ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 10, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

ISOLATION AND CHEMICAL CHARACTERIZATION OF BIOACTIVE ALKALOID FROM *ARGYREIA SPECIOSA* LINN. HAVING ACTION ON ISOLATED RAT LEYDIG CELLS

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Received: 05 July 2019, Revised and Accepted: 13 August 2019

ABSTRACT

Objective: The present study was aimed to isolate and characterize bioactive constituent from alkaloid fraction, prepared from roots of *Argyreia speciosa* (AS) Linn.

Methods: Literature review revealed the presence of ergoline of alkaloids in roots. Alkaloidal fraction was prepared and screened for its action on testosterone biosynthesis, *in vitro,* using isolated rat Leydig cells. Dehydroepiandosterone was used as positive control. This bioactive fraction was subjected to open column chromatography followed by flash chromatography, to isolate constituent. One compound (A1) was isolated and its purity was ascertained using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) studies. A1 was characterized by IR, mass, and ^{1}H-NMR studies. A1 was screened for action on testosterone synthesis *in vitro.* The fraction was standardized with respect to the amount of A1 present.

Results: Alkaloidal fraction (1000 µg/ml) incubated Leydig cells showed 22-fold increase in testosterone content as compared to untreated cells. TLC studies indicated that A1 might possess ergoline moiety in the structure. IR, mass, and ^{1}H-NMR spectral studies suggested that A1 might be N-methylergometrine. This was the first report included isolation and characterization of N-methylergometrine from AS. A1 (1000 µg/ml) was found to stimulate testosterone content, by 14.62-fold, in culture media of Leydig cells after incubation.

Conclusion: The results of *in vitro* studies confirmed that the standardized alkaloid fraction as well as A1 had ability to stimulate Leydig cells to secrete testosterone. A1 might be N-methylergometrine and being ergometrine derivative, it might act through oxytocin receptors expressed on the Leydig cells and stimulates testosterone synthesis.

Keywords: Aphrodisiac, Spermatogenic, *Argyreia speciosa,* Ergoline alkaloids, Leydig cells.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i10.34789

INTRODUCTION

Argyreia speciosa (AS) Linn. is a woody creeper plant belonging to the Convolvulaceae family. The plant is commonly known as *Samudrasosha* in Indian traditional medicinal system. The plant is used as *Vajikarana Dravya* in *Ayurveda* and known to possess spermatogenic properties [1]. Ayurvedic text describes the roots of the plant to be spermatogenic, aphrodisiac and increases the quality of semen [2-4]. The root powder of the plant is included in numbers of Ayurvedic and herbal preparations used to treat debilities related to male reproductive system [5].

Chemical investigations revealed the presence of ergoline type of alkaloids and flavonoids in the roots of plant. Several esters of fatty acids along with flavonoids such as scopoletin and liqueritin were isolated from the roots. Preliminary phytochemical investigation of aqueous and methanolic extracts of root revealed the presence of alkaloids, glycosides, amino acid, tannins, and flavonoids [6-12].

Previously, alcoholic extract of roots showed aphrodisiac activity in male rats with evident from stimulated mounting behavior in a dose-dependent manner [13]. Some alkaloids such as apomorphine, L-DOPA [14], and arecoline [15] showed spermatogenic and aphrodisiac activity when tested individually as well as in the form of fractions. Therefore, the present set of studies was aimed to isolate and characterize bioconstituent responsible for action on testosterone synthesis evaluated using rat Leydig cells, through bioactivity guided fractionation.

MATERIALS AND METHODS

Apparatus

Soxhlet extraction apparatus, consisting of extraction chamber, round bottom flask, and condenser, was used for the extraction of the plant material. Flash chromatographic system (Isolera-I, Biotage) was used for the separation of compounds from fraction. Rotary vacuum evaporator (Heidolph, Germany) was used to concentrate the extract while preparing alkaloid enriched fraction from roots.

Reagents, solvents, and chemicals

Analytical grade reagents, solvents, and chemicals used in the studies were purchased from LOBA Chemie, India. Media and other materials for *in vitro* studies were procured from Hi-Media, India; Acros Organics, India; and Sigma-Aldrich, USA.

Plant materials

The roots of AS were collected from tribal region. Roots were identified and authenticated by Taxonomist, J and J College of Science, Nadiad, and certificate of authentication was issued with voucher (Reference No.: 2011/NV/AS) submitted to Pharmacognosy Department. The roots were cleaned manually to remove adhering dirt and sand. They were cut manually and subjected to drying at room temperature under shade for 15 days and subsequently in tray dryer for 3 days at 60°C and then milled to 70# using laboratory grinder. The powder was stored in an airtight container for further process of extraction.

Preparation of alkaloidal fraction (ASALK)

ASALK was prepared as per the method described by Vyas and Raval [16]. Briefly, 5 kg of root powder was treated with methanolic (00196,

Loba Chemie) and 10% potassium hydroxide (05378, Loba Chemie) in with sufficient quantity. Moist powder was dried and subjected to Soxhlet extractor for extraction with 2 L of chloroform (00076, Loba Chemie) for 48 h at 60°C. Chloroform extract was reduced to 500 ml through concentration and recollection in rotary vacuum evaporator at 30°C. Concentrated extract was subjected to partitioning with equal amount of aqueous sulfuric acid (10%). Aqueous layer was collected and basified with aqueous potassium hydroxide solution (10%) to pH ~9. Basified aqueous portion was subjected to partitioning with chloroform. Pooled chloroform layers were evaporated completely to yield dry mass. Yield of alkaloid fraction was determined and found to be 0.02 % w/w. Further, thin-layer chromatography (TLC) studies were carried out for the detection of alkaloids present in the fraction.

Alkaloid detection in ASALK

ASALK was subjected to TLC studies to confirm the presence of alkaloids. Petroleum ether:chloroform:methanol:ammonia (1.0:9.5:0.5:0.07 v/v/v/v) was used as an optimized mobile phase to separate alkaloids on stationary phase composed of silica gel (Silica gel G60 $F_{254,}$ Merck) coated TLC plates. Chamber was saturated with mobile phase for 10 min while run distance was set to 80 mm at 25±2°C. After development, the plate was seen at 254 nm and 366 nm wavelength in UV cabinet. The plate was then derivatized with Dragendorff's reagent, dried, and scanned using Scanner IV at 520 nm using reflectance absorbance mode. The photograph of TLC plate and picture of chromatogram are shown in Fig. 1.

***In vitro* studies of ASALK**

In vitro studies were performed as described previously [16,22-25]. Protocol for *in vitro* studies was duly approved by the Institutional Animal Ethics Committee formed as per the Committee for the Purpose of Control and Supervision of Experiments on Animals norms (Protocol number: RPCP/IAEC/2011-12/R7). Healthy male Wistar rats of weight between 250 and 350 g were used. Animals were received from Anand Agricultural University, Gujarat, India. All animals were housed in institutional animal house at temperature 25°C±2°C and relative humidity of 75%±5% in 12 h light/dark cycle. A basal animal diet and water were provided *ad libitum*.

Preparation of positive control and test solutions for in vitro screening

10 mg dried fraction was dissolved in dimethyl sulfoxide (DMSO) and diluted appropriately to make 10, 100, and 1000 µg/ml concentration. Dehydroepiandrosterone (DHEA) served as positive control for *in vitro* studies. 10 mg DHEA was dissolved in DMSO appropriately to produce 10, 100, and 1000 µg/ml concentration. These solutions were used for *in vitro* studies.

Preparation of rat Leydig cells

Aseptically testes of male rats were dissected out and Leydig cells were isolated using collagenase dispersion method described previously [16,17,22-25]. Viability of cells was determined by trypan blue cell exclusion method while the purity of cells was checked by positive staining of 3β-hydroxysteroid dehydrogenase. Finally, Leydig cell concentration was set to 1 × 10^6 cells/ml by dilution with incubation medium.

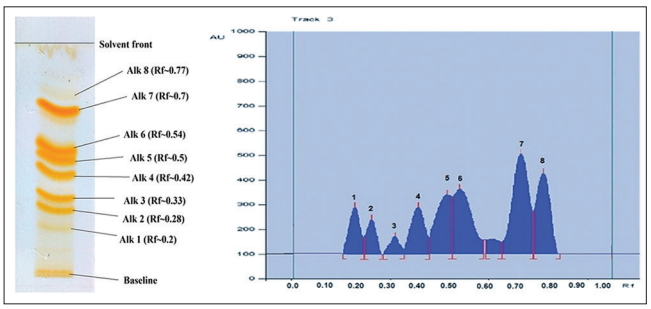

Fig. 1: (a) TLC profile of ASALK after derivatization with Dragendorff's reagent (b) Chromatogram of ASALK recorded at 450 nm

Treatment of cells with fractions and incubation

In vitro experiment was divided into four groups: Blank: Incubation medium without cells; control: 1 ml cells mixed with incubation medium to make final volume up to 1 ml; positive standard: 1 ml cells mixed with 0.1 ml of 10, 100, and 1000 µg/ml DHEA concentration and diluted up to 2 ml with incubation medium; and test: 1 ml cells mixed with 100 µl of 10, 100, and 1000 mg/ml ASALK concentration and diluted up to 2 ml using incubation medium.

All the cells were incubated at an atmosphere of 95% CO_2 using CO_2 incubator at 37°C for 3 h. Triplicate samples were put in the assay (n=3). After incubation, tubes were centrifuged at 4000 g for 10 min at 4°C temperature. The supernatant from each tube was collected and partitioned with 1 ml chloroform, individually. Chloroform layer was the amount of testosterone present in culture tube which was measured immediately using HPTLC method. Testosterone was separated on stationary phase composed of Silica gel GF_{254} aluminum-coated TLC plate with mobile phase of benzene:ethyl acetate (1:1 v/v) with 15 min of chamber saturation time. Testosterone was detected at 240 nm using deuterium as a light source in Scanner IV and data were integrated using WinCATS1.4.7 software. The amount of testosterone measured is expressed as mg% as shown in Fig. 2. Results of *in vitro* studies are expressed as Mean±SEM. One-way ANOVA test followed by Dunnett's test was performed to calculate the difference between the mean values of results. In all statistical tests, $p<0.05$ was considered as statistically significant. All analyses were performed using Microsoft Excel 2007.

Isolation and characterization of compound from ASALK

Isolation of compounds using column chromatography

1 g ASALK was dissolved in chloroform and then mixed with 5 g silica (60–120#) for column chromatography. Glass column (25 mm diameter, 90 cm long) was packed with silica (60–120#, 50g) slurry, prepared in hexane. The mixture was evaporated to dryness using water bath. Alkaloidal fraction loaded on silica was subjected to column chromatography to isolate individual compound/s. Elution was started with 500 ml of chloroform. Subsequently, elution was carried out by gradual increase in concentration (0.5%, 1%, 2%, 5%, 7%, 9%, and 10% V/V) of methanol (100 ml of each fraction). The flow rate was set to 10–15 drops per min during elution. Each fraction was concentrated using rotary vacuum evaporator and subjected to TLC. Fractions with similar TLC profile were combined together. The fractions collected from column chromatography were subjected to flash chromatography for further separation and purification of alkaloidal compound/s.

Isolation of compounds from subfractions using flash chromatography

The fractions showing similar TLC profile collected using column chromatography were subjected to flash chromatography for isolation of individual constituent. It was loaded in SNAP KP-Sil plastic cartridge contained with 50 g silica as stationary phase. Elution in flash chromatography was started with mobile phase of chloroform:methanol (linear gradient system) with flow rate of 10 ml/min. Elutes were scanned continuously at 238 nm and 333 nm throughout the experiment. The fractions (each of 20 ml) were collected using fraction collector in test tubes unless a drift observed in absorbance at the set wavelengths. Each fraction was concentrated and subjected to TLC, individually. The fractions yielded single spot on TLC were mixed together and evaporated to dryness. The detailed report of flash chromatogram is shown in Fig. 3. One alkaloidal compound (A1 ~200 mg) was isolated from subfraction of ASALK.

Purity assessment of A1

Purity of A1 was confirmed by performing TLC studies using three different mobile phases. Plates were sprayed with Dragendorff's reagent. Sample solution (1000 ng/spot) of A1 yielded sharp, single spot in three different mobile phases. A1 was also subjected to purity

analysis using HPLC. TLC pattern of A1 in three different mobile phases is shown in Fig. 4.

Spectral studies of compound A1

A1 was subjected to spectral studies using infrared (IR), nuclear magnetic resonance (1H-NMR), and mass spectroscopy (MS). The sample pellet consisting of A1 and anhydrous potassium bromide was placed in sample holder and IR spectrum was recorded in the range of 4000 cm^{-1} to 400 cm^{-1} using IR spectrophotometer (Nicolet 6700, Thermo Scientific). IR spectrum of A1 was compared with that of IR spectrum of ergometrine as plant was reported to contain ergoline type of alkaloid. ^{1}H-NMR ($CDCl_3$) spectroscopy was performed on a Bruker Avance II spectrometer (500 MHz). Electrospray mass spectroscopy (ES-MS) analyses were performed using API-2000 MS-MS (MDS SCIEX, Toronto, Canada) mass spectrometer. The probable structure of A1 was elucidated by comparing recorded spectroscopic data with the reported data of ergoline alkaloid (ergometrine) in the previous reports [18].

In vitro studies of A1

Isolated Leydig cells were incubated with 1, 10, 100, 500, and 1000 µg/ml concentration of A1, prepared in DMSO. DMSO served as blank while DHEA solution in DMSO served as positive control. The experiments were performed by adopting the protocol described earlier in section 2.6.3. The results of the studies are shown in Fig. 2.

RESULTS AND DISCUSSION

Detection of alkaloids

ASALK was subjected to TLC studies. Mobile phase was optimized to separate maximum number of spots on silica layer. Post-chromatographic derivatization using Dragendorff's reagent yielded eight prominent orange spots. The results are shown in Fig. 1(a) confirmed that fraction contained at least eight alkaloidal compounds. Developed TLC plate was scanned using scanner to record chromatogram. Recorded chromatogram, as shown in Fig. 1(b), may serve as identification parameter for ASALK.

In vitro studies of ASALK

Being terminal precursor in testosterone biosynthesis, DHEA was used as positive control. Amount of testosterone present in incubation media, after incubation period, was estimated using HPTLC method. The results indicated that the cells incubated with DHEA showed increased concentration of testosterone. Concentration of testosterone was increased by 2.8 -fold and 18.7-fold, as compared to control, for 10 µg/ml and 100 µg/ml of DHEA, respectively. The results confirmed that the incubated cells were metabolically active and capable of synthesizing testosterone. Similarly, different concentrations of ASALK were incubated with isolated rat Leydig cells. The results indicated that testosterone concentration, in incubation media of cells treated with ASALK solutions, was increased by 3.7-fold, 20.25-fold, and 22.1-fold, in case of 10 µg/ml, 100 µg/ml, and 1000 µg/ml concentration, respectively. As shown in Fig. 2, increase in testosterone production was dose dependent as well as found statistically significant too, when the mean value for each dose level was compared with that of control.

Isolation of alkaloidal compound from ASALK

ASALK was subjected to column chromatography. Fractions were collected and monitored for the presence of alkaloidal compounds using TLC separation followed by derivatization with Dragendorff's reagent. Fractions having similar TLC separation pattern of alkaloidal compounds were mixed together and subjected to flash chromatography for isolation of individual phytoconstituent. Flash chromatography was performed on Biotage Isolera™ One flash chromatographic system using SNAP KP-Sil plastic cartridge (50 g silica gel) with mobile phase: Chloroform:methanol (linear gradient system) with flow rate of 10 ml min. Elutes were scanned continuously at 238 nm and 333 nm. These wavelengths were selected after scanning spots of constituents obtained on TLC plate using TLC

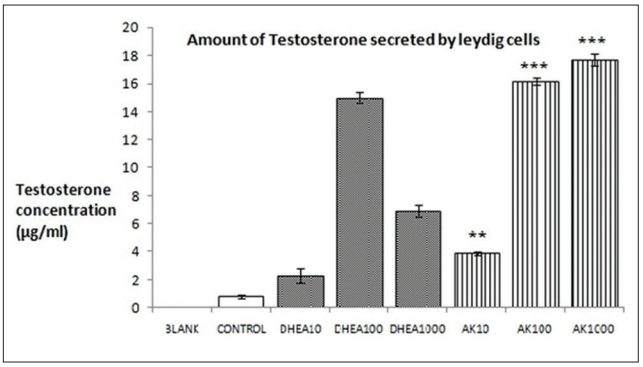

Fig. 2: Results of *in vitro* studies. Values are mean±SEM, *p<0.05, **p<0.01, ***p<0.001 as compared to control. DHEA: Dehydroepiandrosterone, AK: Alkaloidal fraction of AS

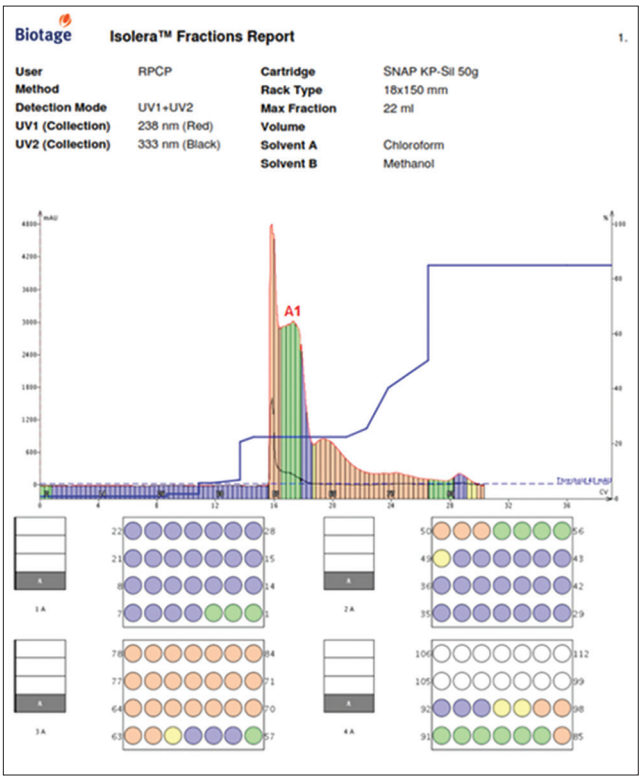

Fig. 3: Flash chromatogram for isolation of compounds from ASALK

scanner. The fractions were collected using fraction collector, operated to collect all the fractions at 238 nm and 333 nm. Gradient for methanol proportion was set using trial and error. Each fraction was collected, concentrated, and subjected to TLC to detect the presence of alkaloidal compounds. Fractions with similar TLC profile were combined together. The fractions containing single spot on TLC were mixed together and evaporated to dryness. The detailed report of flash chromatogram is shown in Fig. 3. One compound (A1 ~200 mg) was isolated from bioactive alkaloidal fraction prepared from roots of AS.

Purity assessment of isolated compound

Purity of isolated compound (A1) was confirmed by performing TLC studies using three different mobile phases. It was revealed from literature review that AS contained ergoline type alkaloids. A1 when subjected to react with Van Urk's reagent, yielded violet color. It was positive test for the presence of ergoline alkaloids. Hence, plates after development were sprayed individually with Dragendorff's reagent as well as Van Urk's reagent. Results showed that A1 yielded sharp

single spot in all three different mobile phase systems at different R_f values. There were no other spots observed on TLC plates. Results of purity assessment performed using TLC studies are shown in Fig. 4. Purity of A1 was also ascertained using HPLC studies. Compound was subjected to reverse-phase HPLC using acetonitrile:phosphate buffer pH ~2.5 (15:85 v/v) as mobile phase on C_{18} column (250 × 4.6 mm, 5 µm) at temperature of 25±2ºC. Flow rate was set to 1 ml per min and scanning was performed at 313 nm. Thus, by TLC and HPLC studies, it was confirmed that the compound A1 was pure and devoid of any impurities.

Spectral characterization of isolated compound [19]

The structure of isolated and purified compound was elucidated using IR, ^{1}H-NMR, and MS spectral studies.

IR (KBr disc): 1351.57 (**C-N** str), 1584.39, 1611.79 (**CO-NH** str), 2114.43, 3416.96 (Aromatic -**NH** str), 3731.12 cm^{-1}.

IR spectrum showed the presence of C-N stretch for N-CH_3 group (1351.57 cm^{-1}), indicating probable presence of N-methyl group in the structure of A1.

^{1}H-NMR (500 MHz, $CDCl_3$) δ ppm: 0.9 (t, -C**H**-CH_2-); 1.286 (s, -CON**H**-); 1.9 – 2.2 (m,-C**H**$_2$-); 3.6 (s, N-C**H**$_3$); 3.31 (d, -C**H**$_2$-OH); 3.4 (s, -O**H**); 2.27 (s, -N-C**H**$_3$); 4.7 – 4.8 (t,-C**H**-CH_2); 6.25 (s, -C**H**$_2$- ring); 7.08 (s, N-C**H**=C); 7.14 (t, CH=C**H**=CH); 7.2 (d, C**H**=CH); 7.3 (d, C**H**=CH).

NMR spectrum showed all characteristic δ values for peaks as reported for ergoline ring. Recorded NMR spectrum showed chemical shift at 3.6, corresponding to three hydrogens of N-CH_3 group in pyrrole ring while chemical shift at 2.2 corresponding to three hydrogens of N-CH_3 group might be attributed to tetrahydropyridine ring (Fig. 5). We have observed only one singlet corresponding to three hydrogens of N-CH_3 group at chemical shift 2.2 in reported^{1}H-NMR of ergometrine. This suggested the presence of N-methyl substituted ergometrine.

ESI-MS m/z $[M+H]^+$340.1;calcd. 339.4314, $C_{20}H_{25}N_3O2$; m/z 325.0 $[M-CH_3]^+$

Molecular weight of ergometrine is 325.4 g/mol while mass spectrum of A1 showed base peak at m/z 340.1 ($[M+H]^+$~340.1). A peak was obtained at m/z 325.0 too. This suggested removal of methyl group from A1 and formation of ergometrine molecular ion.

Considering the results of comparative spectral studies, structure of A1 was predicted as N-methylergometrine, as shown in Fig. 6.

***In vitro* studies of A1**

Previously, ergoline alkaloids were found capable of reducing conversion of testosterone and DHT into less active metabolite *in vivo* [20]. However, when tested *in vitro* using isolated Leydig cell in another independent study, oxytocin was found to stimulate basal testosterone production by Leydig cells, without interacting LH-dependent testosterone production. It was reported that oxytocin receptors are expressed on Leydig cells and oxytocin interacts with Leydig cells through these receptors [21]. As A1 was ergometrine derivative, it was assumed that A1 might interact with oxytocin receptors and stimulated testosterone synthesis. The studies, thus, suggested that ergoline alkaloids from ASALK might act directly on Leydig cells in testis and increased testosterone concentration. Testosterone plays an important role in initiation and maintenance of spermatogenesis process. Thus, inflated testosterone concentration due to the effect of alkaloids on Leydig cells might be attributed to spermatogenic potential of AS.

CONCLUSION

A1 was isolated from ASALK through bioactivity guided fraction using column chromatography and flash chromatography. Spectral studies revealed A1 to be N-methylergometrine. A1 and ASALK stimulated testosterone concentration in culture media of the cells, in a dose-dependent manner. It was further proposed that, being ergometrine derivative, A1 might interact with oxytocin receptors and stimulated testosterone biosynthesis in Leydig cells.

ACKNOWLEDGMENT

Authors are thankful to Gujarat Council on Science and Technology, Gujarat, India, for their financial support for the work.

AUTHORS' CONTRIBUTIONS

Niraj Vyas – Preparation of extracts, *in vitro* and *in vivo* activity.

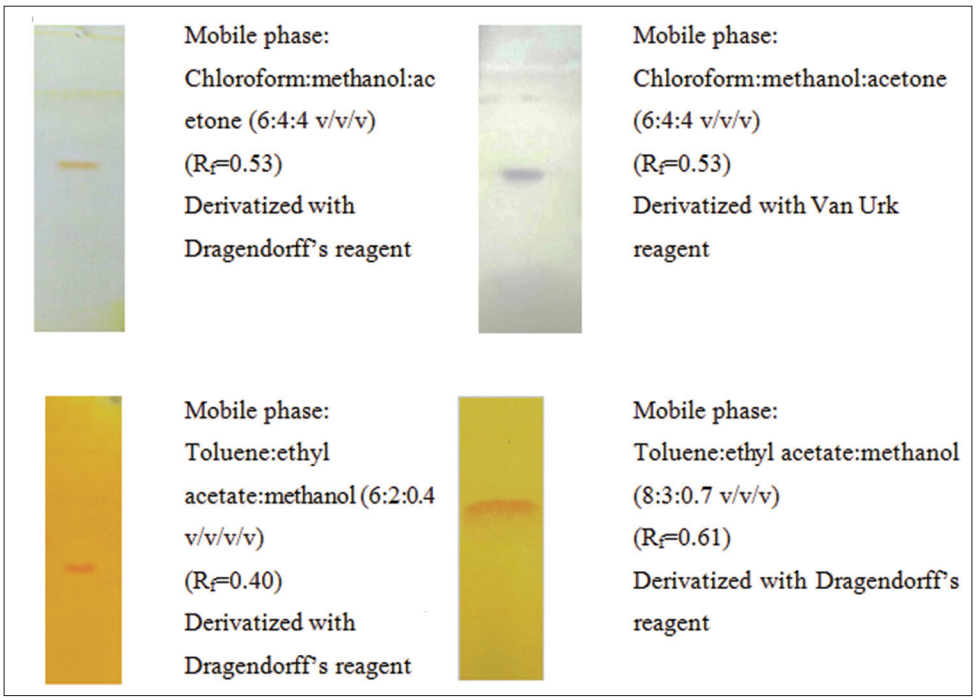

Fig. 4: Purity assessment of isolated compound by TLC

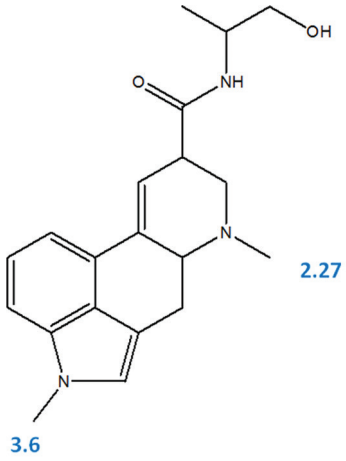

Fig. 5: ^{1}H-NMR shifts of hydrogens in N-CH_3 groups in A1

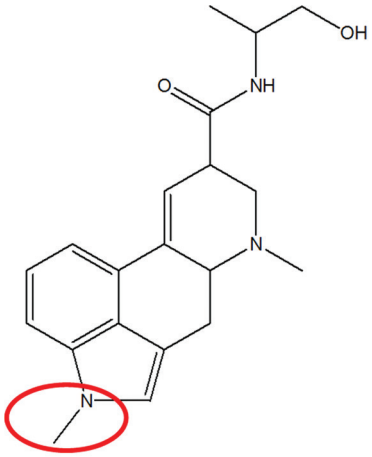

Fig. 6: Probable structure of A1 (N-methylergometrine) (Calculated mol wt. 339.4314 g/mol)

MananRaval – Isolation of alkaloids by chromatography.

Samir Patel – Spectral studies of isolated compounds.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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