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Der Pharma Chemica, 2011, 3(1): 327-337
(http://derpharmachemica.com/archive.html)

ISSN 0975-413X
CODEN (USA): PCHHAX

Phytochemical extraction, optimization and physico-chemical characterization of two bioactive isolates from the leaves and stem of *Cissampelos pareira*

[1]K. Nagarajan, [1]Nithish Chauhan, [1]Ashu Mittal, [1]Vijendar Singh, [2]Ramesh B. Bodla and [3]Raj Kumar Tiwari

[1]Department of Pharmaceutical Chemistry, KIET School of Pharmacy, Ghaziabad
[2]Department of Medicinal Chemistry, Delhi Institute of Pharmaceutical Sciences & Research (DIPSAR), New Delhi
[3]IIMT College of Medical Sciences, Meerut

ABSTRACT

In the traditional Indian medicine, the roots and leaves of Cissampelos pareira are administered for the relief of diarrhoea and antiseptic against inflammation. The present investigation is to optimize the suitable extraction method and thereby isolating and characterizing various bioactive leads from the leaf and stem portion. Preliminary phytochemical screening reveals the presence of maximum alkaloids and moderate quantity of flavonoids and saponins with dichloromethane as solvent. In leaf portion, the hot extraction method was suited as the alkaloid content seems to be maximum, whereas in stem portion, the cold maceration was suited with more flavonoid content. This was evidenced further with higher yield values obtained for leaf extract (56% w/w) rather than stem extract (39.5% w/w). Two pure bioactive leads were isolated, each one from leaf (CP) and stem (CP**). CP* of leaf (80% $CHCl_3$-MeOH) was identified as alkaloid by qualitative analysis (Dragendroff's reagent); TLC analysis (R_f: 0.88, Toluene: EtoAc: GAA (7:2:1)); U.V analysis (λ_{max}: 265nm, Chromophore: C=N; Transition: π-π* (forbidden)); I.R spectra (2919-2850 cm^{-1}; 2346 cm^{-1}; 1724 cm^{-1}; 1166 cm^{-1}; 720 cm^{-1}) and Mass spectra (M-229) fragmentation peak for the presence of Bebecrine after elucidation. In addition CP** of stem (60% $CHCl_3$–MeOH) was identified as isoquinoline alkaloid by qualitative analysis (Hager's reagent); TLC analysis (R_f : 0.95; Toluene: EtoAC: Diethylanine (7:2:1); U.V analysis (λ_{max}: 265nm); I.R. spectra (3774–3378 cm^{-1}; 2926–2974 cm^{-1}; 23.47–2132 cm^{-1}; 1927 cm^{-1}; 1728–1665 cm^{-1}; 1378–1256 cm^{-1}; 1089–1049 cm^{-1}; 880–758 cm^{-1}); and Mass spectra (M+1 607) for the presence of cissampareine, which is reported for the first time from stem portion.*

Key words: *Cissampelos pareira*, Leaf isolate, Stem isolate, Infra red spectra, Mass spectra.

INTRODUCTION

Natural products have been one single most successful source of medicines. Each plant is like a chemical factory capable of synthesizing unlimited number of highly complex and unusual chemical substances whose structures could otherwise escape the imagination forever (1).

Cissampelos pareira Linn, family Memispermaceae, is commonly known as the midwives herb due to its long history of use in South America for women's ailments. The roots are administered against dyspepsia, diarrhoea, dropsy, cough, urinary difficulties like cystitis, dysentery, asthma and heart diseases (2). The leaves are used as an antiseptic against inflammation (3). The root is a well known tonic and diuretic, exerting a specific influence over the mucous membrane which lines the various passages. The active ingredient of pareira is an alkaloid. Buxine (4). The ethanolic extract of the roots are useful for relieving diarrhoea (5), pain and arthritis (6), ulcer (7) and prostaglandin mediated inflammation (8). The alkaloidal fraction of roots was reported to have significant *in-vitro* antioxidant activity and immunomodulatory activity in mice (9).

The active chemical constituents of C. pareira are Cissampelo flavone (10), alkaloids like hyatin, Bebeerine , Cissampareine (11), Cissamine (12), Pareirubrine A and B (13, 14). Since most of the root parts belonging to the family Menispermaceae are toxic, a study was designed to isolate and characterize the bioactive pure components only from stem and leaves, after optimizing the stem and leaves, after optimizing the suitable extraction procedure.

MATERIALS AND METHODS

1. Plant Material

The leaves and stem portion of *Cissampelos pareira* were collected from an ayurvedic shop in Meerut and authenticated by an acknowledged Botanist, Dr. Surbhi Singhal, Head, Department of Microbiology, IIMT College of Medical Sciences, Meerut, India and the voucher specimen was deposited thereafter at the department (IIMT/BD/08/02/2010/06/Tech. 2005).

2. Preliminary Phytochemical Screening

One gram of the powdered leaf and stem were macerated separately with 10ml of various solvents such as Petroleum Ether, Chloroform, Benzene, n-butanol, Ethanol, Ethylacetate, Methanol, Water, Dichloromethane and n-Hexane in different test tubes. The powdered drug mixture along with solvents are closed with a cotton plug and allowed to stand for 48 hours. The cotton plug was removed and they are filtered. The filtered filtrate was used for qualitative chemical analysis. (15). The results are expressed in Table 1 and Table 2.

3. Pilot Level Extraction for Method Optimization

In this method, various plant parts of *Cissampelos pareira* were subjected to heating at high temperature to check whether the chemical constituents degrade or not. Here, separating funnel was used for the method of optimization. The results are expressed in Table 3.

4. Mass Extraction and Extractive Yield Value

Both the leaves and stem of *Cissampelos pareira* were dried at room temperature and reduced to a coarse powder. The leaf powder (88.8 gm) was subjected to soxhlet extraction with dichloromethane (DCM) solvent separately for 72 hours at a temperature of 60°–80°C. The extracts were concentrated and the solvent was completely removed by Rotary Vacuum Evaporator (Buchi). Dark green waxy residue was obtained and the extractive yield value in % w/w was calculated subsequently thereafter. In contrast, the stem powder (70.05 gm) was subjected to cold maceration by soaking the powder with dichloro methane (300 ml) in a 1 litre bottle and cotton was plugged, leaving it aside for 7 days protected from light. The extracts were made concentrate by removing the solvent after decantation and filtration. The % extractive yield value was calculated and noted.

5. Isolation and Preliminary Identification of Bioactive Constituents

The concentrated DCM leaf extract and stem extract (each 0.5 gm) were taken in a china dish separately and Silica gel (for column chromatography, 30–70 mesh size) was then added double the quantity of extract) slowly with continuous mixing by adding sufficient quantity of dichloro methane, till the desired consistency of mixtures were obtained. It was air-dried and larger lumps were broken to get a smooth free flowing mixture.

A column of 5.0 ft. length and 16 mm of internal diameter was taken separately for leaf and stem extract and dried. The lower end of the column was plugged with absorbent cotton wool. The columns were clamped and fitted in vertical position on a stand. The column was then half-filled with n-hexane, silica gel (10.5 gm) was then poured and allowed to settle gently with the immiscible solvent n-hexane, until the necessary length of the column was obtained. The dried silica gel slurry containing the DCM extract of leaf and stem were poured in the columns separately and then eluted successively with different solvents, in the order of chloroform, chloroform: methanol (9:1), chloroform: methanol (8:2), chloroform: methanol (7:3), chloroform: methanol (6:4), chloroform: methanol (5:5), chloroform: methanol (4: 6), chloroform: methanol (3:7), chloroform: methanol (2:8), chloroform: methanol (1:9), methanol, ethylacetate: methanol (1:9), ethyacetate: methanol (2:8), ethylacetate: methanol (3:7), ethyl acetate: methanol (4:6) ethyl acetate: methanol (5:5), ethyl acetate: methanol (6:4), ethyl acetate : methanol (7:3), ethyl acetate: methanol (8:2), ethyl acetate : methanol (9:1) and ethyl acetate. Twenty one fractions were collected in the conical flask and marked. The marked fractions were subjected to TLC to check homogeneity of various fractions (16). Chromatographically identical various fractions (having same R_f values) were combined together and concentrated. They were then crystallized with suitable solvent systems. Two pure bioactive leads, each one from leaf and stem were isolated and the isolated pure fractions were tentatively identified by qualitative chemical analysis. The results of TLC analysis and qualitative analysis are expressed in Table 4 and 5.

6. Physico Chemical Characterization of Bioactive Leads

The isolated 2 bioactive leads are further identified by U.V analysis, I.R, NMR and Mass spectral analysis.

U.V Spectral Analysis

The λ_{max} of a sample was determined by U.V Shimadzu double beam model (UV-1700 Pharmaspec) at the Roorkee research and analytical laboratory. Chromophores can be identified with the 2 isolated compounds, which gives some basic information on Conjugation and groups present in any unknown compound. The results are shown in Table 6.

I.R Spectral Analysis

The infra-red spectra was recorded on Perkin Elmer 1310 model at Roorkee research & analytical laboratory. The isolated test compounds were subjected to I.R by solid potassium bromide-pelleting technique with the study of absorption of infrared radiation functional groups were identified clearly (17) and it is a powerful fool for structure elucidation. The I.R results interpreted for the 2 isolated leads are expressed in Table 7.

^{1}H NMR Analysis

The 'H NMR spectra of 2 compounds were recorded on Bruker's 300 MHz instrument using $CDCl_3$ as the solvent at Sophisticated Analytical Instrument facility (SAIF), Central Drug Research Institute, Lucknow. The test compounds nucleus can be revealed and the number of

protons predicted the structure of bioactive isolate with the help of 'H NMR analysis. Several natural products are very well studied by modern NMR methods (18).

Mass Spectral Analysis

The electrospray mass spectra for the isolated 2 test compounds were recorded on a Thermo Finnigan LCQ Advantage max ion trap mass spectrometer at SAIF, CDRI, Lucknow. The 10µl samples (dissolved in solvent such as methanol/acetonitrile/water) were introduced into the ESI source through Finnigan Surveyor autosampler. The mobile phase (90:10 MeoH/ACN: H_2O) flowed at the rate of 250µl/min by Ms pump. Ion spray voltage was set at 5.3 KV and capillary voltage 34V. The MS scan run up to 2.5 min and the spectra print outs are averaged of over 10 scan at peak top in TIC. The mass spectra gives information on various types of peaks and determining the molecular formula for the isolated compounds after successful interpretation (19).

RESULTS

1. Preliminary Phytochemical Screening

The results of the preliminary phytochemical screening of stem and leaf of *Cissampelos pareira* are expressed in Table 1 and Table 2.

Table 1. Preliminary Phytochemical Screening of *Cissampelos pareira* Stem Extract

Solvents	Alkaloids (Dragendroff's)	Glycosiades (Borntragers)	Steroids (Salkovaski)	Terpenoids (Rochan Test)	Flavonoids (Shinoda)	Carbohydrates (Molisch)	Saponins (Foam)
Petroleum ether	++	–	–	–	+	++	+
Chloroform	–	–	–	–	+	+	+
Benzene	–	–	–	–	–	–	–
n-Butanol	+	+	–	–	+	++	+
Ethanol	++	–	–	–	+	++	+
Ethyl acetate	++	–	–	–	+	++	+
Methanol	++	–	–	–	+	++	–
Water	++	–	+	–	–	++	++
Dichloro methane	++	–	+	–	–	++	++
n-Hexane	–	–	–	–	+	++	–

++ Presence of more active constituents *+Presence of moderate active constituents*
–No active constituents present.

2. Pilot level extraction for method optimization

The results of optimization in pilot level extraction study of leaf and stem portion in dichloromethane of *Cisampelos pareira* are given in Table 3.

Table 2. Preliminary Phytochemical Screening of *Cissampelos pareira* Leaf Extract

Solvents	Alkaloids (Dragendroff's)	Glycosiades (Borntragers)	Steroids (Salkovaski)	Terpenoids (Rochan Test)	Flavonoids (Shinoda)	Carbohydrates (Molisch)	Saponins (Foam)
Petroleum ether	++	–	–	–	+	+	+
$CHCl_3$	++	–	–	–	–	–	+
Benzene	–	–	–	–	–	–	–
n-Butanol	++	+	–	–	–	++	+
Ethanol	++	–	–	–	–	++	–
Ethyl acetate	+	–	–	–	+	+	+
Methanol	+	–	–	–	++	++	–
Water	–	–	–	–	+	++	++
Dichloro methane	–	–	–	–	–	+	++
n-Hexane	–	–	++	–	–	+	–

++ Presence of more active constituents +Presence of moderate active constituents
–No active constituents present.

Table 3. Pilot Level Extraction Study for suitable method optimization in leaf and stem of *Cissampelos pareira* Linn.

Chemical Constituents	Solvent	Leaf	Stem
Alkaloid (Dragendroff Test)	Dichloro methane	++	++
Glycosides (Borntragers Test)	Dichloro methane	–	–
Flavonoid (Shinoda Test)	Dichloro methane	+	+
Steroid (Salkovaski Test)	Dichloro methane	–	–
Saponins (Foam Test)	Dichloro methane	+	+
Carbohydrates (Molisch Test)	Dichloro methane	++	++

++ Presence of more active constituents, +Presence of moderate active constituents
–No active constituents present.

3. Phytochemical Extraction

Based on the method of optimization, it was found that the stem extract was subjected to cold maceration to avoid the degradation of active constituents and subsequently, the leaf extract was subjected to hot percolation as well as to cold maceration in order to compare their extractive yield values. The extractive yield value of stem was found to be 39.5% w/w. The extractive yield value of leaf (Hot percolation) was found to be 56.6% w/w, whereas the extractive yield value of leaf (Cold maceration) was found to be 45.19% w/w, which clearly shows that more active constituents are present in leaf irrespective of the method used for extraction.

4. Isolation and preliminary identification of bioactive leads from *Cissampelos pareira*

Elution of leaf drug in column with 80% chloroform-methanol, i.e. (Fraction 3) yielded dark green amorphous powder, R_f: 0.88 (Toluene: ethyl acetate : Glacial acetic acid: 7:2:1) and positive with Dragendroff's reagent for alkaloid and was designated as CP*.

Similarly, the elution of stem drug in column with 60% chloroform-methanol, i.e. (Fraction 5) yielded yellowish green amorphous powder, R_f: 0.95 (Toluene:ethyl acetae:diethylamine, 7:2:1) and positive with Hager's reagent for the presence of isoquinoline alkaloid and

designated as CP**. The two bioactive leads (CP* and CP**) are identified as alkaloids by TLC analysis and Qualitative Chemical Analysis.

5. Physico-chemical Characterization of Bioactive Leads from *Cissampelos pareira*.

The final structure of the isolated bioactive leads, CP* and CP** were confirmed by U.V, I.R, 'H NMR and Mass spectral datas. Preliminary phytochemical screening and TLC results have revealed that the pure components (CP* and CP**) were basically an *alkaloid class* of compound.

Test Compound 1 (CP*)

The λ_{max} obtained by U.V. analysis for the test compound CP* was found to be 265 nm, which clearly indicated π-π* forbidden transition having the chromophore C=N. I.R spectra shows characteristic functional group bands for the presence of Bebeerine at 2919–2850 cm^{-1} (C–H St. alkanes), 2346 cm^{-1} (N–H St. amino acid), 1724 cm^{-1} (C=C St. Cyclic alkenes), 1166 cm^{-1} (Ar C–H St. aromatic hydrocarbon, C–O–C St. ether) and 720 cm^{-1} (CH_2 γ). 'H–NMR spectra reveals the presence of aromatic proton at 7.27 δ; N-methyl proton at 2.32δ; O-methyl proton at 3.41δ; OH-aromatic proton at 5.36δ; C_6H_5O CH ring proton at 3.88δ; Saturated cyclic CH_2 ring proton at 1.25δ, which clearly indicates the evidence of Bebeerine structure. Mass spectral analysis also indicates the evidence of Bebeerine as the final structure of isolated alkaloid pure component with the fragmented ion peak at (M–229). All these spectral data suggests that the alkaloid leaf isolate (CP*) eluted from column was found to be Bebeerine.

Fig. 1 Chemical structure of Bebeerine

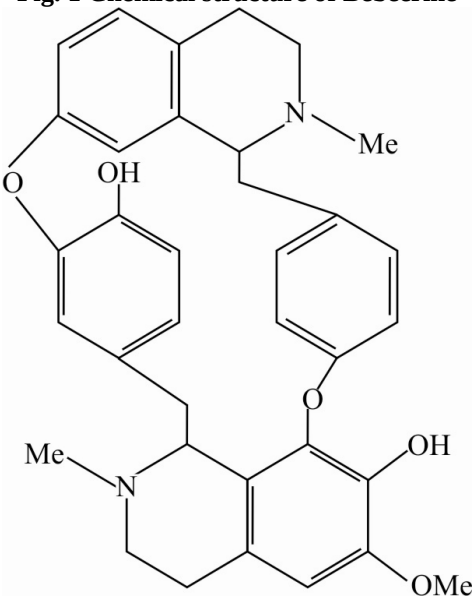


Fig. 2 Slice through the graph representing IR Spectra of bioactive lead CP* from *Cissampelos pareira*.

Test Compound 2 (CP)**

The λ_{max} obtained by U.V-analysis for the test compound CP** was found to be 265 nm, which clearly indicated π-π* transition having the chromophore C=N. I.R spectra shows characteristic functional group bands at 3774–3378 cm^{-1}, 2926–2974 cm^{-1}, 2347–2132 cm^{-1}, 1927 cm^{-1}, 1728-1665 cm^{-1}, 1378–1256 cm^{-1}, 1089–1049 cm^{-1} and 880–758 cm^{-1} for the presence of O–H St. of alcohols, C–H of halogens, $–N^{+}\equiv C^{-}$ of isonitriles, C=0 St. of amide, C=O St. of aldehydes, OC–OH St. of Carboxylic acid, C=C=C St. of alkenes and C–H St. of aromatic hydrocarbons respectively.

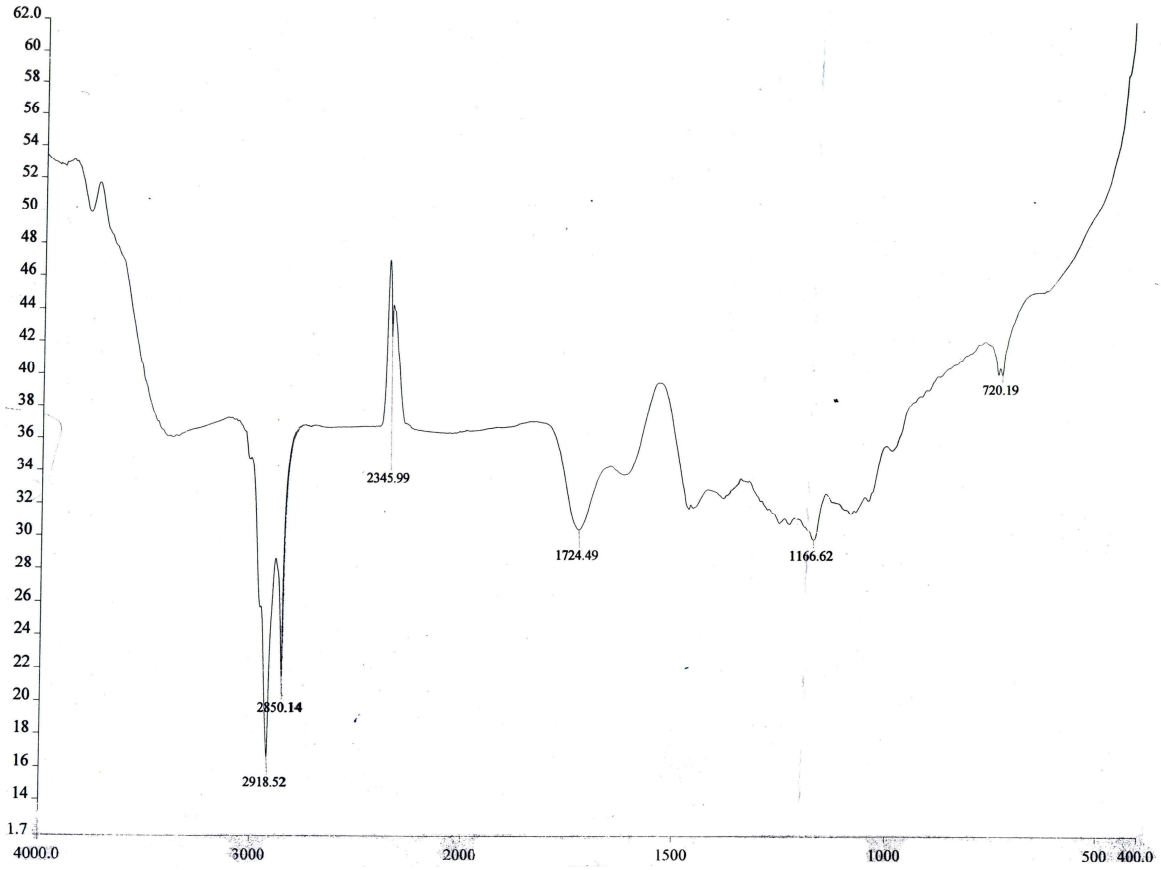


Fig. 3 'H–NMR Spectra of pure alkaloid CP* from *Cissampelos pareira*.

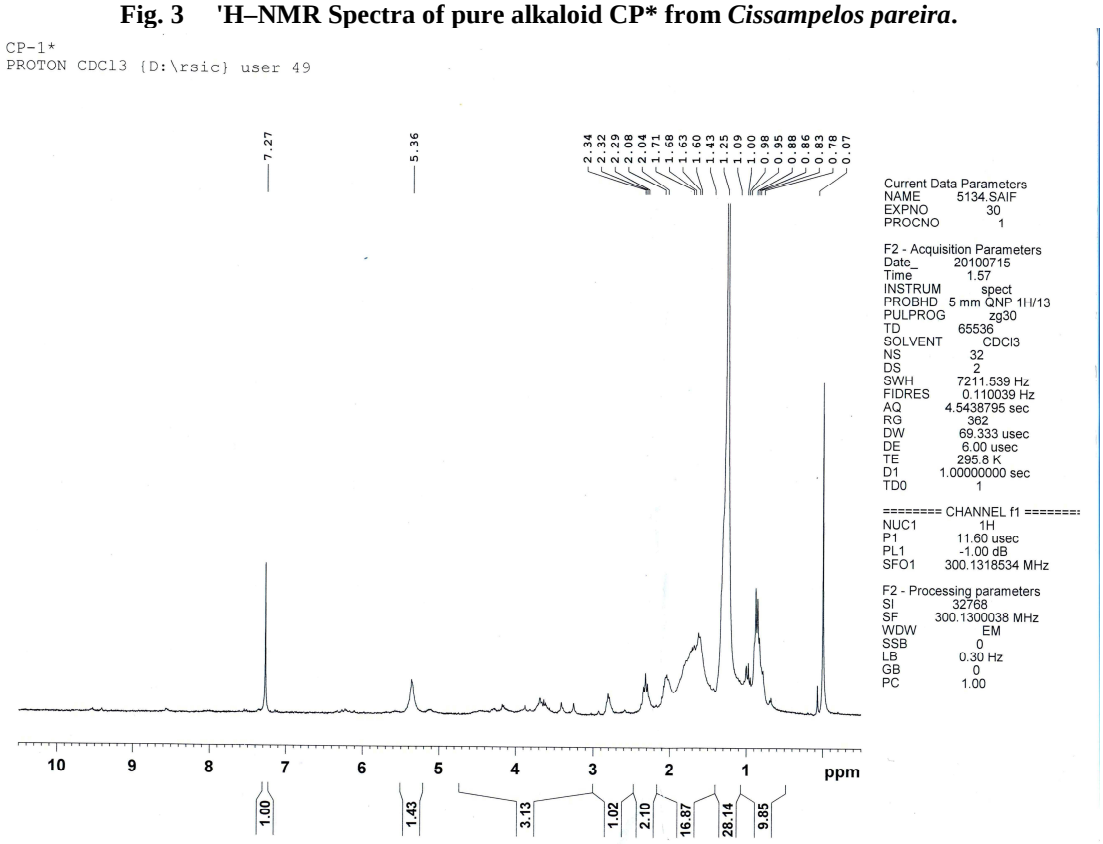


Fig. 4 Mass spectrum of an alkaloid component CP* of *Cissampelos pareira*.

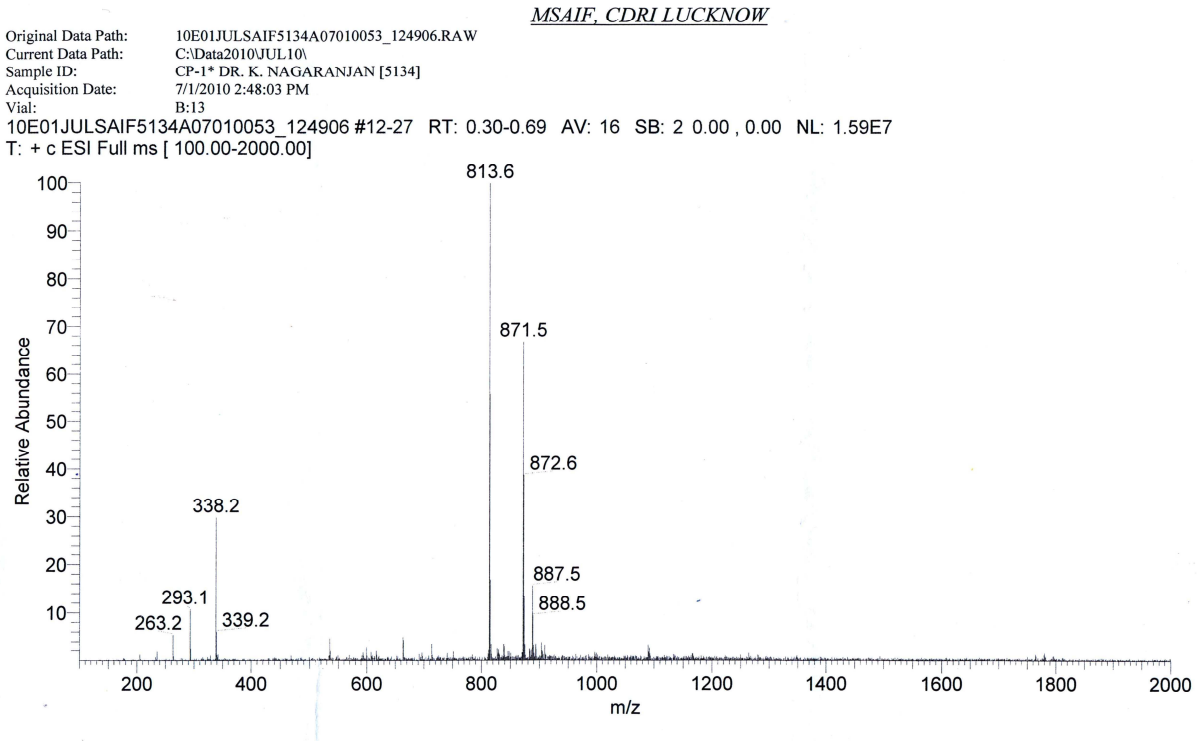


Fig. 5 Slice through the graph representing IR Spectra of bioactive lead CP from *Cissampelos pareira*.**

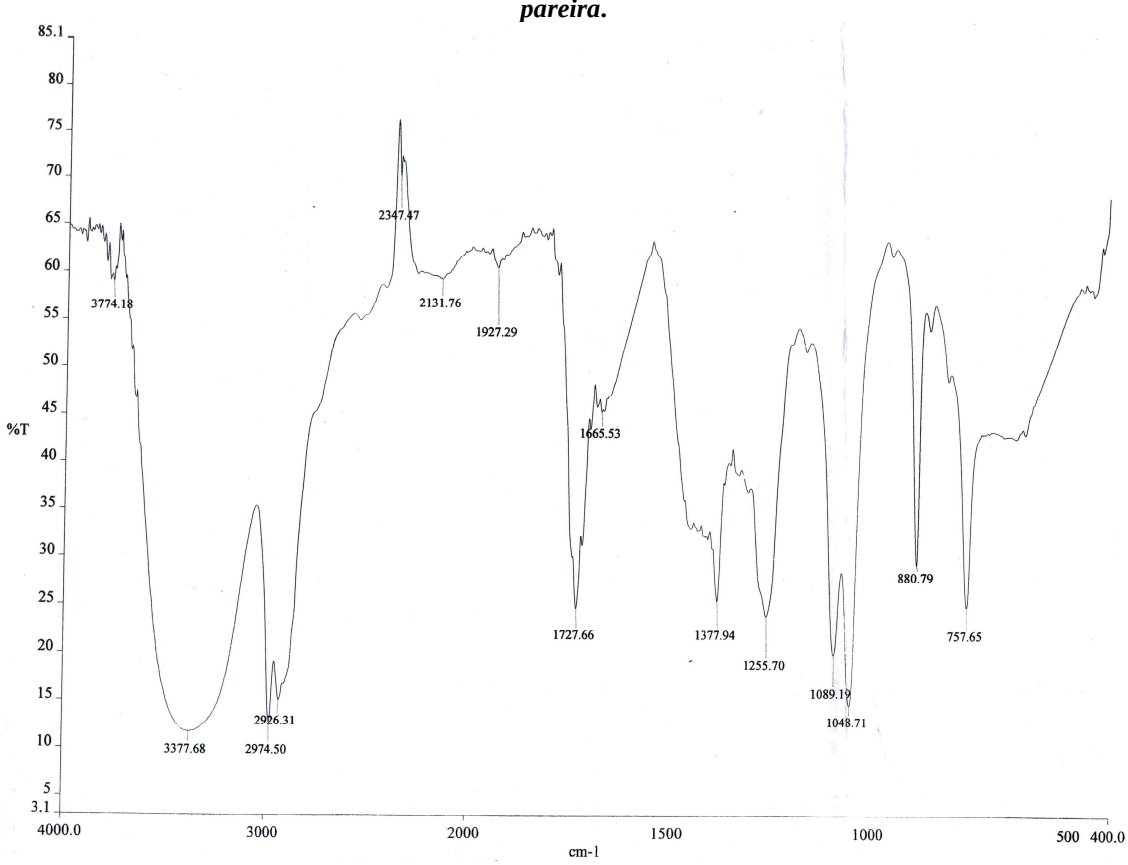


Fig. 6 'H–NMR Spectra of pure alkaloid CP from *Cissampelos pareira*.**

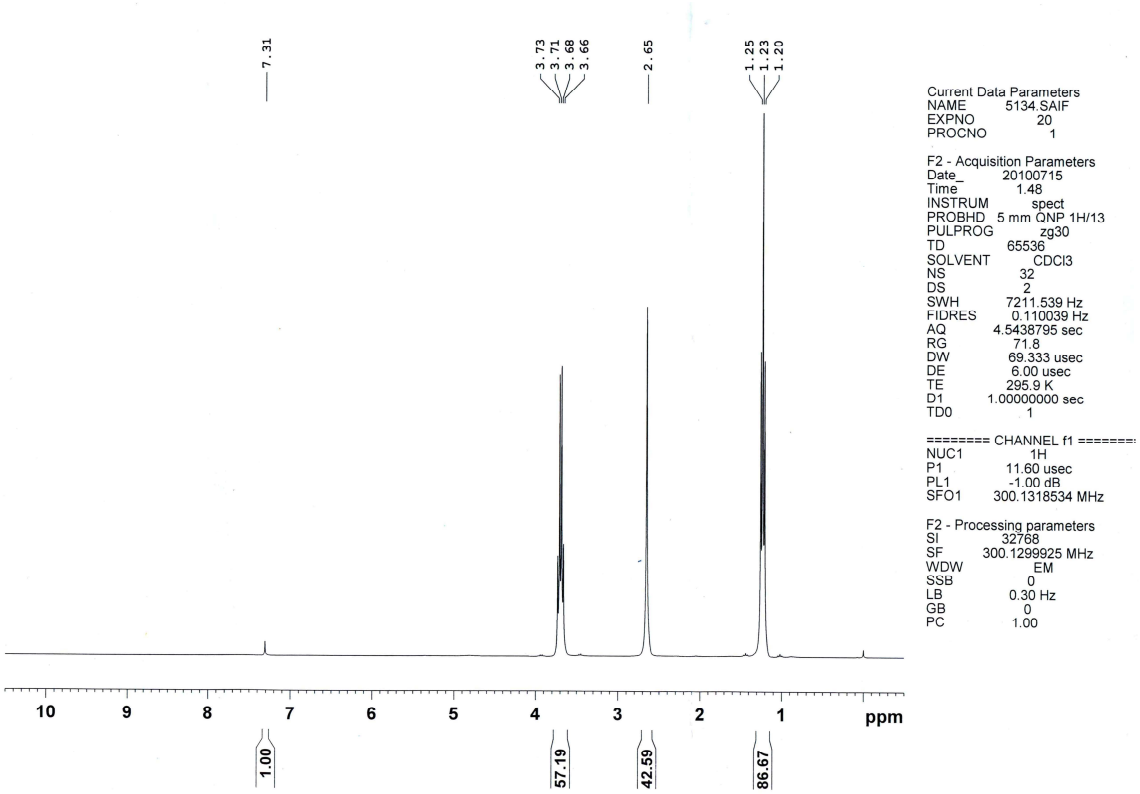
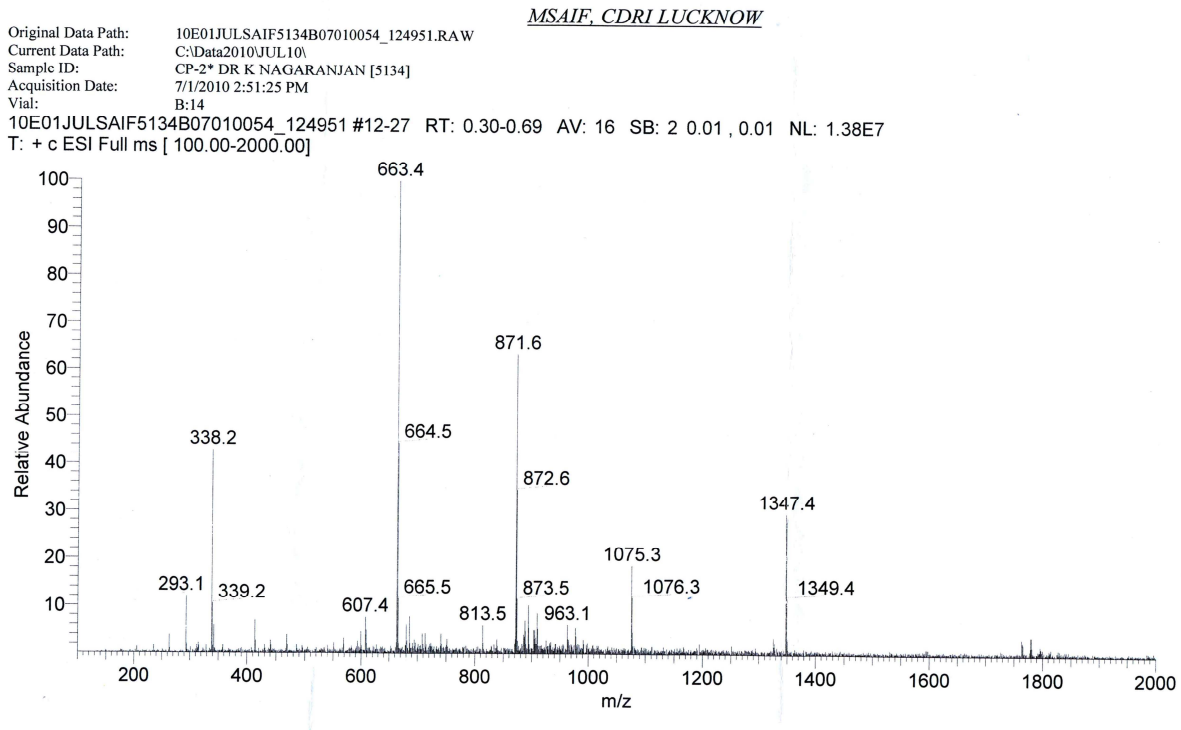


Fig. 7 Mass spectrum of an alkaloid component CP of *Cissampelos pareira*.**



'H NMR spectra reveals the presence of aromatic proton at 7.31δ; N-methyl proton at 2.65δ; Benzyl proton at 3.71δ; Cyclic ring proton at 1.23δ, which clearly indicates the presence of Cissampareine, having bisbenzoyl isoquinoline nucleus. Mass spectral analysis also indicates the evidence of Cissampareine as the final structure of isolated isoquinoline alkaloid pure isolate with the corresponding (M+1) ion peak at 607, bearing the molecular formula $C_{37}H_{38}N_2O_6$. All these spectral data suggests that the isoquinoline alkaloid stem isolate

(CP**) eluted from column was found to be Cissampareine. This is the first evidence based report of Cissampareine in Stem portion.

DISCUSSION

Preliminary phytochemical screening of powdered leaf and stem of *Cissampelos pareira* indicates the presence of alkaloidal active constituents in various organic solvents used. Based on this, solvent selection was made for leaf and stem and extraction was performed in Soxhlet apparatus and cold maceration using dichloromethane as solvent. The method of optimization was followed for the leaf and stem. In case of leaf, the hot extraction method was suited because there is a maximum alkaloid content in leaf, flavonoid was degraded while heating, saponins were also slightly degraded and the carbohydrates were also present in larger quantity. In case of stem, the cold maceration showed the presence of flavonoid and saponins with slight degradation, complete degradation of Steroids and the presence of larger quantity of Carbohydrates.

Hence with this, we confirm that for leaf, the maximum alkaloid and carbohydrate can be obtained by hot extraction of the leaf part. In case of stem part, alkaloid and flavonoid seems to be maximum by cold maceration. With the extractive yield values obtained from the stem and leaf extract of *Cissampelos pareira*, it is evident that leaf extract contain more active constituents when compared with stem extract with corresponding extractive yield values as 56.6 % w/w and 39.5% w/w respectively.

Two fractions (CP* and CP**) were isolated with the help of column chromatography and preliminary identification by TLC using different mobile phases for the isolation of alkaloids. The R_f values of the fractions CP* and CP** were also determined to know tentatively the presence of alkaloids, which were already explained in results section. Later on, the isolated 2 compounds were subjected for U.V, I.R, 'H NMR and Mass spectral analysis. The results of spectral datas suggests that 2 alkaloidal bioactive leads were obtained, namely Bebeerine from leaf and Cissampareine from stem portion. This is the first evidence based report for the presence of Cissampareine in stem portion. As most of the root portions belonging to the family Menispermaceae are toxic with thorough study of literatures cited, we excluded the root and taken the stem portion for isolating and identifying the bioactive lead, along with the leaf portion, since major alkaloidal constituents are in leaf. This evidence based approach of thorough optimization, and chemical confirmation of two alkaloidal bioactive leads from *Cissampelos pareira* is very much essential for further exploration of comparing bio-potency of both the plant parts investigated for various experimental animal studies and clinical studies.

Acknowledgement
The authors are very much thankful to Dr. D.K. Dikshit, Deputy Director and Head, Sophisticated Analytical Instrument Facility (SAIF), Central Drug Research Institute (CDRI), Lucknow for providing all spectral datas in time.

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