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Extraction, Purification and Characterization of Indole Alkaloids from *Strychnos wallichiana* L. – an Endangered Medicinal Plant from India

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ABSTRACT

Strychnos wallichiana (Loganiaceae) is a critically endangered medicinal plant, commonly used in several native medical practices. The majority of members of the *Strychnos* genus shares a similar chemical composition and contains common indole alkaloids. Purification of strychnine and brucine from a few species of *Strychnos* have already been reported; however, we have purified and quantified these alkaloids for the first time from this native Indian species by isocratic RP-HPLC from roots and seeds. The structural identity of HPLC-purified strychnine and brucine was reaffirmed by IR, ^{1}H-NMR, ^{13}C-NMR and LC-MS spectral analysis. The present study reveals that South Indian *S. wallichiana* possesses indole alkaloids in a significant proportion, and that it is an alternative source for strychnine and brucine.

Keywords: alkaloids, Loganiaceae, RP-HPLC, TLC

INTRODUCTION

Strychnos wallichiana is a wild, large woody climber of the family Loganiaceae. It is commonly used in Ayurveda. In traditional ethnomedicine it is used as an antidote for snakebite, aphrodisiac, diarrhoea, paralysis and externally in cutaneous diseases (Prajapathi *et al*. 2003). Due to its exploitation the plant is critically endangered and included in the red listed category (IUCN) (Anonymous 2001). Several members of the *Strychnos* genus contain indole alkaloids exhibiting anti-cancer, anti-viral, anti-fungal, anti-bacterial and anti-inflammatory properties (Philippe *et al*. 2003; Deng *et al*. 2006; Prasad *et al*. 2007). The alkaloids from African and Asian *Strychnos* species have been characterized and structurally elucidated (Bisset 1970; Bisset and Phillipson 1971; Bisset 1972; Marini-Bettolo *et al*. 1972; Bisset 1974, 1976; Neuwinger 2004). However, the complete chemical composition of many species is still unknown. Studies with *S. wallichiana* leaves collected from Bangladesh suggest the presence of icajine and novacine as the major alkaloids while strychnine, brucine, pseudostrychnine, *N*-methyl-*sec*.-pseudo-β-coubrine, 14-hydroxyicajine, strychnine-*N*-oxide and brucine-*N*-oxide as the minor alkaloids (Bisset and Choudhury 1974; Bisset *et al*. 1974).

Earlier, TLC (Thin Layer Chromatography) and colorimetry were widely used to isolate and quantify indole alkaloids in *Strychnos*; however, due to inefficient resolution of all the alkaloids, ambiguous results were obtained, hence the application of HPLC (High Performance Liquid Chromatography) as a qualitative and quantitative tool (Zhang *et al*. 2003). The alkaloids strychnine and brucine are of great commercial value: they are used as stimulants of the nervous system. Strychnine is used in physiological and neuroanatomical research and at an excessive dosage leads to violent muscular convulsions. Brucine also has similar effects but 50 times weaker when compared to strychnine (Ito *et al*. 2001; Anonymous 1976; Duverneuial *et al*. 2003). Several methodologies were reported for the effective separation, purification and quantification of strychnine and brucine from various sources (De and Bisset 1991; Biala *et al*. 1996; Gu *et al*. 1996; Wang *et al*. 2004; Li *et al*. 2006; Zang *et al*. 2006). Among the 190 species of *Strychnos* only six were found to be the primary source for strychnine and brucine namely, *S. nux-vomica*, *S. lucida*, *S. ignatti*, *S. wallichiana* from Asia, *S. icaja* from Africa and *S. panamensis* from Central America, while the African *S. tabescana* contains only strychnine (Bandopadhyay and De 1997; Philippe *et al*. 2003). The alkaloid composition of *Strychnos* species varies due to their geographical distribution, seasonal variations and other environmental factors (Bandopadhyay and De 1997).

No studies on the alkaloid composition of the South Indian *S. wallichiana* have been carried out yet. This work was carried out to identify this rare plant from South Indian forests and to evaluate the alkaloid profiles from their roots and seeds. In this paper, we report the isolation of strychnine and brucine by isocratic RP-HPLC (Reverse phase) and structure determination based on IR, ^{1}H-NMR, ^{13}C-NMR and LC-MS spectral analysis from the roots and seeds, an alternative source for these alkaloids.

MATERIALS AND METHODS

Experimental plant material were collected in August 2002 from Eastern Ghats (Veligonda hills) in Kutlamarri valley along the border of Kadapa and Nellore districts of Andhra Pradesh in Southern India and deposited in our field laboratory at the University of Hyderabad.

Extraction of alkaloids and sample preparation

A general alkaloid extraction method was followed (Bandopadhyay and De 1997). Five g of seeds and roots were shade dried, finely powdered, moistened with 25% NH_4OH and extracted in a Soxhlet apparatus with $CHCl_3$ (200 ml) for 24 hrs. The $CHCl_3$ was extracted with 2% aqueous H_2SO_4 (50 ml x three times). The combined acid extracts were alkalized with 25% NH_4OH solution to pH 10 and then extracted with $CHCl_3$ (50 ml x three times). The combined organic layers were dried over anhydrous sodium sulfate to dryness and concentrated with a flash rotary evaporator

Received: 13 April, 2008. Accepted: 9 June, 2008.

Original Research Paper

(Model: Heidolph, Laborota-4001). The yield of dried crude root extract was found to be 62 mg/5 g while dried seed extract showed 58 mg/5 g dry wt, respectively. The dried plant extract was dissolved in 2 ml $CHCl_3$ and filtered through a 0.2 mm pore size syringe filter and subjected to TLC and HPLC analysis.

Chemicals

Strychnine and brucine were purchased from Fluka. HPLC grade acetonitrile, chloroform, 1-heptanesulfonic acid sodium salt, glacial acetic acid were purchased from Qualigens, 0.45 and 0.2 filters from Sartorius, disposable 2 ml syringes, TLC silica gel aluminum sheets F_{254} 20 × 20 cm from Merck.

Apparatus

HPLC analysis was performed using a Waters Model 1525 pumping system, a Waters Dual lambda 2487 Absorbance detector fitted with a Zorbax ODS C18 RP analytical column F-35993 [4.6 mm × 25 cm] packed with 5 µm particles. A 20 µl injection loop was used. Breeze software (Waters Corporation, USA) was used to analyze the data.

Standard solution, mobile phase and calibration

Approximately 5 mg of each alkaloid was precisely weighed and dissolved in 5 ml of chloroform and stored at 4°C. The mobile phase was aqueous 1-heptanesulfonic acid sodium salt + acetonitrile: water (1:1). Initially 1.1014 g 1-heptanesulfonic acid sodium salt was dissolved in 980 ml of acetonitrile: water (1:1) and pH was adjusted to 3.5 with glacial acetic acid and diluted to 1 L with acetonitrile: water (1:1) and filtered through 0.45 µm filters and refrigerated at 4^0C. The flow rate was 0.75 ml/min. The eluate was monitored at 254 nm. Stock solution was used for making serial dilutions. Calibration was done ranging from 0.2 to 3 µg. Regression equations for strychnine and brucine were obtained from peak area [y] and concentration [x] of authentic alkaloids.

Peak identification from extracts

Identification of peaks from crude plant extracts was established by comparison of the UV spectra and retention times with those of authentic alkaloids. The sample peak purity determination was based on the absorbance at 254 nm and compared to authentic standards. Some samples were analyzed as is or spiked with a known amount of standards.

Statistical analysis

Sigma plot was employed to establish means, standard deviation [SD] of the distributions of compounds' concentration in vegetative parts. The coefficient of variance or relative standard deviation (RSD) was calculated by expressing the standard deviation as a percentage of the relative mean. The capacity factor K was defined as follows $K = (V_a\text{-}V_0)/V_0$ where V_a is the retention volume of the analyte A and V_0 is the void volume of the column (Masuda *et al*. 2001). The selectivity factor ($\propto$) is the measure of the relative retention of the two compounds and denoted by $\propto = K_2/K_1$ (de Beer *et al*. 1991).

Spectral analysis

The IR (KBr) spectra were recorded on a JASCO FT-IR spectrophotometer (Model 5300). ^{1}H-NMR (200 MHz), ^{13}C-NMR (50 MHz)) spectra were recorded on a Bruker-AC-200 with chloroform-d as solvent and TMS (internal standard) as reference ($\delta = 0$ ppm). The chemical shifts are expressed in δ downfield from the signal of internal TMS. The LC-MS (Liquid Chromatography and Mass Spectroscopy) analyses were performed on a SHIMADZU-LC-MS machine (Model: 2010A) using the solvent system of methanol and water (1:1) and RP-C18 analytical column [240 mm× 2 cm] with a flow rate of 0.5 ml/min. The samples were nebulized with nitrogen gas and the ion mass (Electro Spray Ionization) were generated in positive mode.

RESULTS AND DISCUSSION

A general alkaloid extraction procedure was followed. Initially alkaloids were separated from the crude plant extracts i.e. roots and seeds by TLC (E-Merck F-256 plates) using a solvent system (EtOAc: iso-PrOH: 25% NH_4OH = 80:15:5) and run once. Three bands were separated. The most polar band on further TLC (system EtOAc: iso-PrOH: 25% NH_4OH = 90:7:3) run once separated into strychnine and brucine. The alkaloids were detected by spraying with Dragendorff's reagent. TLC analysis revealed the separation of alkaloids from the plant extracts was similar to that of standards. The R_f values of the commercially purchased strychnine and brucine were 0.35 and 0.5 respectively. The crude extracts of roots and seeds that were analyzed with the above standards gave an R_f values of 0.35 and 0.51, respectively. However, TLC analysis of the two alkaloids is limited by lack of accuracy in terms of purity and quantification.

Therefore, quantitative and qualitative studies were carried by RP-HPLC. All the authentic alkaloids were further studied and first characterized individually to record their UV spectrum. Optimal chromatographic conditions were obtained after testing different mobile phases with a reverse-phase C_{18} column. Isocratic elusions resulted in good separation however the separation of strychnine and brucine was shown to be pH dependent. Accuracy of the pH was critical to achieve the separation. The analysis time is a key factor in analytical work and the run time should be reduced to a minimum in order to optimize equipment use and solvent consumption.

The optimized RP-HPLC fingerprinting method was developed for quick analysis of organic extract. The optimal conditions led to good separation of the peaks, which could be identified in the chromatogram, brucine (Rt = 4.3) and strychnine (Rt = 5.1). They were identified by comparison with a chromatograph of the two reference compounds obtained under similar conditions. The eluted peaks of strychnine and brucine from root and seed extracts were matched with reference compounds by internal spiking and overlaying. Retention times were similar for the root and seed extracts. Initially authentic alkaloids were analyzed by increasing concentrations ranging from 0.2 to 3.0 µg. Concentrations of strychnine and brucine were calculated from peak area [y] and concentration [x] of known authentic standards with UV detection at 254 nm. Calibration curves showed linearity in the concentration range used for the standards. The standard solutions were injected in triplicate and the resulting curves had a very good linear correlation coefficient. For strychnine,the regression equation was y = 2548.01x + 888956.6, correlation coefficient = 0.999; for brucine, it was y = 1382.78 x +526350.5, correlation coefficient = 0.999.

Strychnine and brucine showed seasonal variation in vegetative parts examined. The values of the selectivity factor [α] and capacity factor [K] are summarized in **Table 1**. In the roots the amount of strychnine and brucine was very high when compared to other vegetative parts. The amount of strychnine and brucine was 1.2 mg/5 g and 5.3 mg/5 g dry wt of root wood. In seeds strychnine content was 0.33 mg/5 g and brucine content 0.22 mg/5 g dry wt, respectively. The results of the quantitative analysis are the average of three samples (**Table 1**). The reproducibility of retention times of the strychnine and brucine were studied and RSD also calculated (**Table 2**).

The pathway of indole alkaloids in *Strychnos* is quite

Table 1 Parameter values and content of alkaloids in *S. wallichiana* by reverse-phase isocratic-elution chromatography.

Alkaloid	Capacity factor (K)	Selectivity factor (∝)	Root mg/ 5 g dry wt	Seed mg/ 5 g dry wt
Brucine	0.11	-	5.3	0.33
Strychnine	0.33	3	1.2	0.22

*N = 3 samples

Table 2 *S. wallichiana*, reproducibility of retention times and RSD for brucine and strychnine by reverse-phase -HPLC.

Alkaloid	RT mean (min.)	RSD (%)
Brucine	4.3	1.02
Strychnine	5.1	3.6

*N = 12 samples

complex (Heimberger and Scott 1973). In higher plants secondary metabolites are produced from the shikimate pathway, where tryptophan is synthesized and converted to tryptamine, which is in turn condensed with secologanin to yield strictosidine, the common precursor of all indole alkaloids (Nagakura *et al*. 1978; Weaver and Herrmann 1997). In this process the enzyme strictosidine synthase catalyzes a stereoselective Pictet-Spengler condensation between tryptamine and secologanin to yield strictosidine, which is the branch point from which biosynthesis routes diverge towards the various alkaloids found in different indole alkaloid producing plants (Kutchan 1998). The biosythesis of strychnine from trytophan and geraniol has been demonstrated in full accord with the seco-iridoid pathway in *S. nux-vomica* (Heimberger and Scott 1973). Tryptophan decarboxylase also plays an important role in formation of simple indole alkaloids. Several reports suggests that in Asian *Strychnos* species the root is the main site of alkaloid biosynthesis and later transported to other vegetative parts and that the alkaloids of the normal series become converted via the N-oxides to corresponding bases of pseudo-N-methyl-sec-pseudo series due to seasonal variations and environmental conditions (Bisset 1976; Bandopadhyay and De 1997). Secologanin can be a limiting factor in alkaloid production (van der Fits and Memelink 2000) and geraniol 10-hydroxylase, a cytrochrome P450 enzyme, has a regulatory effect on alkaloid biosynthesis, as documented in *Catharanthus roseus* (Collu *et al*. 2001). Here our results are in agreement with the existing literature suggesting that the presence of strychnine and brucine is higher in roots than in seeds.

The structure, composition and identity of strychnine (formula: $C_{21}H_{22}N_2O_2$ M.W. 334.42) and brucine (formula: $C_{23}H_{26}N_2O_4$ M.W. 394.47) were already reported (Wang *et al*. 2004). However, the present investigation was carried out in order to reconfirm and elucidate the structure and identity of the HPLC-purified strychnine and brucine from *S. wallichiana* based on IR, ^{1}H-NMR, ^{13}C-NMR and LC-MS spectral analysis.

The LC-MS spectral data of crude organic root extract of *S. wallichiana* displays the presence of a molecular ion peak of strychnine at 335 *m/z* (MW: 334.42), while brucine at 395 *m/z* (MW: 394.47), respectively (**Fig. 1A**). The HPLC-purified strychnine and brucine also exhibited similar results. Strychnine showed a molecular ion peak (**Fig. 1B**) at 335 *m/z* while the IR spectra depicted the presence of an amide group (1670 cm^{-1}), phenyl ring group (1597 cm^{-1}) and aliphatic group (2941-2816 cm^{-1}). The ^{1}H-NMR spectrum displayed phenyl ring protons in δ 8.10-7.15 ppm range, alkene proton in δ 5.85 ppm range and aliphatic protons in δ 4.40-1.10 ppm range, respectively. In the ^{13}C-NMR spectrum the peak corresponding to amide carbon was observed in δ 169.3 ppm, aromatic and alkenyl carbons in δ 142.3-116.3 ppm range and aliphatic carbons in δ 64.6-26.92 ppm range (**Table 3**). Similarly, brucine also showed a molecular ion peak (**Fig. 1C**) at 395 *m/z* while the IR spectra demonstrated the occurrence of an amide group (1653 cm^{-1}), a phenyl ring group (1597 cm^{-1} merged with carbonyl peak) and an aliphatic group (2928-2868 cm^{-1}). In the ^{1}H-NMR spectrum phenyl ring protons were observed in δ 7.82-6.70 ppm range, an alkene proton in δ 6.05 ppm range and aliphatic protons in δ 1.0-4.4 ppm range. In the case of ^{13}C-NMR spectrum the peak corresponding to amide carbon was observed in δ 168.9 ppm, aromatic and alkenyl carbons in δ 149.0-101.0 ppm range and aliphatic carbons in δ 64.0- 26.0 ppm range (**Table 4**).

The IR, LC-MS, ^{1}H-NMR and ^{13}C-NMR spectral data analysis of strychnine and brucine suggested that the presence of 22 hydrogen atoms and 21 carbon atoms in strychnine composition with a mass of 335 indicating its molecular formulae as $C_{21}H_{22}N_2O_2$ (**Table 3**) whereas in brucine 26 hydrogen atoms and 23 carbon atoms with a mass of 395 representing its molecular formulae as $C_{23}H_{26}N_2O_4$ (**Table 4**). The spectral analysis correlated with the existing litera-

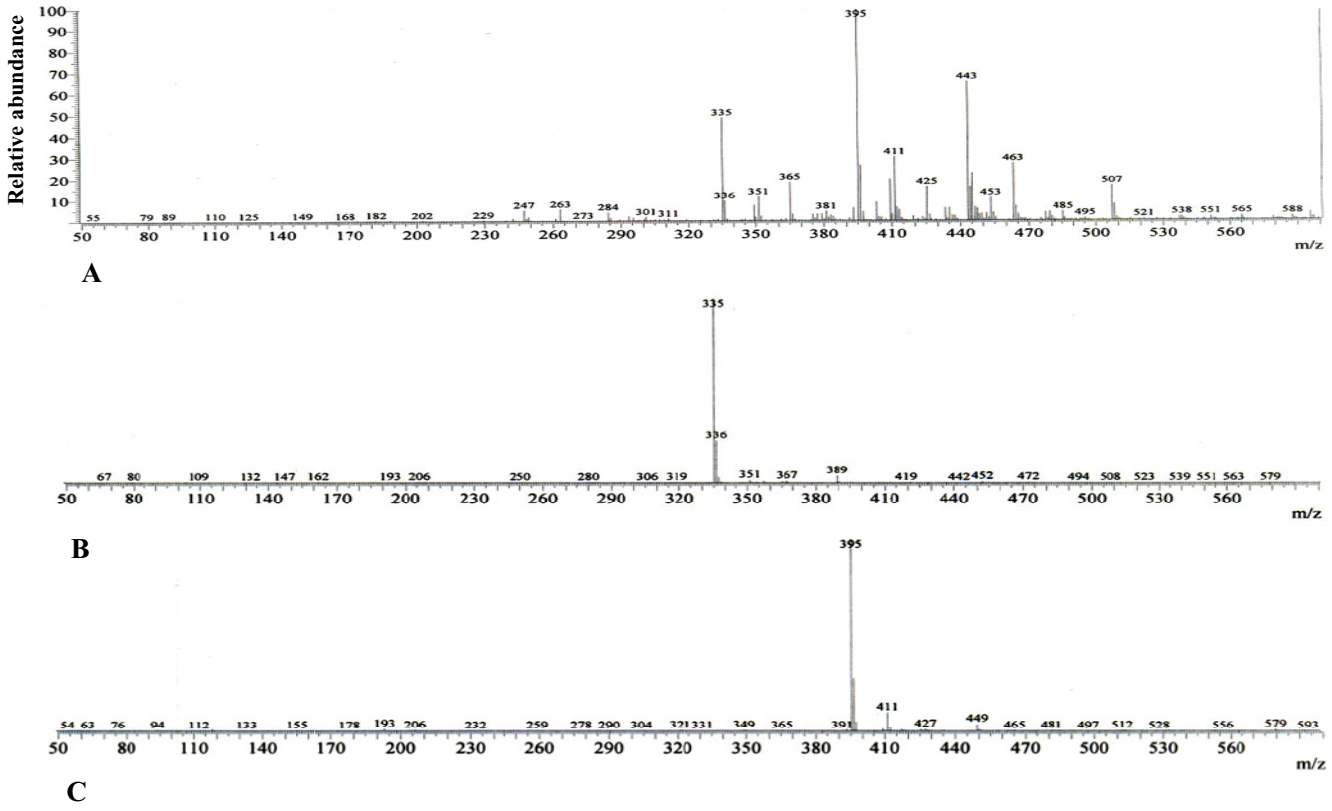

Fig. 1 (**A**) LC-MS analysis of crude organic root extract of *S. wallichiana* showing molecular ion peak of strychnine 335 *m/z* and brucine peak at 395 *m/z*. (**B**) The HPLC purified strychnine showing molecular ion peak at 335 *m/z*. (**C**) The HPLC purified brucine showing molecular ion peak at 395 *m/z*.

Table 3 Spectral details of strychnine.

Instrument	Spectral readings
IR (KBr)	(cm-1) 2941, 2816, 1670, 1597
^{1}H-NMR	(200 MHz, $CDCl_3$, δ ppm): 1.10-4.40 (m, 17H), 5.85(s, 1H, C<u>H</u>=C), 7.15-8.10 (m, 4H, Ar C<u>H</u>)
^{13}C-NMR	(50 MHz, $CDCl_3$, δ ppm) 26.9 (<u>C</u>H_2), 31.7 (<u>C</u>H), 42.5 (<u>C</u>H_2), 42.8 (<u>C</u>H_2), 48.2 (<u>C</u>H), 50.3 (<u>C</u>H_2), 52.0 (<u>C</u>H_2), 52.7 (<u>C</u>H_2), 60.1 (<u>C</u>H), 60.2 (<u>C</u>H), 64.6 (<u>C</u>H), 116.3 (Ar <u>C</u>H), 122.2 (Ar <u>C</u>H), 124.2 (Ar <u>C</u>H), 127.4 (<u>C</u>H=C), 128.5 (Ar <u>C</u>H), 132.7 (Ar <u>C</u>), 140.4 (Ar <u>C</u>), 142.2 (<u>C</u>=CH), 169.3 (N-<u>C</u>=O)
LC-MS(ESI)	*m/z* 335 [M+H$^+$] calcd for $C_{21}H_{22}N_2O_2$: 334.42

* Ar = Aromatic

Table 4 Spectral details of brucine.

Instrument	Spectral readings
IR (KBr)	(cm^{-1}) 2928, 2868, 1653
^{1}H-NMR	(200 MHz,$CDCl_3$, δ ppm): 1.10-4.40 (m, 23H), 5.98 (s, 1H, C<u>H</u>=C), 6.70 (s,1H, Ar C<u>H</u>), 7.82 (s, 1H, Ar C<u>H</u>)
^{13}C-NMR	(50 MHz, $CDCl_3$, δ ppm) 26.8 (<u>C</u>H_2), 31.6 (<u>C</u>H), 42.4 (<u>C</u>H_2), 48.3 (<u>C</u>H), 50.2 (<u>C</u>H_2), 51.9 (<u>C</u>H_2), 52.7 (<u>C</u>H_2), 56.2 (O<u>C</u>H_3), 56.5 (O<u>C</u>H_3), 60.0 (<u>C</u>H), 60.4 (<u>C</u>H), 64.6 (<u>C</u>H), 101.2 (Ar <u>C</u>H), 105.7 (Ar <u>C</u>H), 123.4 (Ar <u>C</u>), 127.4 (<u>C</u>H=C), 136.6 (Ar <u>C</u>), 140.4 (<u>C</u>=CH), 146.3 (Ar <u>C</u>), 149.3 (Ar <u>C</u>), 168.9 (N-<u>C</u>=O)
LC-MS(ESI)	*m/z* 395 [M+H$^+$] calcd for $C_{23}H_{26}N_2O_4$: 394.47

* Ar = Aromatic

ture confirming the structure and identity of these alkaloids as strychnine and brucine (Verpoorte 1980; Frederich *et al*. 2003, 2004).

We finally conclude that the native South Indian *S. wallichiana* possesses indole alkaloids, which are an alternative source for strychnine and brucine. In the present study strychnine and brucine were separated, purified by isocratic RP-HPLC from roots and seeds for the first time from this native Indian species. The structural identity of HPLC-purified strychnine and brucine was confirmed by spectral analysis which was correlated with existing literature. Our HPLC procedure could be useful for the qualitative and quantitative analysis of alkaloids of the Loganiaceae family, particularly in the quality control of phyto-preparations containing strychnine and brucine and as well as in chemosystematics. Since these molecules have commercial and pharmacological significance, an active research is required in further assessing the nature of the molecules especially in understanding the structure – activity relationships which will pave the way for drug discovery.

ACKNOWLEDGEMENTS

PSR thankfully acknowledges the award of research scholarship by the "UGC University with potential for excellence". Part of the work also received financial support from the Department of Biotechnology, Govt. of India, New Delhi (to MNVP vide ref. BT/PR2273/PBD/17/117/2000 dt.7-9-01) during 1-10-2001 to 31-3-2005; and the Ministry of Environment and Forest, GOI ref No. 10/03/2003-CS/ BG dt. 8.2.2005 (Botanic Garden scheme to MNVP) from 1-4-2006 which is gratefully acknowledged. Thanks are also due to the Principal Chief Conservator of Forests, officers of the Andhra Pradesh forest Department for permission to collect germplasm from reserve forest divisions (Rc No. 15698/02/U2. dt. 29-10-2002). We wish to acknowledge Prof. V. S. Raju, Dept. of Botany, Kakatiya University, Warangal and retired Prof. Surayanarayana (S.V. University, Vishwodya Degree College, Vekatgiri, Nellore) for suggestions and providing useful information on the taxonomy and nomenclature of the material investigated. We also acknowledge to Department of Animal Sciences and School of Chemistry for providing access to the HPLC instrument and IR, ^{1}H-NMR, ^{13}C-NMR and LC-MS spectral analysis.

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