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Naphthyl Iso-quinoline alkaloids Extracted from Stem of *Ancistrocladus heyneanus* Exhibits Bactericidal Activity.

Anil Kumar Karn, Maheshwar Sharon, Rohit Kumar and Madhuri Sharon*

N.S.N. Research Centre for Nanotechnology and Bionanotechnology, Jambhul Phata, Kalyan-Badlapur Road, Ambernath (W), 421 505, Maharashtra, India

ABSTRACT

Ancistrocladus heyneanus is a tropical liana plant, rich in Naphthyl Iso-quinoline (NIQ) alkaloids. Alkaloids from stem were extracted in both water as well as organic solvent. The organic solvent extract was further separated by HPTLC that showed four distinct bands of alkaloids. Each of them was analyzed by LC/MS and NMR and tested for its antimicrobial activity against three Gram negative and one Gram positive bacteria; using standard agar well method. Aqueous extracts were tested as crude extract without the separation of alkaloids. Crude aqueous extracts showed activity against both gram negative and gram positive bacteria. Whereas different fractions of alkaloids separated by HPTLC exhibited microbe specific activity. Band 2 (H2) of HPTLC separated alkaloid showed bactericidal activity against all the 4 tested bacteria; H1 and H4 could inhibit the growth of three of the four tried bacteria whereas H3 being least bactericidal could kill only one bacteria i.e. S. typhi.

Keywords: *Ancistrocladus heyneanus,* Antimicrobial, Alkaloids, Bactericidal, Naphthyl Iso-quinoline alkaloid

Correspondence author

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INTRODUCTION

A special feature of higher plants is their capacity to produce a large number of organic chemicals of high structural diversity that are not required for the general growth of plants. These compounds are called secondary metabolites. Some of these secondary metabolites are produced for self-defence. Based on their mechanism of function such metabolites could be chemotherapeutic, bacteriostatic, bactericidal or antimicrobial. Over the last 50 years, a large number of plant species have been evaluated for their antimicrobial activity. One of the plants known for having medicinal uses in African traditional system of medicine is Ancistrocladus. Its roots, stem and leaves are used in traditional medicine. The active medicinal ingredient of this genus is a secondary metabolite known as naphthyl iso-quinoline alkaloids, which is not of paramount significance for plant life, rather it functions as defence tools, for detoxification, communication, signalling, safe-guarding and other biological processes.

Ancistrocladus is known to contain many different alkaloids and recently, efforts of WHO programme have found that it has various medicinal applications for diseases like malaria, HIV, leishmaniasis, chagas disease etc. There is only one species of this genus is found in India i.e. *Ancistrocladus heyneanus*, endemic to the Western Ghats. This species produces a number of NIQ alkaloids such as secondary Ancistrocladine, Ancistrocladinine, Acistrocladisine, Ancistrocladidine, Ancisheynine and Ancistroheynine [1-4].

The work reported in this paper is an initial attempt to study the bactericidal activity of stem extracts of *Ancistrocladus heyneanus* using Crude Aqueous extract, four isolated alkaloid fractions of HPTLC (Karn 2014).

MATERIALS AND METHODS

Plant Material: Stems of *Ancistrocladus heyneanus* were collected from Khandala hills of Maharashtra, India and sun dried and grinded to fine powder.

Figure 1: *Ancistrocladus heyneanus* young plant

Test Pathogens*:* three gram negative bacteria (*Salmonella typhi, Escherichia coli & Klebsiella pneumoniae)* and one gram positive organism (*Staphylococcus aureus)* were procured from NCCS, Pune and used for antibacterial testing of plant extracts.

Aqueous Extraction of alkaloids: from stem was done in the acidic medium.10g of stem powder + 500 ml distilled +50 ml of 0.05 N Sulphuric acid was taken in a flask and stirred on a magnetic stirrer for 3 hrs and then boiled for 20 min. To the boiled solution 25 g of heavy magnesium oxide it was added and again boiled for 20 min. Solution was allowed to cool and then filtered through Whatman filter paper no.41. The filtered solution was transferred to Petri plates and the filtrate was evaporated to dryness on a hotplate. The dried residue was collected.

Extraction of alkaloids in Organic Solvent Methanol – was done by adding 10g of the dried stem powder to 90 ml Methanol and 25% Ammonia. Solution was left uncovered overnight. Next day, it was filtered through Whatman filter paper number 12.

Separation of Alkaloids: was done by HPTLC. Sample was loaded onto the Silica Gel 60 F254 HPTLC plates (MERCK) of dimensions 20x10 cm, in the form of uniform bands with the help of a Linomat 5 Applicator. Prior to use plates were marked with a pencil indicating a solvent front and the direction of run in the form of arrows at the upper edge of the plate. Bands were applied maintaining a distance of 10mm from each other and a distance of 15mm from the edge of the plate. After sample application, the plates were air dried with an air drier. After that they were placed very carefully in the twin trough chamber saturated with the mobile phase by placing a filter paper soaked in the solvent system containing
Ethyl acetate: Methanol: 17%Ammonia (8: 1.75:0.75)
After the solvent system covered a stipulated distance, the plates were removed and air dried. These developed plates were then visualized under the UV chamber at 254nm and at 366 nm. The plates were then derivatized with Dragendorrf reagent and again visualized at 254 nm and 366 nm.

Elution of separated alkaloids: After detection of bands, they were carefully cut into strips and then into very small pieces and were transferred to test tubes containing minimum amount of methanol. The tubes were further sonicated for 30-45 minutes. The strips were then separated from methanol and this methanol was collected in separate tubes. Excess of methanol was then dried at 700 C using a water bath. This sample of alkaloid was used for detecting their bactericidal property.

Agar Well Diffusion tests: 'Agar Well Diffusion' tests were used for testing bactericidal activity of stem extracts. In this technique, the zone of inhibition of microbial growth on Nutrient agar medium was measured, which gave a qualitative impression of effectiveness of the alkaloids from the stem of *A. heyneanus* against the selected micro-organisms.
Fresh 24 hr old culture slants of each of these organisms were prepared. A loopful of the culture was suspended in freshly prepared saline solution and the optical density of the suspension was recorded at 0.1 at 540 nm.

0.1ml of the culture suspension was poured on to solidified nutrient agar plates and spread uniformly on to the plate using a glass spreader. A 6 mm wide well was bored into the agar using a cork borer. 100 µl of the crude (i.e. un-separated alkaloids) sample (conc.

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0.5mg/ml) was poured into each well and the plates were kept in the incubator at 37^0C. After the desired growth period the inhibition zone of microbial growth around the agar well was recorded.

The same procedure was performed using 4 separated fractions of the alkaloids. The plates were observed after 24hrs of incubation. All the tests were performed in triplicates.

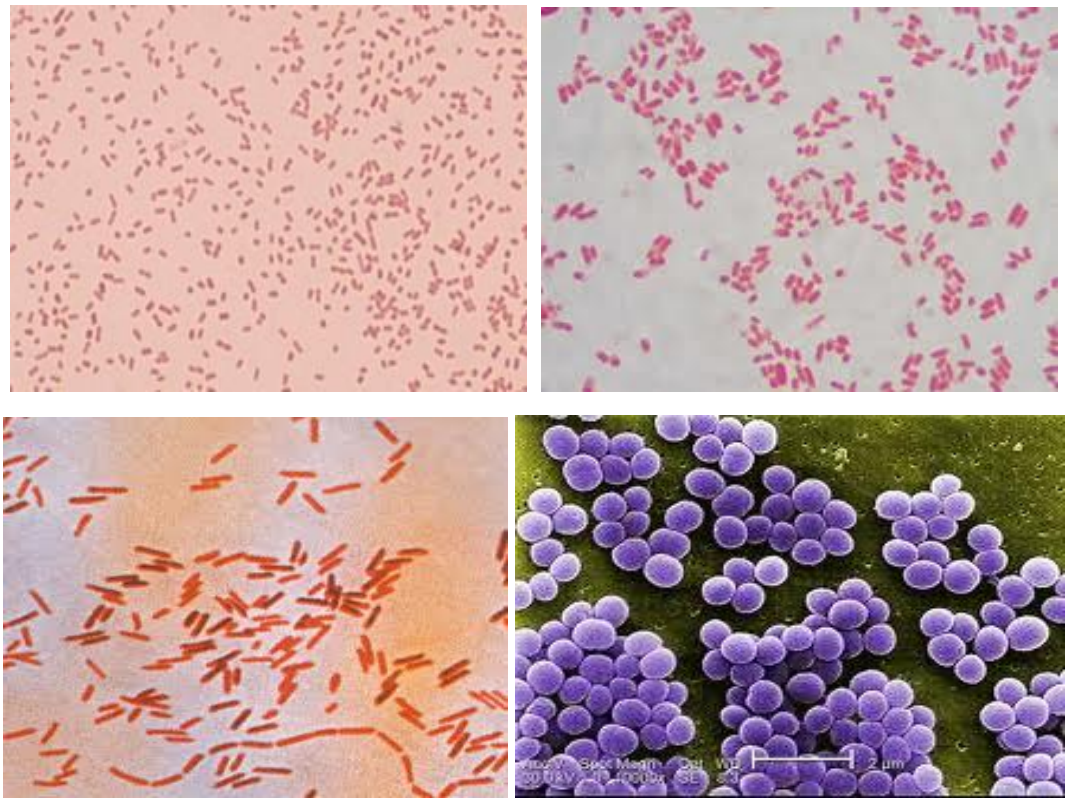

Figure 2: (a) *Klebsiella pneumonia* (b) *Escherichia coli* (c) *Salmonella typhi* and (d) *Staphylococcus aureus*

RESULTS AND DISCUSSIONS

HPTLC Separation of aqueous stem extract

The mobile phase for HPTLC comprising of Toluene: Ethyl acetate: Diethyl amine (7:2:1), exhibited four bands (Figure -3)

Impact of Crude Aqueous & Organic Extract on Bacterial Growth

As mentioned above crude Aqueous extracts of stem taken in water as well as organic solvent extracts, were tested against four pathogenic bacteria. After 24hrs of incubation of bacteria in presence of extracts, the zones of inhibition of bacterial growth around the well containing extract or control was measured. In the inhibition zone bacterial colonies did not grow, hence nutrient agar appeared clear without any microbial colony. The results are presented in figure 3 & 4 and table 1 & 2

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As it can be seen in figure 3a *Klebsiella pneumoniae* showed very slight reduction in growth of the bacterial colony around the well containing Aqueous Crude extract whereas very clear zone of inhibition of 0.4cm was observed when organic solvent extract was used. Irregular inhibition zone was seen when *Escherichia coli* was grown in presence of both Aqueous and organic solvent extracts. However inhibition was more pronounced in organic solvent extract (Figure 3c)

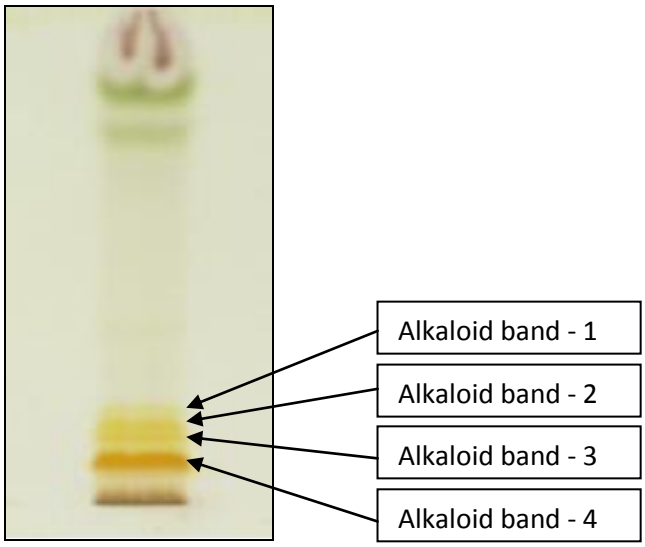

Figure 3: HPTLC of NIQ alkaloids from the stem extract of *Ancistrocladus heyneanus*, using Ethyl acetate: Methanol: Water (10:1.35:1) as mobile phase and derivatized with Dragendorrf's reagent

Table 1: Effect of crude Aqueous and Organic solvent (Me-A) stem extracts of *Ancistrocladus heyneanus* on the growth of four bacteria

Bacteria	Zone of inhibition of Bacterial growth in stem extract of *Ancistrocladus heyneanus* taken in	
	Water	Organic Solvent
Klebsiella pneumoniae (gram negative)	No effect	0.4cm
Escherichia coli (gram negative)	No effect	0,15 cm
Salmonella typhi (gram negative)	No effect	0.2 cm
Staphylococcus aureus (gram positive)	0.5cm	0.8cm

No antibacterial activity was exhibited when *Staphylococcus aureus* was grown in presence of either aqueous or organic solvent extract. Whereas *Staphylococcus aureus* showed inhibition in colony growth when grown in both aqueous as well as organic solvent extract. However, inhibition in bacterial colony rowth was more pronounced by organic solvent (Figure - 4d).

Since Organic solvent extract showed more inhibition in all the bacterial colonies it was decided to grow these microbe in organic solven as control. Results are presented in Figure -5. Both Methanol and Methanol+Ammonia did not inhibit the growth of either *S. aureus or S.typhi*

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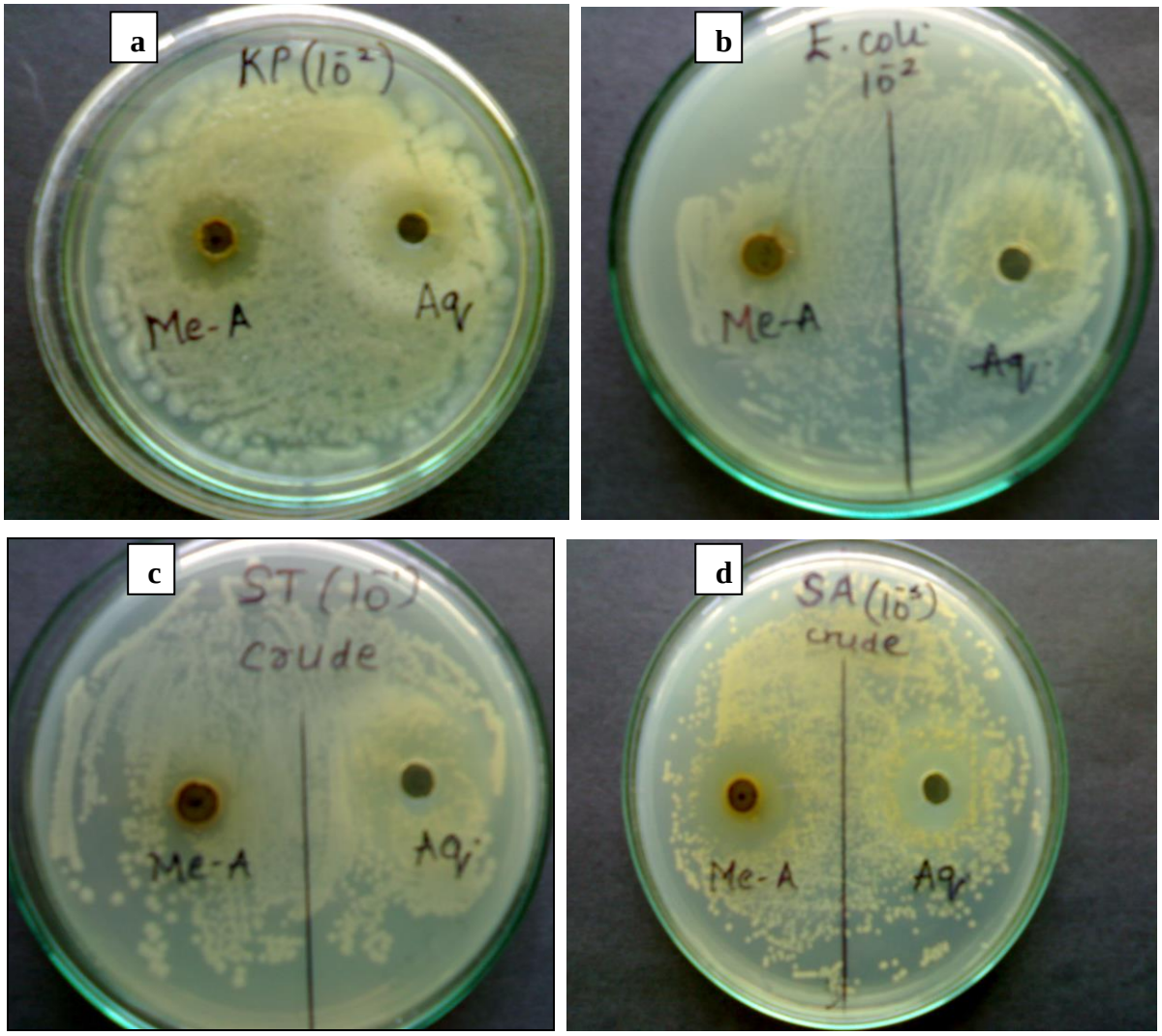

Figure 4: Effect of Crude Aqueous and Organic solvent (Me-A) stem extracts of *A. heyneanus* on the growth of (a) *Klebsiella pneumoniae* (b) *Escherichia coli* (c) *Salmonella typhi* & (d) *Staphylococcus aureus;*as tested by standard Agar-Well method.

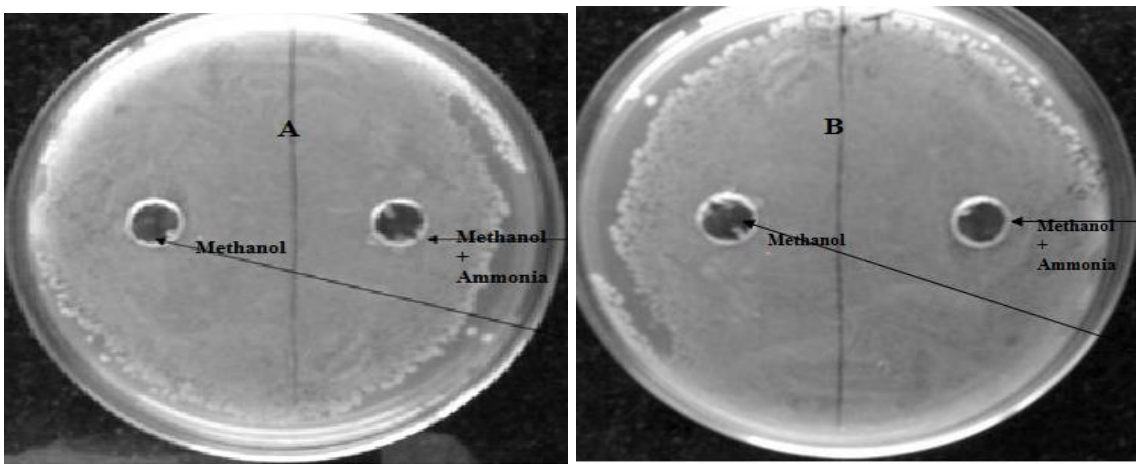

Figure 5: Two bacteria namely (A) *S. aureus* and (B) *& S. typhi* were tested against the solvent (control) i.e. methanol and methanol-ammonia. Both are not showing any bactericidal activity

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Impact of 4- isolated alkaloids by HPTLC on Bacterial Growth:

Organic solvent extracts of leaf were fractionated by HPTLC (Chapter 4). Four fractions of alkaloids were obtained. Since the organic solvent extracts showed better bactericidal activity all the 4 fractions separated by HPTLC were also studied for their antimicrobial activity using same microbes as mentioned above.

Alkaloids from aqueous extract were not used because they were showing less antimicrobial activity and their separation was not proper and bands were not distinct.

The amount of isolated fractions was very less hence the agar wells could not be filled to their brim. The results are presented in Table 2 and Figure 6

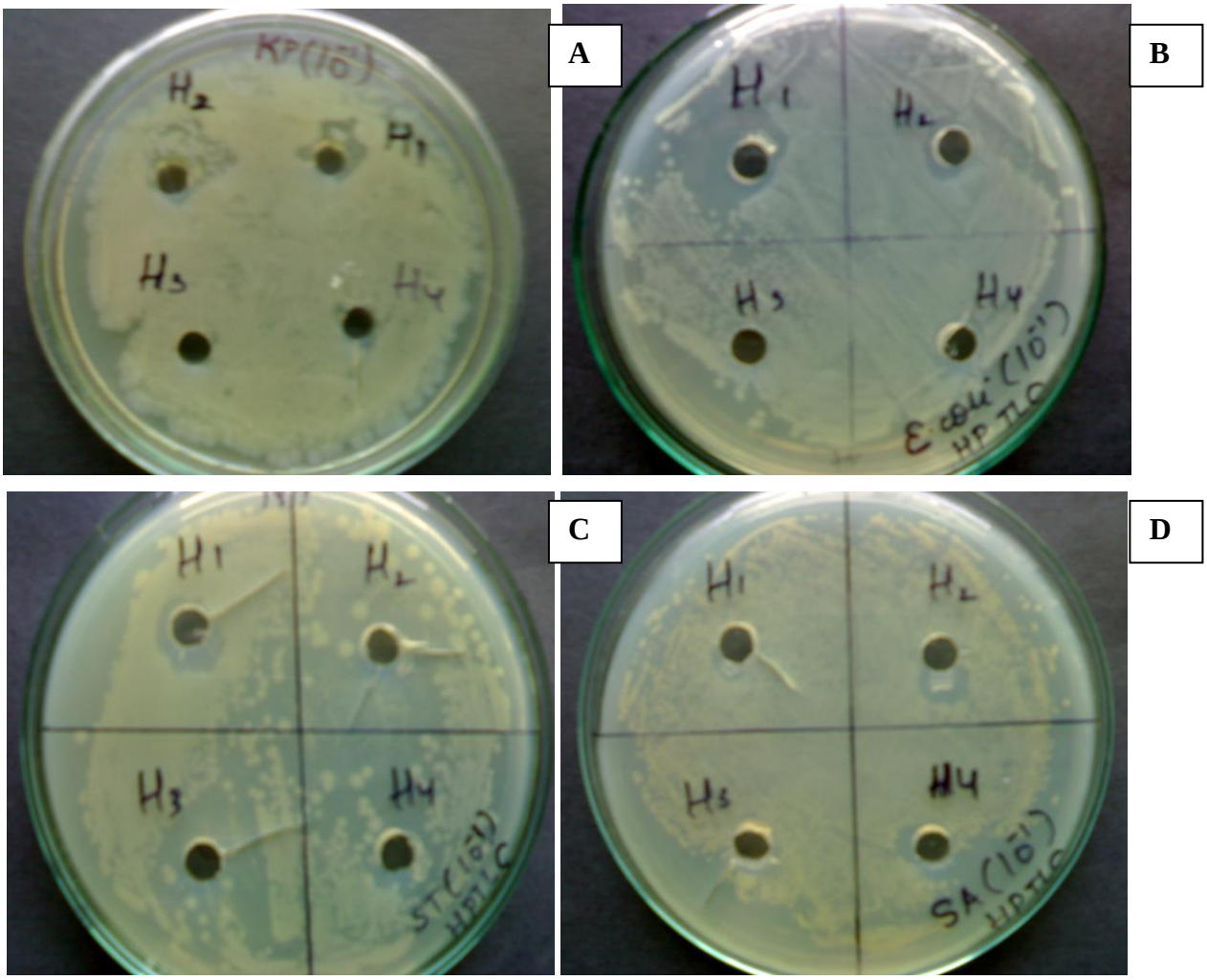

Figure 6: Effect of four isolated alkaloids (by HPTLC) from stem extracts of *A. heyneanus* on the growth of (a) *Klebsiella pneumoniae* (b) *Escherichia coli* (c) *Salmonella typhi* and (d) *Staphylococcus aureus;*tested by Agar-Well method

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Table 2: Effect of four isolated alkaloids fraction (by HPTLC) from Organic solvent (Me-A) stem extracts of *Ancistrocladus heyneanus* on the growth of four bacteria Results of 24hrs of incubation of the inoculated plates is summarized in this table

Bacteria	Bacterial growth in four isolated alkaloids fractions from stem extracts of *Ancistrocladus heyneanus*			
	H1	H2	H3	H4
Klebsiella pneumoniae (gram negative)	+	+	-	-
Escherichia coli (gram negative)	+	+	-	+
Salmonella typhi (gram negative)	+	+	+	+
Staphylococcus aureus (gram positive)	-	+	-	+

As compared to antimalarial activity, anti-HIV activity and anti-cancer activity of NIQ from Ancistrocladus species; comparatively less work has been done on *in vitro* anti-bacterial assay of NIQ from Ancistrocladus species. Only one work has reported moderate anti-bacterial activity of *Root* extract of *A. tectorius* [5] and the other from the *Leaf* extracts of *A. heyneanus* against *Escherichia coli* and *Salmonella typhi, Klebsiella pneumoniae* and *Staphylococcus aureus* [6].The other recently reported work is from the extract of whole plant of *A. heyneanus,* against antibacterial activity of *Escherichia coli* and *Salmonella typhi* [7].

In the present work Stem extract (Taken in water, in organic solvent & extracts separated by HPTLC in to 4 bands denoted as H1, H2, H3 & H4) of *A. heyneanus* was assessed for their antimicrobial activity. Stem is the organ that transported metabolites synthesized from leaves to other parts of the plants e.g. roots, seeds etc, hence was taken into consideration.

H2 band was found to be having antibacterial activity against both gram positive and gram negative bacteria (Figure 4 and table 2). The analysis of the alkaloid band has shown that H2 band is Ancistrocline [8]. First band H1 was also inhibitory to all Gram positive bacterial growth but not to Gram negative bacteria *Staphylococcus aureus.*

Gram positive and Gram negative bacteria are classified in two groups on the basis of structural differences in their cell wall and their response to Gram stain test. Gram negative bacteria do not stain where as Gram positive bacteria gets stained. Compared with Gram-positive bacteria, Gram-negative bacteria are more resistant against antibiotics, because of their impenetrable wall. Variation in cell wall layer might have shown differential response to H1 band.

H3 band of alkaloid could inhibit the growth on all the four tried bacteria; whereas H4 showed bactericidal activity on only one Gram negative bacteria and also on gram positive bacteria.

S. typhi was found to be the most sensitive to NIQ alkaloids as all the four alkaloid showed bactericidal activity against *S .typhi*

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