

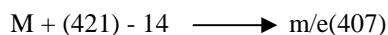
The peak of m/e value 421 is formed by bombardment of electron and formation of (M^+) molecular ion. The abundance of this peak is 3.5%.



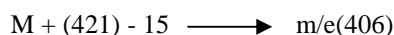
The peak of m/e value 420 is formed by the elimination of hydrogen ($H\cdot$) radical ($M^+ - H$) from molecular ion m/e 421 and the abundance of this peak is 3.1%.



The peak of m/e value 407 is formed by the elimination of $-CH_2$ radical ($M^+ - CH_2$) from molecular ion m/e 421 and the relative abundance of this peak is 39%.



The peak of m/e value 406 is formed by the elimination of $-CH_3$ radical ($M^+ - CH_3$) from molecular ion m/e (421) and the abundance of this peak is 100%.

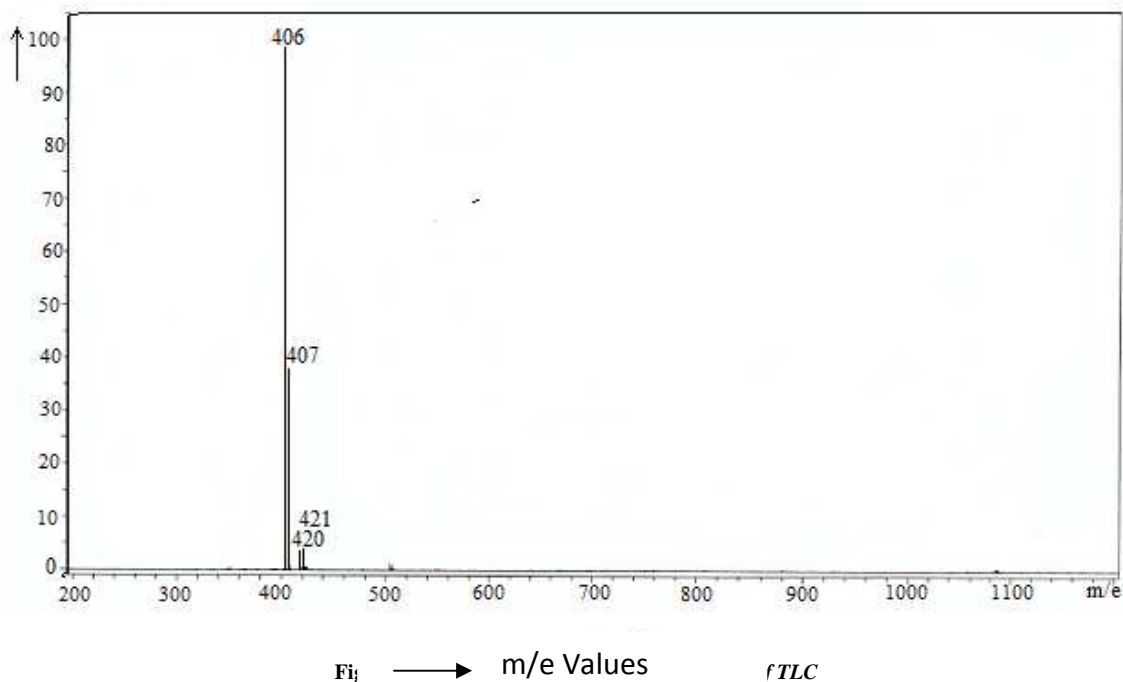


According to Bringmann and Kinzinger (1992) [6] the mass to charge value (m/e) of the molecular peak of extracts taken from the *Ancistrocladus tectorius* was 421 (M^+) and the abundance of this peak was 2.3%;



Moreover, these peaks of m/e value 420, 407 and 406 obtained by them also matches with results obtained (Fig 3) as explained above. In conclusion the tested compound molecular weight matches with ancistroline i.e. 421.52864

Relative Abundance



After confirming that the m/e value of the molecular ion peak is 421, that matches with the Ancistrocline (Bringmann and Kinzinger 1992) [6]; the same TLC band was further characterized by NMR to know its molecular structure.

3.4. NMR analysis of Alkaloid of TLC band 2 (R_f - 0.71)

From the ^1H NMR (Fig 4) it is observed that there are five aryl protons, methoxy groups (δ 4.60), an arylmethyl group (δ 2.29) and doublet for H-4 proton. This arrangement of groups is similar to those observed for monomeric naphthyl-isoquinoline.

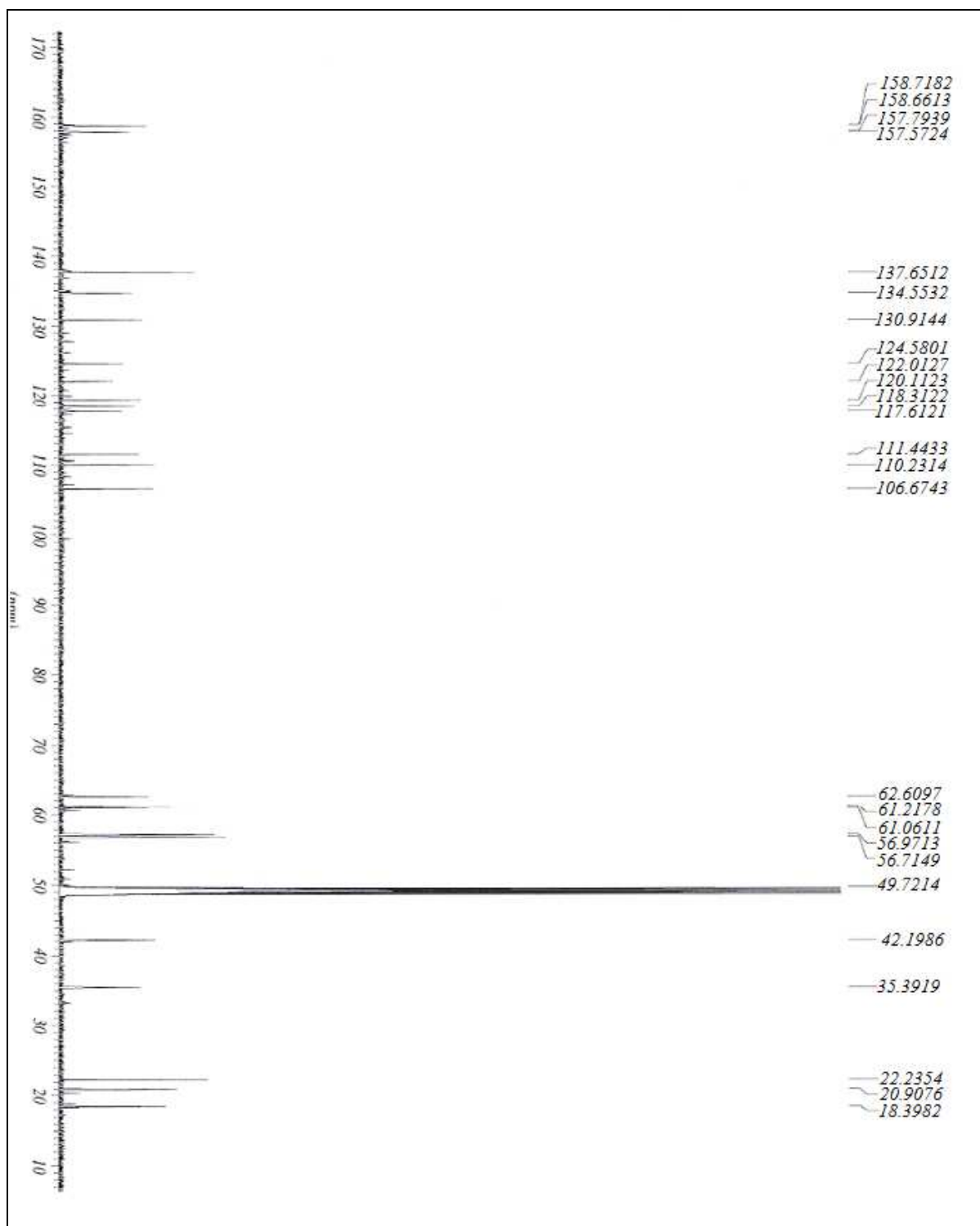


Fig 4: ^{13}C NMR spectrum of Alkaloid from Band 2

The ^{13}C NMR spectrum indicates (Fig 5) presence of 3 methyl groups distinguished individually, later shown peak intensity for two methyl groups at $\delta 20.9$ and $\delta 18.2$ shows one Methylene and five methine resonance quaternary carbons. The correlation from $\delta 3.32$ (H-4ax) and $\delta 3.14$ (H-4eq) to $\delta 120.1$ (C-5) and $\delta 157.7$ (C-8a) and $\delta 122.0$ (C-7') to $\delta 106.6$ (C-7) establishes that C-8' of naphthalene ring is attached to C-5 in the iso-quinoline ring. Quaternary carbons at $\delta 157.7$ in place of sp^3 methine carbon also support the analysis.

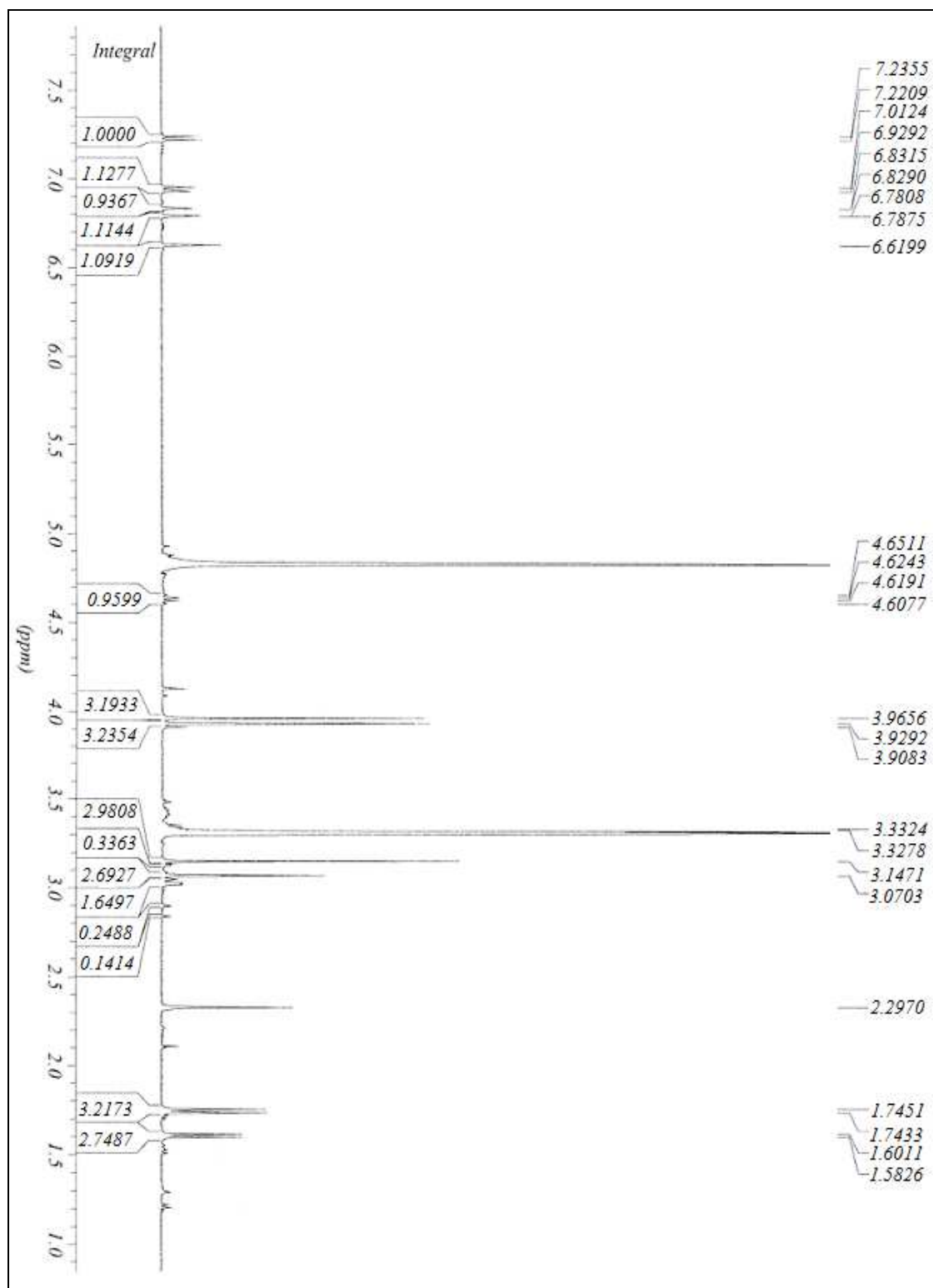
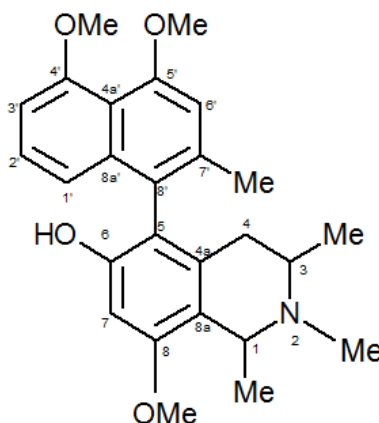


Fig 5: ¹³C NMR spectrum of alkaloid from Band 2

Table 1: Compiled data of ^1H and ^{13}C observations obtained for alkaloid-2

Position	^1H		^{13}C	
1,3	1.74(m)	Alkyl proton	49.7	Methylidene carbon
4ax	3.32(dd)	Alkyl proton	35.3	Methylene carbon
4eq	3.14(dd)	Alkyl proton		Methylene carbon
4a	--		130.9	Methine carbon
5	--		120.1	Methine carbon
6	--		111.4	Methine carbon
7	6.61(s)	Aryl proton	106.6	Methylidene carbon
8	--		157.5	Methine carbon
8a	--		157.7	Methine carbon
1'	6.82(d)	Aryl proton	118.3	Methylidene carbon
2'	6.92(q)	Aryl proton	117.6	Methylidene carbon
3'	7.22(d)	Aryl proton	110.2	Methylidene carbon
4'	--		158.7	Methine carbon
4a'	--		134.5	Methine carbon
5'	--		158.6	Methine carbon
6'	6.78(s)	Aryl proton	106.6	Methylidene carbon
7'	--		122.0	Methine carbon
8'	--		124.5	Methine carbon
8a'	--		137.6	Methine carbon
C1-CH ₃ , C3-CH ₃	1.58(dd)	Alkyl proton	20.9	Methyl carbon
C2-NCH ₃	3.07(s)	Alkyl amine	42.1	Methyl amine carbon
C8-OCH ₃	3.96(s)	Alkyl oxy	61.0	Methoxy carbon
C4'-OCH ₃ , C4'-OCH ₃	4.60(s)	Alkyl oxy	61.2	Methoxy carbon
C7-CH ₃	2.29(s)	Alkyl proton	22.2	Methyl carbon

The NMR results revealed that the second alkaloid band is of Ancistrocline which must have caused anticancer activity. Structure of the compound on the basis of data presented above would be as shown in Fig 6. The results match with the molecular formula of Ancistrocline. It is also known as 5-(4,5-dimethoxy-2-methylnaphthalen-1-yl)-8-methoxy-1,2,3-trimethyl-3,4-dihydro-1H-isoquinolin-6-ol.

**Fig 6: Molecular structure of Ancistrocline**

CONCLUSION

The methanol extract of NIQAs from stem of *Ancistrocladus heyneanus* showed anticancer activity and it is probably due to occurrence of Ancistrocline as one of the alkaloid. NIQAs were separated using TLC. Molecular weight and structural identification were analyzed using LC/MS and NMR. The molecular weight of the second separated band from TLC was found to be 421 by LC/MS and structure was identified as Ancistrocline by NMR analysis. As far as data of R_f value in TLC is concerned, we are the first one to report R_f value of Ancistrocline which was depicted as 0.71. Hence, Ancistrocline extracted from stem of Indian variety *Ancistrocladus heyneanus* is potent anticancer agent which can be used for cancer treatment with minimum cytotoxicity to normal cells.

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