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Ancistrocline: A Naphthyl Isoquinoline Alkaloid from the Leaves of Ancistrocladus Heyneanus: Exhibits Anticancer Property Against Plc/Prf/5 Hepatoma Cells

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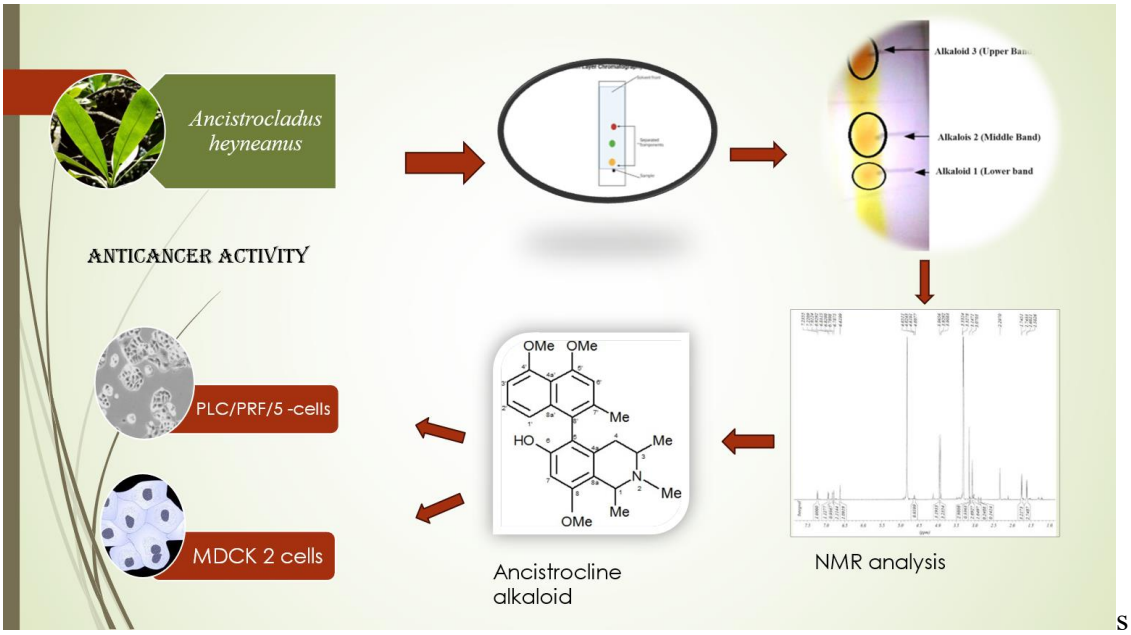
ABSTRACT

From the leaves of an Indian species of *Ancistrocladus heyneanus*, crude extract of mixed Naphthyl Isoquinoline alkaloids were tested against cytotoxicity and anti-cancer activity using MDCK 2 -cells and PLC/PRF/5 -cells. It showed >50% viability of MDCK 2 -cells, at all tried concentrations, showing it to be non-toxic to normal cells. Whereas, response to the viability of PLC/PRF/5 -cells at all concentrations of alkaloid exhibited a decrease in viability with increase in concentration, suggesting anti-cancer property of alkaloids. Since extract from leaves was a mixture of alkaloids, it was thought desirable to separate the alkaloids to see which alkaloid had anti-cancer property. We report anti-cancer property of separated Ancistrocline alkaloid. The alkaloids were separated using TLC, which showed 3-bands. Only middle band of the TLC bands, that has a molecular weight of 421 (as detected by LC/MS) and identified as Ancistrocline by NMR, has exhibited anti-cancer activity against PLC/PRF/5 -cells and was non-cytotoxic against MDCK 2 -cells.

KEYWORDS: *Ancistrocladus heyneanus*, Naphthyl Isoquinoline Alkaloids, Ancistrocline, MDCK 2 -cells, PLC/PRF/5 -cells, Anti-cancer.



Graphical Abstract

INTRODUCTION

The plant of concern for this study is *Ancistrocladus heyneanus*, the only species of *Ancistrocladus* found in India, which is endemic to the Western Ghats. Ancistrocladus is a genus of lianas (a woody vine that uses trees and other vertical support) restricted to Africa (15 species) and South East Asia (5 species). Ancistrocladus is morphologically notable for its climbing habit which is assisted by hooks formed from modified stem; these hooks are one of the identifying characters of this genus (Figure 1). Extensive work has been done mostly on the species of *Ancistrocladus* found mostly in Africa.

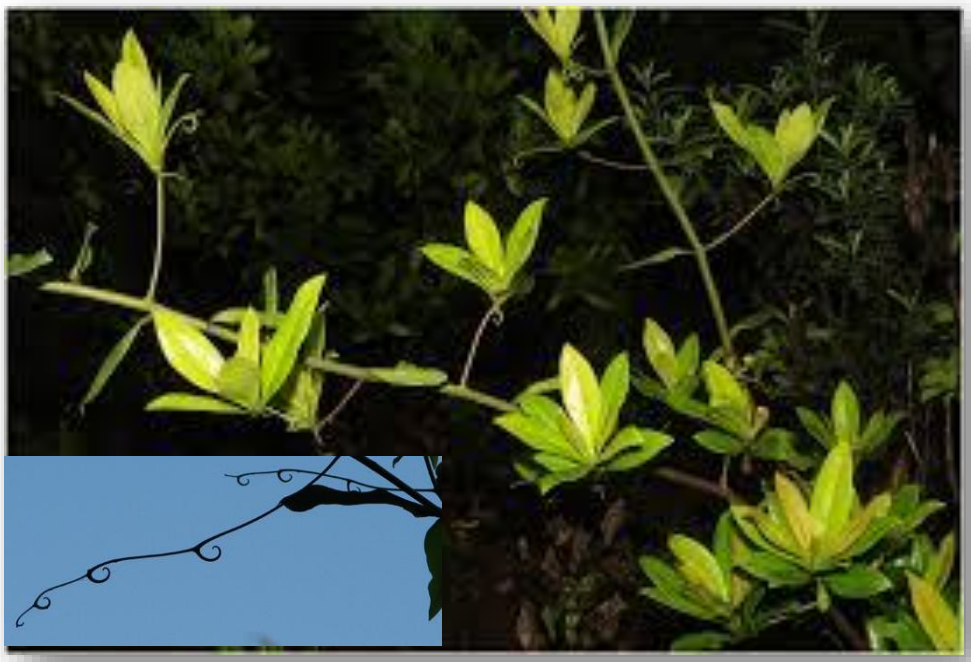
Authentic identification of this plant was done by Botanical Survey of India, Govt. of India, Western Circle, Koregaon Road, Pune-411011. Leaves used for extraction and analysis of alkaloids was first sun-dried.

Ancistrocladus is known to contain many different alkaloids and it has various medicinal applications for diseases like malaria, leishmaniasis, tumor etc. Present work was focused of Naphthyl-Isoquinoline Alkaloid (NIQ) content of leaves. Naphthyl-Isoquinoline alkaloids represent a totally new class of alkaloids that is reported in the last 60 years. As the name suggests, these alkaloids comprise of two moieties; a naphthyl group and an Isoquinoline group. This alkaloid arises from acetic acid units and not from aromatic amino acids like most of the other alkaloids do. Therefore, remarkable biological activities are expected from this alkaloid. Because of serious side effects of chemotherapy and radiation therapy associated with cancer, intensive research is being done on other alternatives like plant extracts that show anti-cancer activity. *Ancistrocladus heyneanus* is known for its many biologically active naphthyl Isoquinoline Alkaloids such as Ancistrocladine, Ancistroheynine, Ancistrocladinine, Acistrocladisine, Ancistrocladidine and Ancisheynine [**1, 2, 3, 4**]. It is the biological activity of these compounds that has earned this plant so much of interest.

Our interest lies in studying whether the Indian species also has any of the clinically important anti-cancer metabolite or not. Many African and some Asian species of Ancistrocladus has been reported to have anti-fungal [**5**], anti-HIV [**6, 7, 8**] anti-leishmaniasis and in particular antimalarial [**9, 10**] anti-bacterial [**11**] and anti-tumor activity that was reported in *Ancistrocladus tectorius* an African species [**12**].

The aim of the present work was to isolate and study the anti-cancer property of alkaloids present in the leaves of Indian species of Ancistrocladus heyneanus and to do the chemical characterization alkaloid responsible for anti-cancer activity. To accept the suitability of the extracted alkaloids their cytotoxicity was also analyzed.

Figure 1: *Ancistrocladus heyneanus* Plant (Inset) showing characteristic hook.



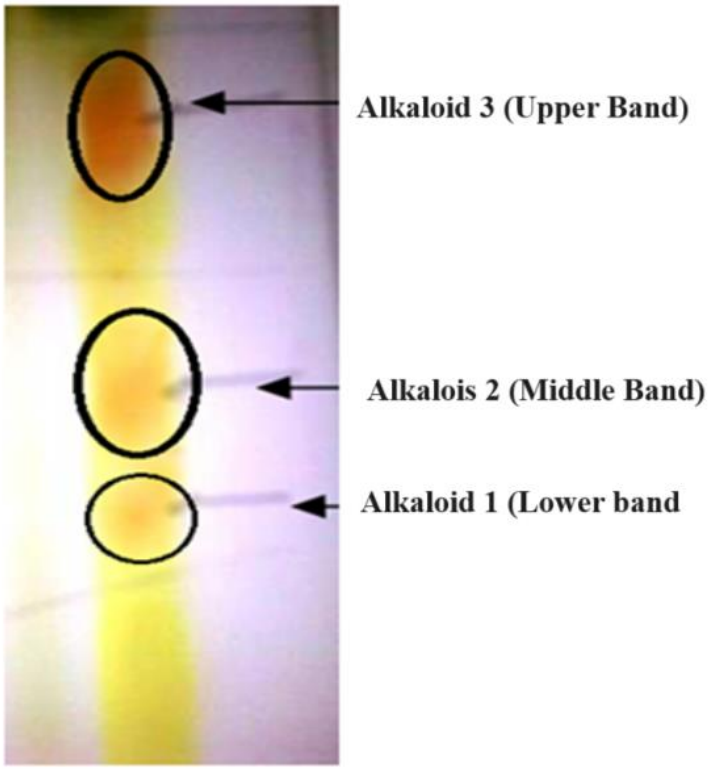
MATERIALS AND METHODS

Collection and Identification of Plant Material. - Plant was collected from the steep cliffs of Matheran Hills forest located in Karjat taluka of the Raigad district of Maharashtra. The Botanical Survey of India, Western Circle, Pune; did the identification and authentication of the plant as *Ancistrocladus heyneanus* (Figure-1).

Extraction of Crude Mixture of Alkaloids from the leaves of Ancistrocladus heyneanus - The collected leaves were sun dried, and powdered. 250 g leaves powder was taken in a thimble and placed in to the extraction tube of Soxhlet apparatus. 250 ml dichloromethane was added to the leaf powder and was placed on heating mantle and heated gently for extraction with recycle time being 20 minutes. The process was continued till the colour of solvent in the extraction tube changed from green to colourless. The collected dichloromethane extract was concentrated to 20 mL.

Separation of Alkaloids by TLC – Concentrated leaf extract taken in dichloromethane was used for TLC separation using 20x20 cm TLC plate (percolated Silica gel 60F254, MERCK), deactivated with NH_3. The plate was dipped in mobile phase contained 1:8:1, Methanol: Ethyl Acetate: Ammonia, to run the chromatogram. It took approximately 45-60 minutes for the solvent system to reach the marked front. Then the plate was removed, air dried and sprayed with Dragendorrf's reagent which showed presence of three orange-coloured bands (Figure – 2). Spots were visualized under UV light.

Figure- 2: TLC plate of leaf extract of *Ancistrocladus heyneanus* carried out using Methanol: Ethyl Acetate: Ammonia solution (1:8:1) as mobile phase, showing three orange-coloured spots of alkaloids when sprayed with Dragendorf's reagent



Elution of Alkaloid from the Second Band Obtained by Scraping TLC – Elution from second (Middle) band was done in Methanol. Elute was dried at 500 C in an oven to remove the solvent.

Analysis by NMR- Although other band of TLC were also analyzed but in this article we are discussing analysis of 2nd band only that has shown anti-cancer activity. For the present study BRUKER NMR model No.400MHz was used for the analysis of 2nd band of NIQ alkaloids extracted from leaves of *A. heyneanus* and separated by TLC. Approximately 5 mg of eluted dried alkaloid-2 sample was dissolved in 0.5mL pure dry deuterated chloroform. Using a pipette the solution of alkaloid-2 was carefully transformed into the NMR tube through the Kimwipe filter and NMR tube was capped.

Maintenance of Normal Cell line and Cancer Cell Lines: For Normal cell MDCK 2 cells (an epithelial-like cell isolated from the distal renal tubule of dog); and for cancer cell PLC/PRF/5 cell (a human liver cancer or hepatoma cell line) were used. Both cell lines were procured from nsnRc, Ambernath. In order to maintain a healthy population of cells, regular subculture was done in fresh medium. As the cells became confluent, they were collected via trypsin treatment and subcultured. The cell population were of two types; the adherent population, which grows and become confluent as an attached layer and the non-adherent population is found suspended in the supernatant. The cell wall proteins which aid in attachment of the cells is dissolved by trypsin, which got inactivated on addition of serum.

To Maintain a Healthy Population of Cells: The *non–adherent* cells were decanted in fresh centrifuge tubes and centrifuged at 2000 rpm for 10 minutes. The supernatant consisting of the medium was discarded and the pellet was re-suspended in fresh medium. Re-suspension was done in Nunc Culture Bottles with

10 mL final volume of medium. Prior to re-suspension, the cell count was calculated so as to monitor the growth of cells.

For removal of *adherent-cells*, filter sterilized 0.25% trypsin solution (1mL) was added to the culture bottle from which non-adherent cells had been decanted. It was allowed to stand for 10 minutes with gentle shaking from time to time. This was to ensure trypsin carries out efficient detachment of the cells. 1mL of fresh medium was then added to the bottle and all the contents were then decanted to a fresh centrifuge tube and centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded and the pellet re-suspended in a new culture bottle with fresh medium (procedure same as described above for non – adherent cells). Once appropriate cell population was attained with lesser number of dead cells, instead of the separate procedures for non-adherent and adherent cells subculture, all of the cells were pooled in one culture bottle.

Calculation of Cell Viability - Cell viability was determined using dye exclusion test with 0.4% trypan blue. Trypan blue selectively colours the dead tissues or cells. Live cells or tissues with intact cell membranes are not coloured. Cell viability was determined using the following formula every 24 hours:

Viable cell number /mL = n×5×104

Where n = average number of cells

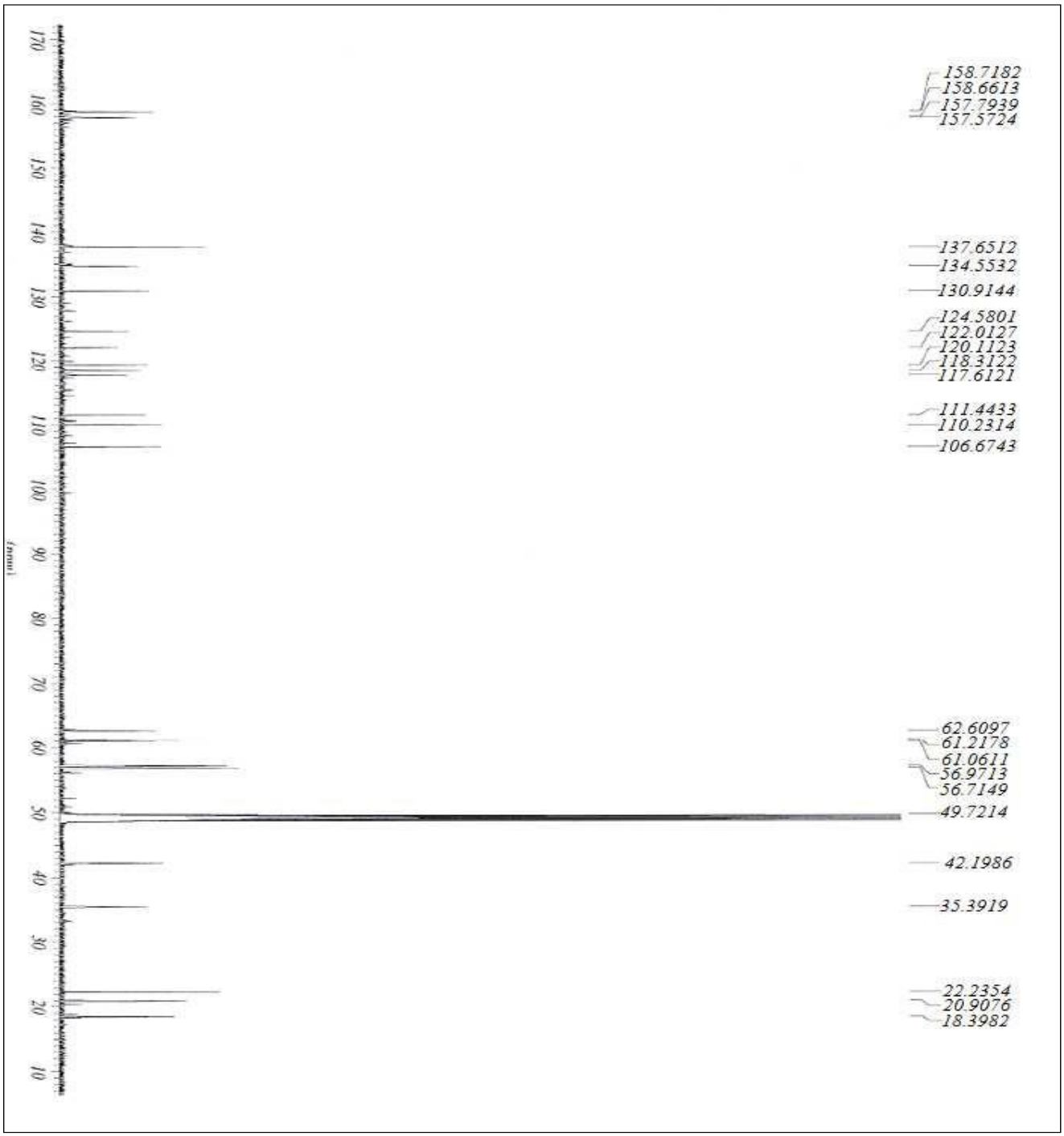
Addition of Plant Extracts to Both the Cell Lines - As mentioned earlier, second distinct bands obtained after TLC of organic solvent comprising of Ancistrocline was used as plant extract. The plant extracts were all filter sterilized using a 0.22 µM Millipore filter. The extracts were inoculated into the 6-well plates containing the cell cultures such that the concentrations in the wells were in the series of 0.05, 0.1, 0.2, 0.5, 1.0 µg/5mL of medium in case of MDCK 2 cell line cultures, and 0.1, 0.2, 0.5, 1.0 µg/volume of medium; for PLC/PRF/5 cell line cultures. Viable cell counts were taken after every 24, 48 and 72 hrs.

RESULTS AND DISCUSSION

As shown in figure 2 there are three alkaloids (Alkaloid-1, Alkaloid-2, and Alkaloid-3) separated by TLC. They were characterized by NMR to find out their structures (figure 3 and 4). Of these three alkaloids the NMR analysis data of only Alkaloid-2, are discussed here; because data matches with the Ancistrocline that was reported to be having anticancer property, as demonstrated in rats by Bringmann and Kinziger **[12].** NMR analysis of Alkaloid eluted from the second band obtained on TLC sheet had Rf value of -0.71. Typical Chemical Shift in ^{1}H NMR ^{13}C NMR spectra were recorded.

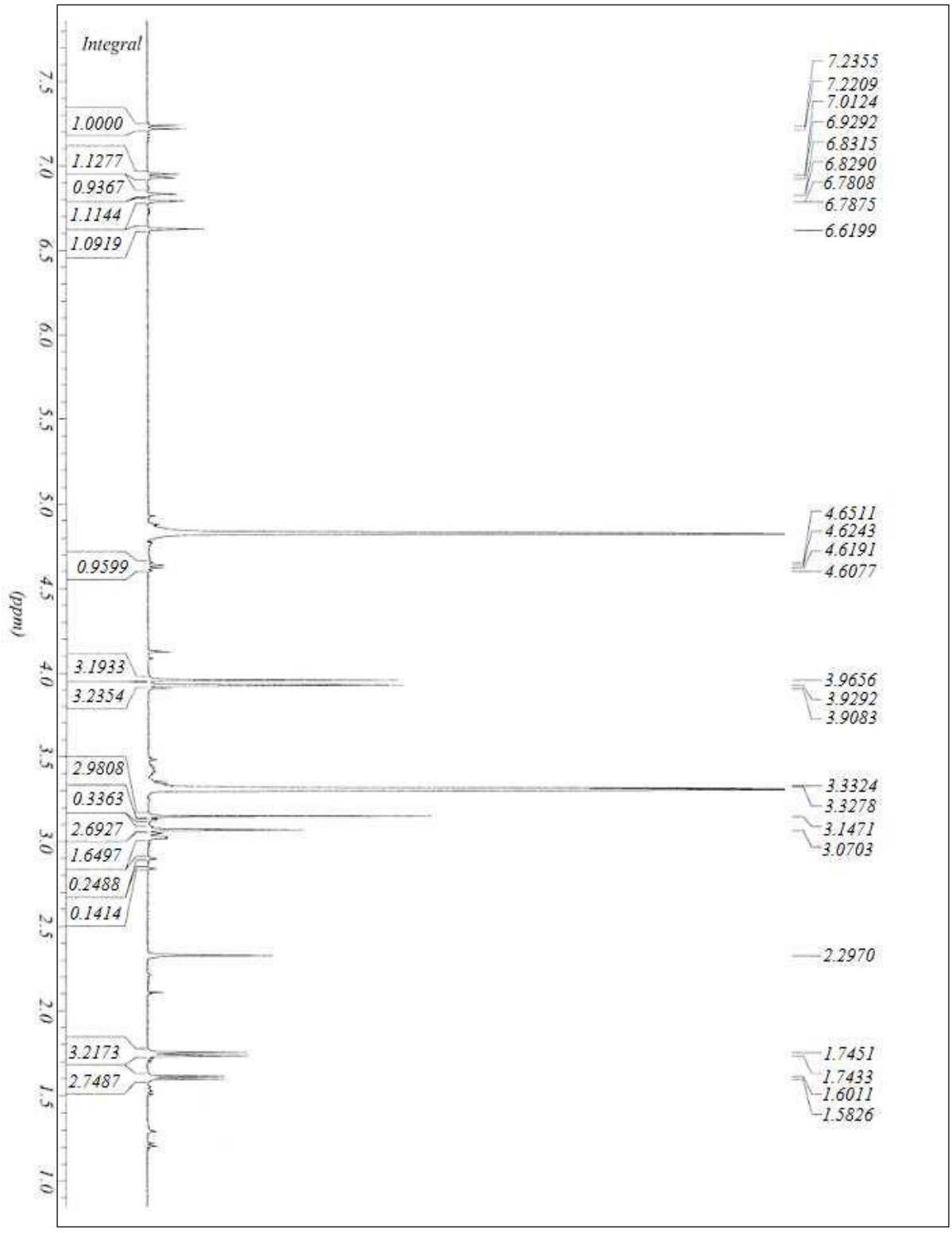
The ^{1}H NMR (Figure 3) shows five aryl protons, methoxy groups (δ4.60), an arylmethyl group (δ 2.29) and doublet for H-4 proton. This arrangement of groups is similar to those observed for monomeric NIQ alkaloid.

Figure 3: ^{1}H NMR spectrum of Alkaloid from second band.



The ^{13}C NMR spectrum shows (Figure 4) 3 methyl groups distinguished individually, later shown peak intensity for two methyl groups at δ20.9 and δ61.2 shows one methylene and five methine resonance quaternary carbons. The correlation from δ3.32 (H-4ax) and δ3.14 (H-4eq) to δ120.1 (C-5) and δ157.7 (C-8a) and δ122.0(C-7') to δ106.6(C-7) establishes that C-8' of naphthalene ring is attached to C-5 in the iso-quinoline ring. Quaternary carbons at δ157.7 in place of sp3 methine carbon also support the analysis.

Figure 4: ^{13}C NMR spectrum of Alkaloid from second band.

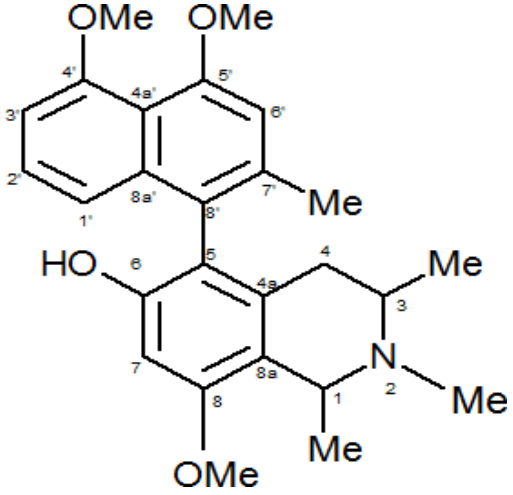


First report of two alkaloid ancistrocladisine and ancistrocladidine, extracted from the powdered roots of *Ancistrocladus heyneanus* Wall form India was by Govindachari et al. (1975) [**13**]. Our efforts were to extract Ancistrocline from leaves was based on results presented by Bringmann and Kinzinger [**12**], who showed anti-tumor activity of Ancistrocline extracted from the roots of *A. tectorius* on Rats. However, this work in about Ancistrocline extracted from leaves of *Ancistrocladus heyneanus* and its impact on human cancer cell line.

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

POSITION	^{1}H		^{13}C	
1,3	1.74(m)	Alkyl proton	49.7	Methylidine 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	Methylidine carbon
8	--		157.5	Methine carbon
8a	--		157.7	Methine carbon
1'	6.82(d)	Aryl proton	118.3	Methylidine carbon
2'	6.92(q)	Aryl proton	117.6	Methylidine carbon
3'	7.22(d)	Aryl proton	110.2	Methylidine carbon
4'	--		158.7	Methine carbon
4a'	--		134.5	Methine carbon
5'	--		158.6	Methine carbon
6'	6.78(s)	Aryl proton	106.6	Methylidine carbon
7'	--		122.0	Methine carbon
8'	--		124.5	Methine carbon
8a'	--		137.6	Methine carbon
C1-CH3, C3-CH3	1.58(dd)	Alkyl proton	20.9	Methyl carbon
C2-NCH3	3.07(s)	Alkyl amine	42.1	Methyl amine carbon
C8-OCH3	3.96(s)	Alkyl oxy	61.0	Methoxy carbon
C4'-OCH3, C4'-OCH3	4.60(s)	Alkyl oxy	61.2	Methoxy carbon
C7'-CH3	2.29(s)	Alkyl proton	22.2	Methyl carbon

Figure 5: Molecular structure of Ancistrocline.



Based on the compiled data of ^{1}H and ^{13}C NMR observations obtained for alkaloid-2, presented in table – 1; it is disclosed that the analyzed alkaloid is Ancistrocline (Figure 5), 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

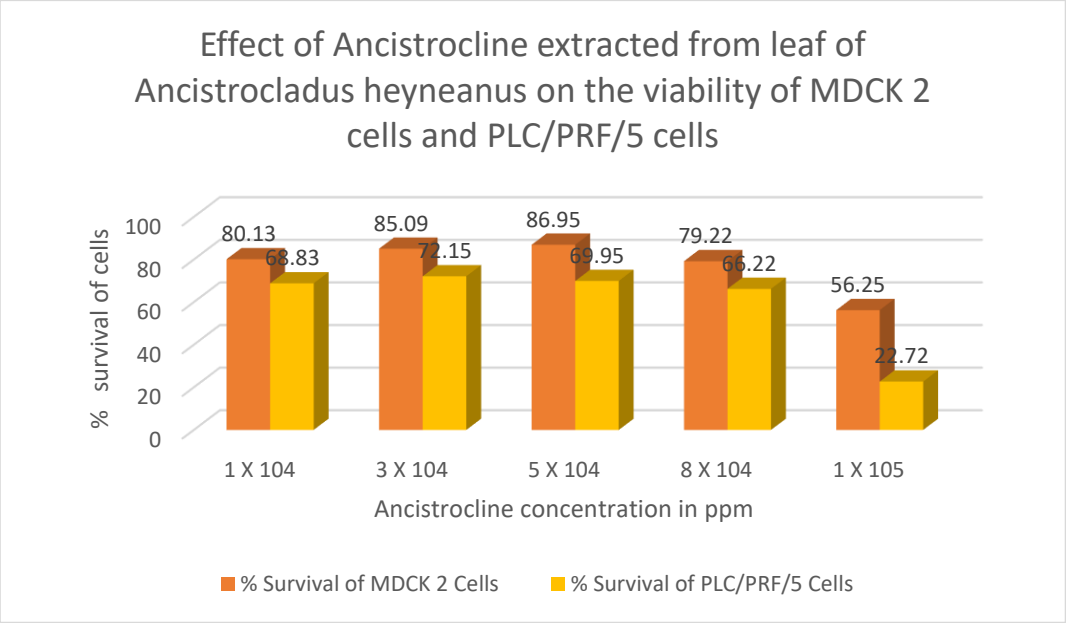
results match with the molecular formula of Ancistrocline. It is also known as 5-(4,5-dimethoxy-2-methylnaphthalen-1-yl)- 8-methoxy-1,2,3-trimethyl-3,4-dihydro-1H-isoquinolin-6-ol.

As mentioned above from the crude extract of NIQ alkaloids, a TLC separated alkaloid eluted from the second band and analyzed by NMR to be Ancistrocline was used for cytotoxicity and anti-cancer assay using MDCK 2 cells and PLC/PRF/5 cells respectively. The work reported here is primary study on the *in vitro* cytotoxic /anti-cancer cancer activity of Ancistrocline extracted from leaf of *Ancistrocladus heyneanus*.

Results of five tested concentration of Ancistrocline on MDCK 2 cells (normal epithelial cells) and PLC/PRF/5 cells (renal cancerous cells) are presented in figure -6. It was found that survival of PLC/PRF/5 cell lines was always less than MDCK 2 cell lines. Usually survivals of 50% cells depict non-toxicity. It was found that with increase in concentration of Ancistrocline survival of PLC/PRF/5 cell lines decreased and at a concentration of 1 X 10^5 ppm hardly 22% cells survived. In 1992, Bringmann, Kinzinger[12], have also studied the anti-tumor activity of Ancistrocline extracted from the roots of *A. tectorius*. They found that Ancistrocline present in the roots of this plant has an antitumor activity. However, no specific reference of Ancistrocline extracted from leaves of Ancistrocladus has been reported. With little reluctance we can conclude that Ancistrocline is the alkaloid responsible for exhibiting anti-tumour activity.

Alkaloids derived from plants are known for their anti-cancerous activities e.g. vinblastine, vincristine, vindesine, vinfosiltine and vinorelbine from *Catharanthus roseus* are being commercially used as therapeutic agent against many cancers (leukaemia, lymphoid leukaemia, hepato-cellular cancer, non-small cell lung cancer, advanced malignant melanoma, and advanced breast cancer). Camptothecin from *Camptotheca acuminata,* Taxol or paclitaxel from *Taxus brevifolia* are anti-cancer drug.

Figure 6: Effect of Ancistrocline extracted from leaf of *Ancistrocladus heyneanus* on the viability of MDCK 2 cells and PLC/PRF/5 cells



CONCLUSION

Ancistrocladus. heyneanus procured from Western Ghat of Maharashtra is an NIQ alkaloids containing plant. From a mixture of NIQ alkaloid obtained from the extract of dried leaf, Ancistrocline was isolated

using TLC method and confirmed using NMR. Various concentration of Ancistrocline was tested against the viability of a normal epithelial cell-line of dog MDCK-2 and a human renal cancer cell-line PLC/PRF/5. Even at the highest tested concentration MDCK-2 showed non-toxicity by having > 56% survival of cells. Whereas, PLC/PRF/5 cells showed increased cell death with increasing concentration. At highest tried concentration of 1 X 10^5 ppm only 22% cells survived. Thus showing anti0cancer property of this alkaloid. A further detailed analysis is needed to comprehend the anti-cancer efficacy of Ancistrocline found in *Ancistrocladus heyneanus*.

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