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Targeted isolation of alkaloid from *Cyclea Peltata* and determination of structural formula of Tetrandrine alkaloid based on NMR studies

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Abstract

Alkaloids are group of chemical entity with proven medicinal properties. This work consolidates the procedure in isolation and structural determination of a bisbenzyl isoquinoline alkaloid from *Cyclea peltata* with related NMR values and data. Dried routes of *Cyclea peltata* was extracted using chromatographic techniques monitored using TLC and proton NMR studies. The pure compound isolated was subjected for proton NMR, carbon NMR, HSQC, HMBC, NOESY and COSY spectral experiments. Goal is to document the methods of isolation and NMR experiments involved in structural determination. The compound isolated were bisbenzyl isoquinoline alkaloid with head to head ether bond connection and identified as "Tetrandrine". Eventhough tetrandrine is a known compound and is reported from many other plants, this time it is isolated and reported from *Cyclea peltata* (H.f & T).

Keywords:- *Cyclea peltata*; bisbenzyl isoquinoline; NMR; HSQC

Introduction

As per Ashtanga Hrdaya (600 AD), "Jagatyevam anoushadham na kinchit vidyate dravyam vashaannaarthayagayoh" (Sutra Sthana Ch. 9 - verse 10). This translates as "There is nothing in this universe, which is non-medicinal, which cannot be made use for many purposes and by many modes" [1]. The present work reports on experimental methods for targeted isolation of medicinal plant *Cyclea peltata* (H.F&T), family Menispermacae. The plant is used traditionally for many medicinal purposes but only few documented evidences are available. The species Cyclea and the family Menispermacae are reported to have different alkaloids and this is an effort to create evidence for targeted isolation of alkaloid from *Cyclea Peltata* and methods for structural determination of pure compound isolated.

Botanical description of *Cyclea peltata*

As per flora of India the plant *Cyclea peltata* was identified by Hooker f & Thomson in the year 1855 [2]. The genus Cyclea was proposed and characterized by Arnott which was adopted by Miers. The plant *Cyclea peltata* of Menispermacea family is a slender twining shrub with sparingly pilose stems and branches. The leaves are 3 - 6 with 2 - 4.5 inches long, deltoid or ovate, acute, truncate or slightly sinuate at the base with rounded angles. Flowers

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are minute, smaller than the preceding with axillary male panicles, slender but much branched. The branches are remote and divaricate, and gradually becomes shorter upwards, the uppermost part are very short or obsolete. Flowers are subsessile, interruptedly spicate or collected into heads. Calyxes are campanulate and divided nearly to the base into 4 (rarely 5) segments without pilose and four fid corolla. Female panicles are racemose and much shorter than the male. Sepals are oblong and glaborus, petals are orbicular and shorter than the sepals. Ovary and drupe are often pilose (Hooker and Thomson, 1855) [2].

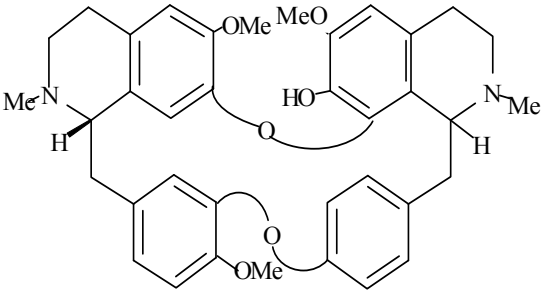
Medicinal values of *Cyclea peltata*

A group of Asian indigenous people (Nagas) use this plant for fighting against evil spirits [3]. *Cyclea peltata* is reported to be used for skin diseases such as allergies, burns, cuts, wounds, inflammation, leprosy, leucoderma, scabies, smallpox and certain sexually transmitted diseases (STD) [4]. The roots of *Cyclea peltata* have been tested for inhibitory properties on nephrolithiasis in rats, induced by treating with 1% ethylene glycolated water for 35 days [5].

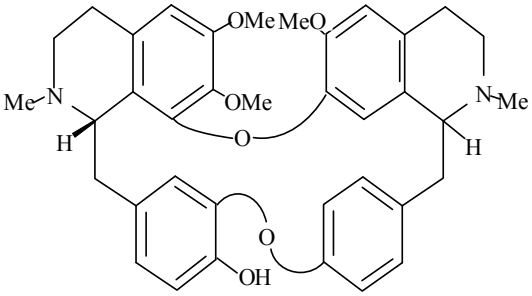
Research Organization for Oriental Traditions and Sciences (ROOTS) in India shows *Cyclea peltata* as one of the ingredients in an ayurvedic preparation called diabe drink to manage diabetes mellitus. A review article on medicinal plants and natural products by Dahanukar, S.A. et al (2000) explains the CNS activity of *Cyclea peltata* along with 25 natural products from Nilgiri hills in India [6].

Bis-benzyl isoquinoline alkaloids from *Cyclea peltata*:

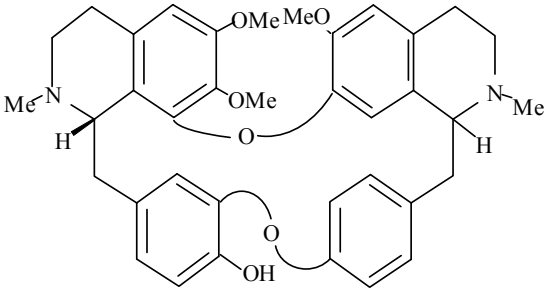
Five bisbenzyl isoquinoline alkaloids were isolated by Kupchan, S.M. et al (1962) from *Cyclea peltata* Diels and named as cycleapeltine, cycleanurine, cycleadrine, cycleacurine, cycleahomine chloride (fig 1) [7]. The chemical structures are shown below,



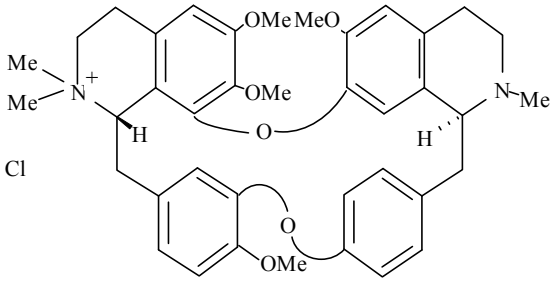
Cyclea peltine



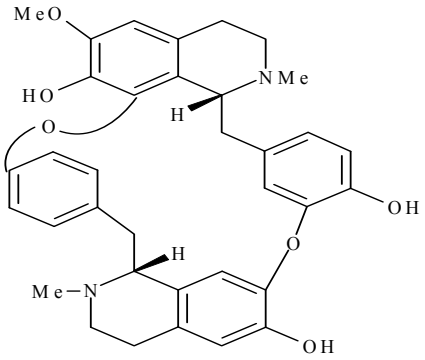
Cycleadrine



Cycleanorine



Cycleahomine chloride



Cycleacurine

Fig: 1, five bisbenzyl isoquinoline alkaloids reported from cyclea pelata Diels. Four of them are connected via head to head and later one with head to tail connectivity.

Alkaloids reported from species Cyclea

Guinaudeau, H. et al (1993) [8] reported two new bisbenzyl isoquinoline alkaloid namely 2'norlimacine and cyclea barbatine along with some known alkaloids like tetrandrine-2-beta-N-oxide, berbamunine, repandine, cycleanorine, daphanandrine, curine, cocularine and N-methyl cocularine from Cyclea barbata. Lin, L. Z. et al (1993) worked on some of these alkaloids for their cytotoxic and antimalarial properties [9]. Two naturally occurring head to tail bisbenzyl isoquinoline-N-oxides were isolated by Lai, S. et al (1993) from Cyclea sutchunesis and named as insularine-2'α- N-oxide and insularine 2-β, 2'-β N, N-dioxide on the basis of spectral analysis [10]. They also isolated Cycleanine, isocycleanine, D-isochododendrine, and sutchuensine from the roots. The latter two alkaloids were epimers of the former two alkaloids and they are examples of bisbenzyl alkaloids with two diaryl ether bridges in between C-8, C-12' and C-12, C-7' [11].

Materials and Methods

Objective of this study is to adopt targeted experimental methods to isolate and separate alkaloids from *Cyclea peltata*, and perform 1 & 2D NMR spectroscopic methods to determine the structural formula for pure compound isolated. The step wise process is described in the following sessions.

Phytochemical Isolation of *Cyclea peltata*

The plant *Cyclea peltata* was collected during winters from hills near botanical garden, Palode, Kerala, India. Voucher specimens were examined and phytosanitary certificate were issued by Directorate of Plant Protection, Ministry of Agriculture, Government of India. The whole plant was dried under sunlight for 2 days and roots were separated from plant material. The roots were further dried in hot air oven with airflow regulated at 40^{o} C. The dried powdered roots were sieved and weighed before starting the experimental procedures. Initial extraction was done using Soxlet apparatus under controlled temperature. The extraction was done with different type of solvent (3 liters each), based on the polarity. Non Polar, Semi Polar and Polar solvents namely Hexane, Chloroform and Methanol were used for extraction. 50% Methanol along with glass distilled water until complete exhaustion were done. The Extracts were concentrated under reduced pressure using rotary evaporator and stored in freezer.

Vacuum Liquid Chromatography

It was decided to work on isolation techniques for alkaloids therefore further work was continued in chloroform extract. The chloroform fraction showed maximum amount of alkaloids in TLC using Dragendroff test for alkaloids. The chloroform fraction was subjected to further fractionation by VLC using 50 grams of 60H

graded silica gel. The extract was mixed with 6 grams of 60A grade silica gel and uniformly distributed over the stationary phase. The mobile phase used was 100% chloroform with adding incremental amounts of methanol for each elution.1 % of ammonia upon chloroform and methanol was added to elute the column [12].

Open Column Chromatography

Open column chromatography was carried out in glass column filled with slurry of silica gel. After plugging the bottom end of column with cotton to prevent silica eluting to the extract the column was filled with mobile phase or solvent mixture and silica gel slurry (sized 60, 230-400 mesh), prepared 10 hours prior to the experiment. The column was subjected to gentle tapping and left for some time for compact packing of stationary phase [13,14].

Table 1 Solvent system

Mixture of mobile phases used	Percentage	Type of compounds
Cyclohexane: Toluene: diethyl amine	75:15:10	Nitrogen bases
Chloroform: Methanol	90:10	Nitrogen bases
Chloroform: Acetone	9:1	Acidic and neutral compounds
Dichloro ethane: Methanol: water	95:5:02	Aqueous fractions
Toluene: Ethyl acetate: diethyl amine	70:20:10	Neutral compounds

Gel Chromatography

Gel chromatography was carried out using Sephadex LH-20, which allows separation of extracts depending on the size of molecule but when using mixed solvent systems the separation occurs by the size of molecules and polarity. Significantly better separation was achieved in chromatographic techniques while using isocratic system of mobile phases, monitored by TLC. The fractions obtained were passed through a cotton wool filter to remove any silica gel coming along with the mobile phase. The extracts were dried under reduced pressure to store in a freezer [13,14].

Monitoring using TLC plates

Pre-coated TLC plates either plastic or aluminas (0.25 mm, silica gel 60 PF254 Merck), were used to analyze the samples and to check the purity of each fractions eluted. The mobile phase was same as used in column chromatography along with slightly more polar solvents. Reverse phase chromatographic plates were used to analyze the higher polarity compounds in the mixture. RF values for each fraction were computed and monitored. A consistent monitoring system based on TLC was adopted for determining the solvent systems and combining similar fractions depending upon the RF values. Mixture of mobile phases was used to enhance maximum separation between compounds in TLC. Different mixtures of mobile phases, at different concentrations were tried (Table 1). The TLC were observed under UV lamp set at 254nm (short wave) and 360 nm (long wave) to detect compounds with fluorescence or quenching properties. The plates were sprayed with selected reagents prior to heating for color development.

Type of reagents used in TLC

Anisaldehyde Reagent

A solution of 0.5ml of anisaldehyde in 50 ml of acetic acid and 1 ml of concentrated sulfuric

acid was sprayed on the plates and heated at 100^0 C for color development. This reagent was usually used for non alkaloid compounds and RF values of each spot were measured to compare the identity of compounds.

Dragendroff's Reagent

The reagents were prepared as two different solutions. Solution A contains 2 gm of bismuth subnitrate, 25 ml of acetic acid and 100 ml of water where as solution B consists of 40 gm of potassium iodide in 100 ml of water. 10 ml of each solution is added to 20 ml of acetic acid and 100 ml of water to complete to reaction. The reagent is sprayed to TLC plates prior to heating in autoclave to develop a dark orange color to confirm the presence of alkaloids.

NMR Spectroscopy

The solubility of purified compound in deuterated methanol, chloroform and pyridine was determined to obtain nuclear magnetic resonance (NMR) spectra. The sample was filtered through a cotton plug into specified NMR tubes and spectra were obtained from 250 MHZ, 400 MHZ and 600MHZ instruments with different runtimes depending on the complexity of compound.

Proton NMR

It is a simple NMR technique to check the purity of sample by exiting the spin state of ^{1}H and evaluating the energy release when ^{1}H returns to its lower energy state. Proton spectra are obtained between 0-10 ppm and can find out chemical shifts, coupling constants and integration of atoms in the structure [15].

Carbon NMR

To establish the carbon skeleton of a compound, with a broad spectrum between 0 to 230 ppm (JMOD). The experiments help to distinguish the carbon types showing C, CH2 and CH, CH3 in opposite directions. The number of scans is increased to reduce noise level for getting better peaks in complex structure. We obtained different set of ^{13}C spectra in 400 and 600 MHZ machines.

Cosy Spectra

Correlation spectra (Cosy) reveal the position of protons in carbon skeleton using pulse sequences and coupling of protons are indicated by a cross peak in spectrum. [16]. A contour plot is obtained for correlation between two perpendicular axes with the diagonal corresponding to normal proton spectrum which helps to build up a picture of the position of protons.

Noesy Specta

It is called as Nuclear Overhauser Enhancement Spectroscopy (NOESY) used to determine the relative stereochemistry of atoms in space. It has a delay interval for mixing period and third pulse, but gives the idea of coupling of protons 3 dimensionally by giving cross peaks to protons which see each other through space.

HMBC spectra

Heteronuclear multiple bond coherence (HMBC) gives long range correlation between protons and carbons usually J^2 or J^3 and rarely J^1or J^4. The technique was used to assign the position of ^{13}C and ^{1}H but because of nature of compound isolated, the peaks were broadened looking at cosy along with neighbourer.

HSQC Spectra

The acronym stands for Heteronuclear single quantum correlation spectrum which shows a unique peak for each proton attached to a heteronucleus (^{13}C and ^{15}N). It is possible to calculate the direct bonding information's eg: -C-H- (^{1}J coupling constants) and also chemical shifts of heteronucleus if protons are known and vice versa.

Mass spectroscopy

Mass spectroscopy analyses the fragmentation of the molecule and gives the mass to charge ratio of the sample by which the molecular weight of compound is determined. The Electron impact, High resolution and fast atom bombardment experiment is carried out to determine the molecular formula of compound isolated in this work.

Results and Discussion

Soxlet extraction was done at 280 gms of *Cyclea peltata*. 17.374 gms of dried Hexane extract, 3.728 gms of Chloroform extract and 27.280 gms of Methanol extract were obtained. On further extraction till exhaustion using 50% Methanol and glass distilled water, 23.320 gms of dried extract were obtained. A total of 37 fractions was isolated and were scrutinized by TLC. The identical fractions with same RF values were combined, concentrated, weighed and stored in freezer for further chromatographic experiments. Since the study was restricted towards alkaloids the work continued using the chloroform extract. Even though TLC of Methanol and Hexane extracts showed the presence of many compounds but was not under the purview of this study.

Crystals were formed in some of the VLC fractions. The crystallization was done by dissolving the extract in D-chloroform with addition of 2 drops of MeOH slightly heated and allowed to cool under room temperature. Crystals were filtered and subjected to proton

NMR spectroscopy showed the presence of Sitosterol. TLC showed the presence of highly polar compounds which were monitored using reverse phase chromatographic plates. Presence of ammonia in mobile phase increased the movement of alkaloid compounds as free bases.

Pure Compound Isolation

The isolated fractions showing highest density in dragendroff test was selected for further study. Fraction 10 obtained from VLC separation was subjected to silica gel column chromatography using graded system of Chloroform and Ethanol as mobile phase. The pure compound was isolated with a mixture of 5% Ethanol in 95% Chloroform and was named as RF-8. This fraction showed was tested for positive results with Dragendorff's reagent confirming the presence of an alkaloid. The sample was carefully filtered and dried and subjected for ^{1}H NMR. Presence of silica was detected in the spectrum in D-Chloroform but on dissolving with deuterated pyridine this left the impurity undissolved and gave pure compound in solution. The yield obtained was 7 mg after final purification and the compound was brown amorphous solid with a pungent odor.

A range of NMR spectra were obtained using different solvents like D-Chloroform, DMSO-d5 and Pyridine-d5 to try to resolve the confusion that arose due to broadening of peaks in some solvents.

Structural Elucidation for pure compound in D5- Pyridine

A whole set of NMR spectra in d5-Pyridine at 400 MHz frequency were used to assign the position of protons and to determine the coupling constants. Initially we used d-chloroform and DMSO as NMR solvents and tried in different frequencies at 400 and 600 MHz. Because of broadening of peaks and less solubility the spectra's were confusing but d5-Pyridine in 400 MHz gave a comparatively good set of spectrum which is used for structural elucidation.

A strong solvent peak was observed at 7.21 ppm and 4 related singlets at ppm 7.01, 7.16, 7.26 and one in the range between 7.4 - 7.45

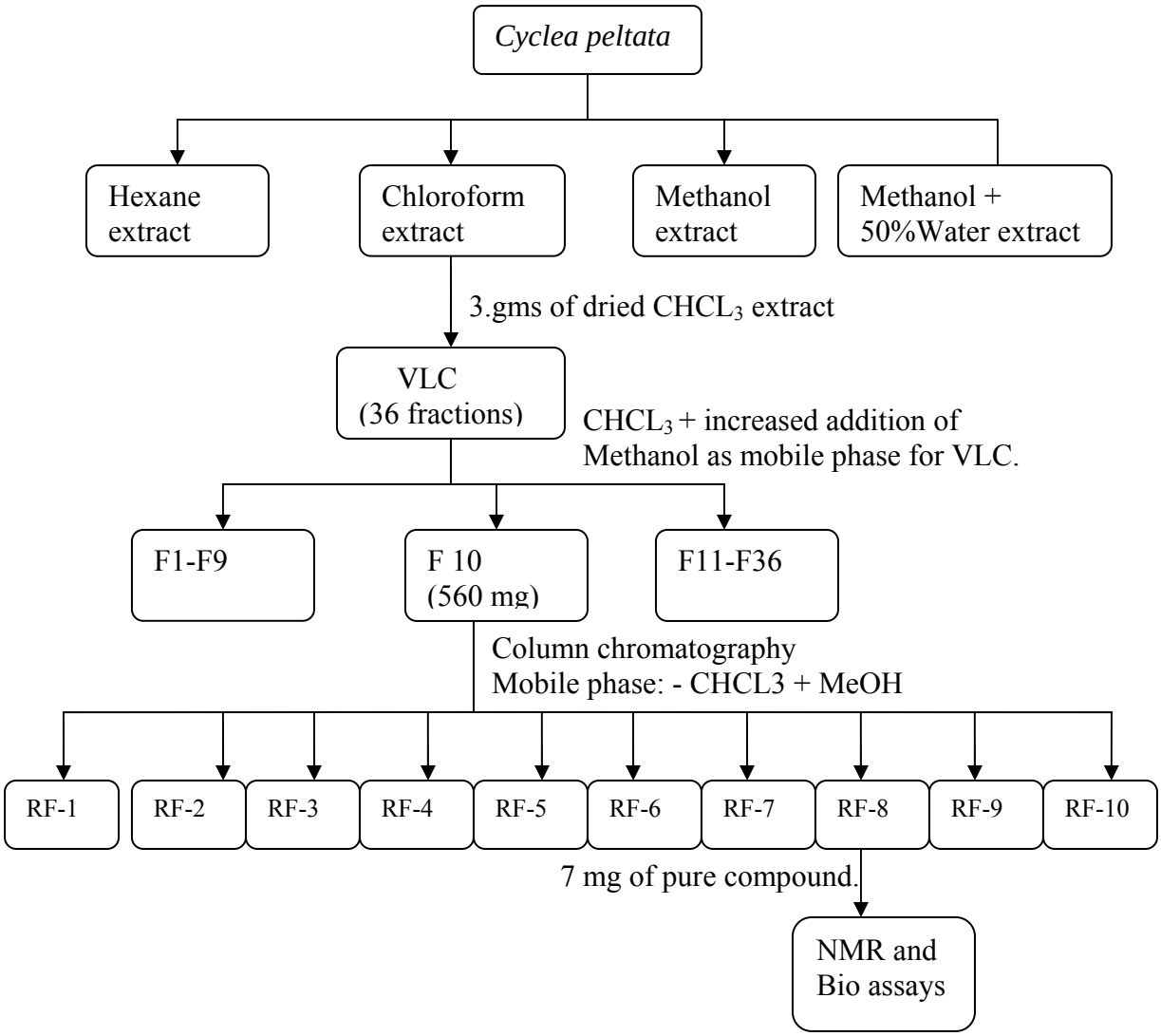


Fig: 1 flow chart for isolating the alkaloids in this work

δ ppm was assigned to ^{l}H of pyridine. Four strong singlets at 3.37, 3.43, 3.67 and 3.82 (aromatic region) was assigned to O-Me groups and singlets at 2.44 and 2.81 δ ppm assigned to protons at 2 and 2^{l}. The doublets identified at 6.95, 7.10 ppm goes to 10 and 13 protons respectively as well as doublets of doublets (dd) at 6.40, 6.70, 7.30 ppm is assigned to 10^{l}, 11^{l},13^{l} positions. The most down fielded proton is at 14th position which is a doublet whose J-value is 8.2, 1.6 Hz resonating at 7.40 ppm. Proton at 1st position shows a broad peak at 4.24 ppm which is annoyed by ^{l}H at 10th position whose coupling constants (J values) calculated as 8.6 and 1.6 respectively. 2 ^{l}H each at 3 and 3^{l} positions shows multiplets at 3.63-3.76 and 3.68-3.82 δ ppm. 4th Carbon atom has 2 protons at 3.01 ppm which is a multiplet and at 2.54 ppm which is broad or a quardet, singlet at 6.48 is assigned to ^{l}H at 5th position and 3.67 ppm is of O-Me at 6th position. Multiplets at α position shows 2 ^{l}H which resonates at 3.09 and 3.20 ppm, a singlet for O-Me at 12th position resonates

on 3.83 ppm and a doublet to 13th $^{|}$H at 7.09, whose J-value is calculated as 8.2 Hz. A doublet of doublet resonating at 7.40 ppm is assigned for 14 $^{|}$H whose J-value is 8.2, 1.6. NOESY spectrum shows a correlation between all these protons, 4a is an axial proton which can see 5th proton which also sees 4band proton of O-Me at 6th position. The broadened peak of 4b may be because of pull from 5th $^{|}$H. Proton at 1$^{|}$ is a broadened multiple peak resonating at 4.42, 4.50 ppm which shows a correlation with 8$^{|}$ proton as in NOESY spectrum. Axial proton at 4$^{|}$a resonates at 3.15 which see 5$^{|}$ (singlet) and is annoyed by O-Me at 6$^{|}$ position. 4$^{|}$b is a broad doublet or a quardet resonates at 2.86 and j-value calculated as 14.5 Hz. Protons at 3$^{|}$ position are multiplets 3.68, 3.82 and so as at α $^{|}$ resonates at 2.97, 3.10 ppm. The assignment of Protons to each carbons were done according to HMBC and correlations confirmed using COSY experiments, the resonating peak values assigned to protons and carbons are shown in Table 2.

Compiling all the NMR data the possible molecular structure of pure compound is discussed in Fig: 2, which are confirmed by the results from mass spectrum and comparison with literatures. The Mass spectra shows the molecular weight of pure compound as 623.77 and the molecular formula is $C_{37}H_{42}N_2O_6$, the compound was identified as Tetrandrine (Lin, L.Z et al,1993) [(9)], which is a popular bisbenzyl isoquinoline alkaloid with head to head connectivity.

Table: 2, assignments of $^{|}$H and ^{13}C for pure compound, with respect to the positions.

Positions	Protons	Carbons δ (ppm)
1	4.24 brd	62.7
2	2.44	42.9 (N-Me)
3	3.63,3.76 m	45.2
4	3.01m, 2.54 brd	22.75
5	6.48s	107.97
6	3.67	152.8, [56.3 (O-Me)]
7	3.44	139.2, [60.7 (O-Me)]
8	---	---
α	3.09, 3.20m	42.89
9	---	---
10	6.96 d	118.11
11	---	150.6
12	3.83s	148.9 [56.7 (O-Me)]
13	7.09 d	113.58
14	7.40 dd	---
1$^{|}$	4.42, 4.50 brm	64.4
2$^{|}$	2.8	42.2 (N-Me)
3$^{|}$	3.68, 3.82 m	45.6
4$^{|}$	3.15 m, 2.86 brd	24.51
5$^{|}$	6.81 s	113.58
6$^{|}$	3.38 s	150.1[56.3(O-Me)]
7$^{|}$	---	145.3
8$^{|}$	6.38 s	---
α$^{|}$	2.97, 3.10 m	38.30
9$^{|}$	---	135.46
10$^{|}$	6.30 dd	132.8
11$^{|}$	6.82 dd	122.49
12$^{|}$	---	155.5
13$^{|}$	7.15	122.27
14$^{|}$	---	131.44

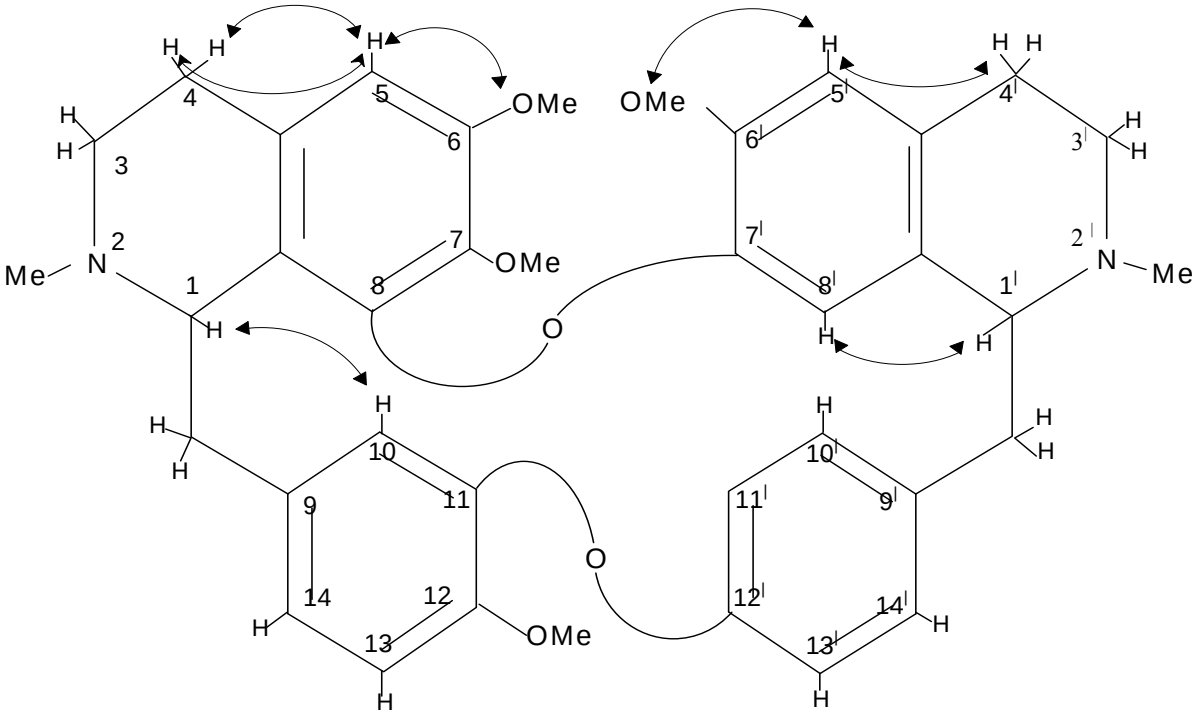


Figure 3: Structural formula for Tetrandrine a bis benzyl isoquinoline alkaloid. The double headed arrows show the NOESY correlations through space.

Conclusion

The identification of the plant *Cyclea peltata* (Hooker, J.D and Thomson, T) belonging to Menispermaceae family was confirmed by literature review and botanical descriptions. A targeted structured methodology was adopted to isolate the alkaloid and sequence of experiments conducted to identify the structure of pure compound isolated. The compound isolated was a bisbenzyl isoquinoline alkaloid, Tetrandrine using chromatographic techniques and the structure of compound confirmed by spectroscopic studies.

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