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Analysis of pyrrolizidine alkaloid from *Crotalaria retusa* L

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are produced by numerous plant species in nature. Numerous intoxications in animals and humans caused by the consumption of certain plants were attributed from the middle of this century to compounds of vegetable origin, the pyrrolizidine alkaloids. Phytochemical studies on the crude extract of the leaves of Crotalaria species (Fabaceae family) were conducted. These plants are commonly used in traditional medicine such as for treatments of uterine hemorrhages, dysentery, and inflamed wounds. Although it has medicinal value, it was also reported that leaves of this plant containing pyrrolizidine alkaloids (PAs) are toxic to the humans and animals, which are hepatotoxic, pneumotoxic, genotoxic, neurotoxic, and cytotoxic. The purpose of current investigation is to study the PAs content of Crotalaria species. The structure of desired phyto constituents were elucidated using Liquid Chromatography-Mass Spectroscopy and a combination of one- and two dimensional 1H and 13C Nuclear Magnetic Resonance spectroscopy. In this study, one potent pyrrolizidine alkaloids, monocrotaline was isolated from Crotalaria retusa. This results suggests that it should be a safety consideration in consuming this plant for traditional medicine because it is also contains toxic pyrrolizidine alkaloids. The structurally elucidated toxic compound, monocrotaline was used for further toxicological investigations.

Keywords: *Crotalaria* species, Phytochemistry, LC-MS, NMR spectroscopy, Pyrrolizidine alkaloid.

INTRODUCTION

Secondary metabolites, especially phytoconstituents, when isolated have pronounced pharmacological actions on animal organs and their system. Several bioactive phytoconstituents were purified and used in the formulation of drugs such as digoxin, morphine, reserpine, vincristine, vinblastine, quercitin [1–3]. The plants contributes their significant phyto constituents in the preparation of drugs belonging to Asteraceae, Caesalpiniaceae, Euphorbiaceae, Fabaceae, Lamiaceae, Malvaceae, Mimosaceae, Moraceae, Moringaceae, Poaceae, Rubiaceae, Solanaceae and Zingiberaceae [4–5].

Several investigations were carried out and many plant compounds are identified to have antibiotic properties [6]. The traditional healing systems utilizing herbal remedies are the significant source for the discovery of new antibiotics [7]. Some of the compounds already produced from herbal remedies have antagonostic activity against multi-resistant bacterial strains [8]. The discovery of unrevealed secrets in already known toxic herbal medicines facilitates the further synthesis of potent drugs with reduced toxicity.

Numerous taxa of the genus Fabaceae are rich in pyrrolizidine alkaloids exhibiting anti-leukemic, anti-neoplastic, anti-diabetic and anti-cardiac properties [9]. Pyrrolizidine alkaloids especially diesters, in addition to medicinal properties, cause hepatotoxic, mutagenic and carcinogenic properties against humans and cattle [10–11]. We aimed at studying the impact of pyrrolizidine alkaloid in causing the toxicity in the organisms. As a part of the structure based toxicological investigations, we standardized the protocols for the purification and structural determination using analytical techniques. During the current investigation, the purification of the putative compound was

conducted through analytical techniques such as preparative and analytical HPLC. The purified putative constituent was further subjected for molecular determination using LC-MS and ^{1}H and ^{13}C NMR spectroscopic analysis. In future, the elucidated compound was about subjecting for structure based toxicological investigation, provides information for designing the potent drugs with less toxicity.

MATERIALS AND METHODS

Plant material

Leaves of *Crotalaria retusa* L. were collected in Rushikonda area, Visakhapatnam, India, in March 2012, and identified by Dr. Lakshminarayana, Andhra University. A voucher specimen (A.U. (B.D.H.) 5527) was deposited at the Herbarium of the Department of Botany, Andhra University, India.

Methods

Spectra were recorded on a Shimadzu UV spectrometer on Liquid Chromatography– Mass Spectrometer (LC-MS) were used for molecular weight determination. 1H and 13C NMR spectra was recorded on Nuclear Magnetic Resonance - AV 400 using TMS as an internal Standard. Thin layer chromatography was carried out on silica gel 60 GF254 plates (Merck, Germany), chloroform-methanol-ammonia (80:10:1) as mobile phase and Dragendorf reagent as spot detection

Extraction

The air dried Crotalaria species (4 kg) was extracted with methanol in a Soxhlet apparatus for 75 hours. Residue after evaporating the solvent added with water was extracted with diethyl ether in a separating funnel to give diethyl ether fraction and aqueous phase I. Aqueous phase I was mixed and extracted with ethyl acetate give ethyl acetate fraction and aqueous phase II. After addition of NH4OH until pH 10, aqueous phase II was extracted with CHCl3, which give organic fraction and aqueous phase III. After solvent evaporation under reduced pressure, the CHCl3 fraction as a yellow brownish solid residue (5.85 g) was separated on Silica Gel 60 H (pro TLC) with hexane-ethyl acetate (50:50) to methanol (100%) in a gradient eluting method by Dry Column Vacuum Chromatography (DCVC). The ethyl acetate- methanol fractions (10:90 to 1: 99) give 100 mg of white crystal named compound 1. The crude extract obtained was dissolved in methanol and then filtered before subjecting to LC-MS and NMR analysis.

Analysis of HPLC– Electro Spray Ionization (ESI) MS experiments

This was performed on Shimadzu HPLC auto sampler system coupled with Shimadzu LCMS-2010A ESI (Electron Spray Ionization) Mass spectrometer (Shimadzu, Japan). The sheath and auxillary nitrogen gas acts as nebulizer gas with flow ratio of 70:30. The Shimadzu HPLC instrument is equipped with a Luna C18 reverse phase column (250mm X 4.6mm, 5µ), an LC-20AT pump and UV/Visible detector SPD-20A (254 nm), for the analysis. A 20 µl Hamilton injection syringe was engaged with the system. The system was controlled by a PE Sciex Mass Chrom data system (version 1.1.1). The sample was analyzed with operational conditions for the ion spray interface having MS mode with 200-1000 u, step size with 10 u and dwell time 5 min. LC–ESI-MS–MS experiments were performed online directly after LC separation. The MS-MS spectra were considered to be significant for signal-to-noise ratio higher than 5.0. The separation of crude sample was performed at a flow rate of 1 ml/min under uniform conditions. MS spectra were recorded in positive ionization mode and analyzed with a capillary temperature and voltage of $225^{0}C$ and 43 V respectively in second spectral scan. The selected daughter ions from initial MS fragmentation were selected for further fragmentation.

Nuclear Magnetic Resonance spectrometry (NMR)

The HPLC purified fraction was dissolved in 7mL of 99.9% $CDCl_3$ (Deuteriated Chloroform) for recording the NMR spectra using Bruker Avance 400 Ultra-shield - AV 400 (Bruker BioSpin, Germany) NMR spectrometer with 400MHz magnet with QNP probe (5mm) for the determination of structure for the putative compound using ^{1}H and ^{13}C NMR. The solvent and Tetramethylsilane (TMS) were used as the internal standards for ^{13}C and ^{1}H signals respectively. The chemical shift values for both ^{13}C and ^{1}H NMR signals were recorded for the elucidation of the structure for the targeted putative compound. The structure of the putative compound was deduced and identified as monocrotaline.

Statistical analysis

All the qualitative tests were conducted and evaluated through Linear regression analysis ($R^2 \leq 1$).

RESULTS AND DISCUSSION

The purification and structural elucidation of the putative alkaloid from *Crotalaria retusa* is the mile stone in the toxicological studies on toxic compounds such as pyrrolizidine alkaloids. During the present investigation, crude methanolic extract of *Crotalaria* species was subjected for purification of putative constituent through HPLC and their structure was elucidated using LC-MS and NMR spectroscopic analysis.

Liquid Chromatography-Mass Spectrometry (LC-MS)

A pyrrolizidine alkaloid in crude extract of *Crotalaria* species was directly analyzed through ESI-MS method. The LC-ESI-MS analysis of eluent showed the ion signals for pyrrolizidine alkaloid at *m/z* 326 $[M+1]^+$, *m/z* 276 $[M+1]^+$, *m/z* 128 $[M+3]^+$ and 102 $[M-5]^+$ (Fig. 1). The results obtained represented that the positive ESI mode is good for the detection and their primary structural characterization of HPAs in a complex mixture. The total ion chromatogram of alkaloid fraction of *Crotalaria retusa* was falls in ESI scan mode. The tandem mass spectrum of authentic standard of Crotaline was first analyzed through direct infusion. The sample chromatogram was analyzed and compared with the reference peak. The Liquid chromatography - Mass analysis (LC–MS) was resulted with an empirical formulae $C_{16}H_{23}NO_6$ having *m/z* = 326 [M+1] in positive ionization. The alkaloids were well separated under the current HPLC conditions, which were confirmed through MS–MS precursor ions scan of *m/z* 276 and *m/z* 256. The elemental analysis was done and the share of each element in the molecular stability is deciphered. The calculated share of elements in putative molecule are C = 59.18%; H = 7.03%; N = 4.23%; O = 29.56%, obtained C = 59.07%; H = 7.07%; N = 4.30%; O = 29.56%. The Mass spectral analysis was also done for the pyrrolizidine alkaloid from *Echium vulgare* and similar success was observed in the elucidation of the compound [14].

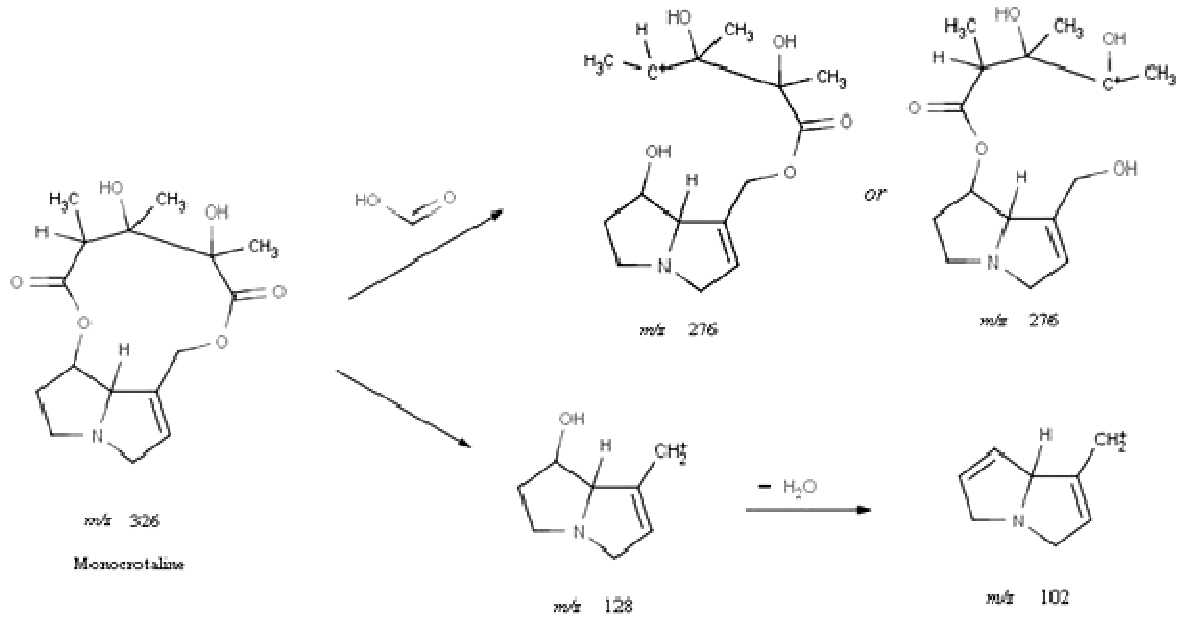

Figure 1: Proposed fragmentation pathway of monocrotaline. The ions at *m/z* 128 and *m/z* 102 are two diagnostic ions for retronecine type of pyrrolizidine alkaloids. The ion at *m/z* 276 corresponding to [MH – CO_2]+ is the most abundant character ion and was selected for analysis of putative alkaloid

NMR spectroscopic analysis

The chemical structure of the putative compound was identified according to their ^1H and ^{13}C – NMR data (Fig. 2-3). The ^{13}C-NMR spectrum of the analyzed alkaloid fraction deciphered the presence of 16 carbons. The signal at δ 174.0 ppm confirmed the presence of the carbonyl group, while the signal at δ 173.5 ppm is evidence for the oxygenated ring carbon. The spectrum revealed three methyl carbons at δ 78.8 ppm, 33.6 ppm and 21.9 ppm in 23, 21 and 15th position. The signal obtained at δ 17.7 ppm indicated the presence of hydroxylated carbon. The ^1H-NMR spectrum revealed that the two doublets at δ 5.04 ppm (*J* = 5.2 Hz) and δ 6.03 ppm for the protons of carboxyl group which is typically located at positions 7 and 9. The singlet at δ 4.9 ppm is due to 3H while one methylene signals at position 1 and 2 appeared as multiplets between δ 2.58–3.50 ppm (Table – 1).

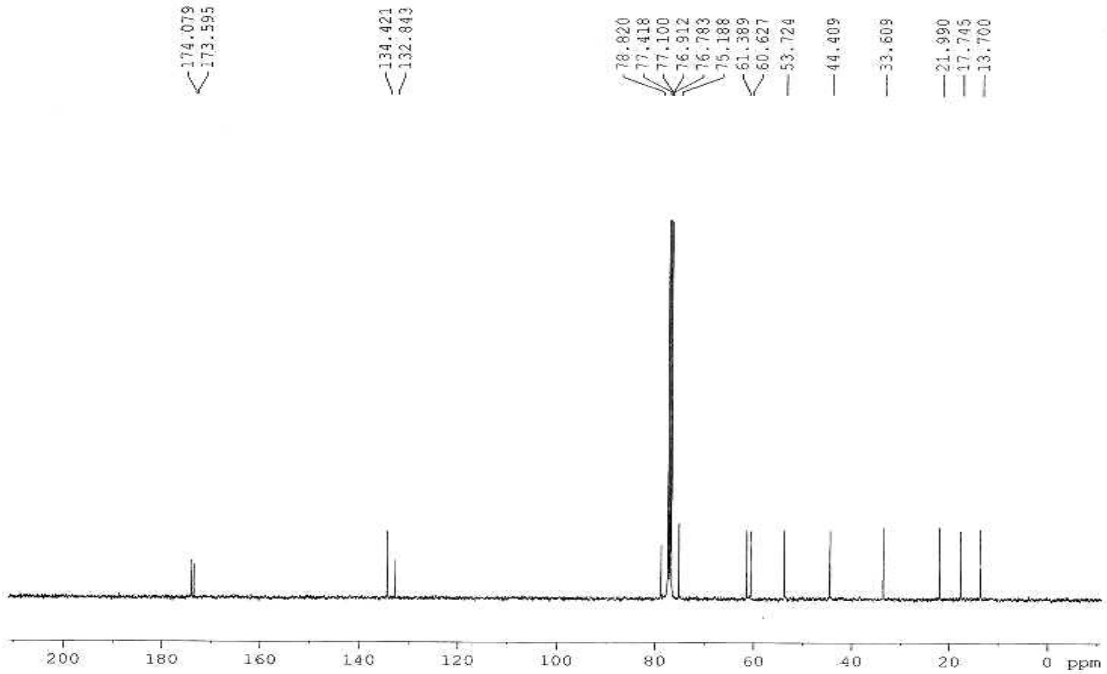

Figure 2: ^{13}C NMR spectra of monocrotaline alkaloid analyzed in Bruker Avance 400 Ultra-shield NMR spectrometer

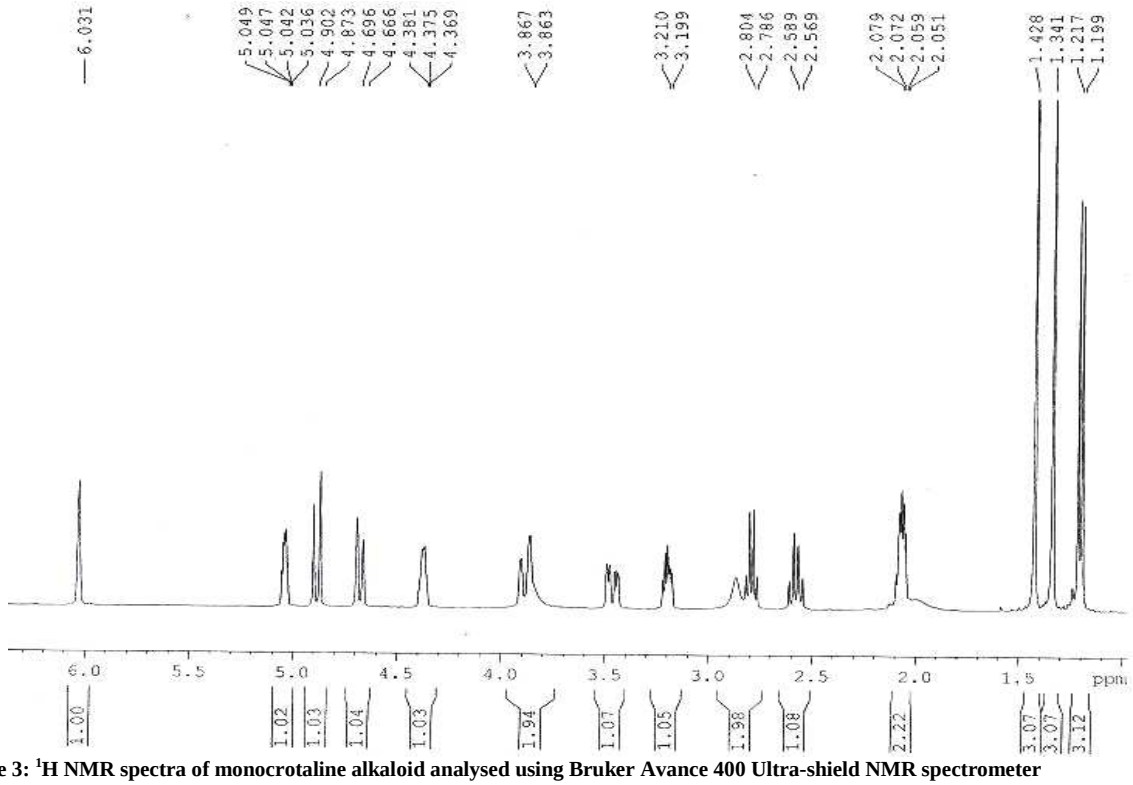

Figure 3: ^{1}H NMR spectra of monocrotaline alkaloid analysed using Bruker Avance 400 Ultra-shield NMR spectrometer

The structure of the putative desired compound was identified based on all the above spectroscopic analysis of alkaloid fraction from methanolic extract of *Crotalaria retusa* (Fig. 4). The structure deduced in present investigation was identified as Monocrotaline; pyrrolizidine alkaloid through comparison with literature data. The compound with 98% similarity was previously reported for *Senecio* species [10]. The NMR analysis for both ^{1}H and ^{13}C was done accordingly through standard protocols and similar success was observed in the structural elucidation of β-glucan polysaccharide isolated from *Auricularia polytricha* [15]. We also standardized the protocol for structure based toxicological studies on structurally determined pyrrolizidine alkaloid, monocrotaline isolated from *Crotalaria retusa*.

Table 2: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (75 MHz) spectral data of alkaloid fraction dissolved in CD_3OD (δ in ppm, *J* in Hz)

Position	^{1}H-NMR (δ ppm)	^{13}C-NMR (δ ppm)
1	–	134.4
2	–	132.8
3	2.58 (q, 1H, *J* = 8.0 Hz)	44.4
4	–	–
5	2.80 (q, 2H, *J* = 7.2 Hz)	53.7
6	–	60.6
7	3.49 (h, 1H, J_o = 4.4 Hz)	61.3
8	1.21 (h, 3H, *J* = 7.2 Hz)	75.1
9	4.90 (s, 1H)	76.7
10	–	–
11	–	173.5
12	–	77.4
12[a]	3.86 (d, 2H, *J* = 1.6 Hz)	13.7
13	6.03 (s, 1H)	78.8
13[b]	5.04 (dd, 1H, J_m = 5.2 Hz)	17.7
14	2.07 (q, 2H, *J* = 11.2 Hz)	21.9
15	1.42 (s, 3H)	174.0
16	1.34 (s, 3H)	33.6

NOTE: s = singlet; h = hextet; q = quartet; d = doublet; J= coupling constant; dd = double doublet; m = middle; o = octet.

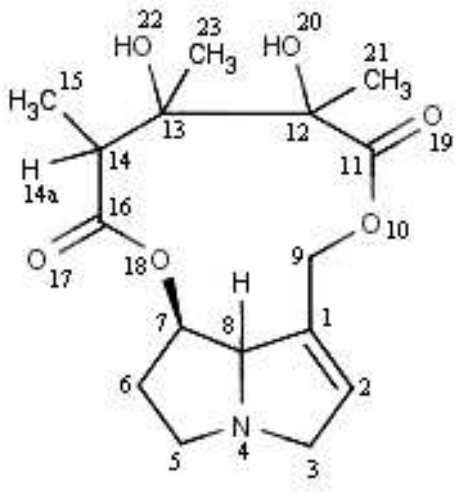

Figure 4: The schematic representation of the putative compound analysed through LC-MS, ^{1}H and ^{13}C NMR analysis [(4R, 5R, 6R, 9S, 16R) – 5,6-dihydroxy – 4,5,6,9 – tetramethyl-2,8-dioxa-13 – azatricyclo (8.5.1.0 ^{13,16}) hexa dec – 10-ene- 3,7-dione] (monocrotaline)

Several analogue were obtained, but only one analogue of monocrotaline was significant for having less toxicity and retaining their bioactive properties without any alteration (data not shown). We are standardizing the protocol for conducting the structure based *in silico* toxicological studies on structurally determined pyrrolizidine alkaloid, monocrotaline isolated from *Crotalaria* species.

CONCLUSION

An efficient, reliable and sensitive LC–MS analytical method with significant precision and accuracy developed in present investigation facilitates the purification of medicinally important pyrrolizidine alkaloids from plant extracts. The ESI–MS experiments fragmented the diagnostic ions for the desired compound from which both the similarity and diversity in structure was detected implies the specificity and sensitivity of the method. The spectra obtained through ESI fragmentation in LC–MS, 1H and 13C NMR may helps in determination of the structure of putative compound. In the present investigation, a pyrrolizidine alkaloid was purified through HPLC and their molecular mass and structure was determined through online coupled LC-MS and NMR spectroscopic analysis respectively. The structural elucidation of the toxic pyrrolizidine alkaloids provides a way for the structure based *in silico* toxicological studies and their modeling in drug design against various potent diseases in addition to the estimation of pharmaceutical formulations.

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