IJPSR (2016), Vol. 7, Issue 8 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 04 March, 2016; received in revised form, 08 April, 2016; accepted, 31 May, 2016; published 01 August, 2016

PHYTOCHEMICAL ANALYSIS OF ACTIVE CONSTITUENTS OF *ALSTONIA SCHOLARIS* AND THEIR CYTOTOXICITY *IN VITRO*

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Keywords:

Alstonia scholaris, ethanolic extract, echitamidine-N-oxide-19-o-β-D-glucopyranoside, cytotoxicity and MTT assay

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ABSTRACT: The non-infected bark of *Alstonia scholaris* R. Br. was carefully peeled off, shade dried, and coarsely powdered with the help of a hand club. Powdered dried bark was extracted in 95% ethanol at room temperature and evaporated in vacuo (40°C). The ethanolic extract was suspended in distilled water and subsequently extracted in hexane, chloroform, ethyl acetate and n-butanol. The solvent of each fraction was removed in a rotary vacuum evaporator under reduced pressure. The crude chloroform extract was mixed with 3% HCl and ethanol. The aqueous layer was collected and the pH was adjusted to 10 by adding NaOH, thereafter it was further extracted with chloroform to get a crude alkaloidal fraction. The alkaloidal fraction was subjected to column chromatography over silica gel and eluted with chloroform: methanol (50:50). The elutes between fractions 40-100 were collected and subjected to preparative HPTLC in the solvent system of ethyl acetate: benzene (1:1). The HPTLC showed the presence of two alkaloids, which were evaluated for their cytotoxic effects in neoplastic cell lines by MTT assay. The compound which showed greater cytotoxicity was subjected to UV, NMR, Mass, and IR spectra and identified as echitamidine-N-oxide-19-o-β-D-glucopyranoside (EOG). The presence of EOG in the chloroform fraction was further confirmed by semipreparative RPHPLC (H_2O/CH_3CN-0%-80%; CH_3CN in 80 min, flow rate 3 mL/min, UV detector - λ 250 nm) and was also identified based on the literature.

INTRODUCTION: The indigenous knowledge of use of plants for medicinal purposes has been passed from generation to generation in the various parts of the world and it has significantly contributed to the development of various traditional systems of medicine. The use of plants as a source in modern medicine started in early 19th century when Friedrich Serturner isolated morphine from the opium poppy (*Papaver somniferum*) in 1806, that subsequently led to the isolation of early drugs including cocaine, codeine, digitoxin and quinine, and some of them are still in use [1-3].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.7(8).3262-73

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (8).3262-73

Isolation and characterization of pharmacologically active compounds from medicinal plants continue till today in the hope to search better non-toxic medicine and cure, which has resulted in the application of state of art techniques to standardize the herbal medicines and isolate bioactive compounds that can serve as analytical markers. This phytotherapy research led to generation of so many compounds like alkaloids, polyketides, terpenoids and even flavonoids [4].

The success of drug discovery from plant sources has resulted principally in the development of anticancer and antibacterial agents, During the period 1983-2002, there are nine drugs approved by FDA for the treatment of cancer which are directly isolated from the natural products and twenty one of them are natural product derivatives[5]. The saptaparana or *Alstonia scholaris*

(Apocynaceae), popularly known as Devils tree, grows to a height of hundred meters and has been used in the Indian system of medicine to treat various ailments [6]. Its ripe fruits are used to treat syphilis insanity and epilepsy. The milky juice of *A. scholaris* has been used to treat ulcers. The bark is extensively used part of the plant and is used in many compound herbal formulations. It is also used as a tonic, antiperiodic and anthelmintic [7]. It is a bitter tonic, alternative and febrifuge and it is reported to be useful in the treatment of malaria, diarrhea and dysentery [8-9]. Recently, the leaf extract of saptaparana has been reported to possess antimicrobial properties [10]. The alcoholic extract of the stem bark of *Alstonia scholaris* has been found to possess anticancer activity in HS1 human sarcoma and embryonated egg [9]. The earlier studies have shown the anticancer and chemopreventive activities of stem bark extract of *A. scholar* [11-12].

The pharmacological activity of the bark is attributed to the presence of several alkaloids. The alkaloid corialstonine and corialstonidine present in the plant have been found to be active against *P. falciparum* [13]. The various alkaloids including lagumamine (19 - hydroxytubotaiwine), angustilobine B acid, losbanine (6,7-seco-6-nor-angustilobine B), tubotaiwine, its oxide, 6,7-secoangustilobine B; 17-0-Acetyl echtamine, echitamine, manilamine, N4-methyl angustilobine B, vallesamine, angustilbine B N4-oxide , 20 (s) tubotaiwine and 6,7- seco angustilobine B have been isolated from the leaves and bark of *Alstonia scholaris* [14-15].

Similarly, it has been found to contain other alkaloids like 19-epischolaricine, Nb-methylscholarcine, Na-methylburnamine and vallesamine Nb-oxide, picrinine, nareline, alschomine, and angustilobine [16]. The *Alstonia glaucescens* have been reported to consist indole alkaloids such as 17-O-acetyl- Nb- demethyl echitamine, echitamidine-N-Oxide, echitaminic acid, sweroside, echitamidine, Nb-demethyl echitamine, 20-epi-19-echitamidine, N-b-demethylechtamine-N- oxide and echitamine [17].

The new alkaloids obtained from bark of *Alstonia scholaris* are akuammiginone, echitamidine N-oxide-19-O-ß-D-glucopyranoside, echitaminic acid, echitamidine-N-oxide, Nb-demethylalstogustine–N-oxide, akuammicine-N-oxide, Nb-demethyl-alstogustine. Likewise, *Alstonia scholaris* has been reported to contain echitamine chloride in its root and root bark [18]. It also contains other minor alkaloids including akuammicine, akuammicine-Nb-methiodide, akuammicine - Nb-oxide, akuammigine, Nb-demethyl ehitamine, tubotaiwine and venoterpine [19]. The neutral constituents of the root and root bark of *Alstonia scholaris* are α-amyrin, lupeol acetate, stigmasterol, β-sitosterol, campesterol [20]. Earlier study on the chloroform fraction of 85% ethanolic extract was found to be most cytotoxic than the other extract [21]. Therefore, the present study was undertaken to isolate the bioactive components of chloroform extract of *Alstonia scholaris* & study their antineoplastic activity *in vitro*.

MATERIALS AND METHODS:

Collection and extraction of the stem bark*:*

The saptaparana, *Alstonia scholaris* R. Br. (family Apocynaceae) was identified by Dr. G. K. Bhat (a well known taxonomist of the area), Department of Botany, Poorna Prajna College, Udupi, India. The non-infected stem bark was carefully peeled off, shade dried and coarsely powdered with the help of a ball mill. The powdered material was extensively extracted with 85% ethanol using a Soxhlet apparatus. The fractionation of ethanolic extract was carried out using standard procedures. The semisolid extract of *Alstonia scholaris* was serially washed with cold methanol until the methanol turned colorless.

The methanol fraction was pooled, freed of solvent by distillation until a white powder was obtained. The residue left after the methanol was suspended in a fixed volume of distilled water and mixed with ethanol. The ethanol was filtered under suction and concentrated in vacuo. The concentrated solution was evaporated at room temperature so as to obtain a mass of semisolid consistency.

Fractionation of 85% ethanol extract:

The ethanol residue (450 g) of *A. scholaris* was resuspended in 1,500 ml distilled water and subsequently extracted in hexane (8 X 500 ml), chloroform (8 X 500 ml) ethyl acetate (8 X 500 ml) and n-butanol (8 X 500 ml). The solvents were

removed from all the fractions with the help of a rotary vacuum evaporator under reduced pressure. The ethanol extract was thus fractioned into hexane soluble fraction (45g), chloroform soluble fraction (55g) and ethyl acetate soluble fraction (70g) and n-butanol (148 g) soluble fraction. Out of which chloroform soluble fraction was further subjected to chemical analysis.

Preliminary Phytochemical Screening for Alkaloids: The following methods were utilized to ascertain the presence of alkaloids in the chloroform soluble fraction of *A. scholaris*

(a) Dragendorff's test: Five ml of distilled water was added to 2 mg chloroform soluble fraction of the extract, followed by the addition of 2 M Hydrochloric acid until initiation of an acid reaction. Thereafter, 1 ml of Dragendorff's reagent was added to this reaction mixture and the formation of orange or orange red precipitate indicated the presence of alkaloids.

(b) Hager's test: A few drops of Hager's reagent was added to 2 mg chloroform soluble fraction of the extract. The formation of yellow precipitate confirmed the presence of alkaloids.

(c) Wagner's test: Two mg chloroform soluble fraction of the extract was acidified with 1.5% v/v of hydrochloric acid. This was followed by the additon of a few drops of Wagner's reagent and appearance of yellow or brown precipitate indicated the presence of alkaloids.

(d) Mayer's test: To a few drops of the Mayer's reagent, 2 mg of chloroform soluble fraction of the extract was added and the formation of white or pale yellow precipitate showed the presence of alkaloids.

Determination of Foreign Matter:
Medicinal plant materials should be entirely free from visible sign of contamination by moulds or insects, and other animal contamination, including animal excreta. The collected stem bark of *A. scholaris* did not show any abnormal odour, discoloration, slime or signs of deterioration.

Procedure:
100 g of the saptaparna stem bark was weighed and spread out in a thin layer. The foreign matter was detected by inspection with the unaided eye. Separated and weighed and the presence of foreign matter present if any was examined.
The dried stem bark powder was subjected to following analysis:

Determination of Moisture Content:
Determination of the amount of volatile matter (i.e., water drying off from the drug) in the drug is a measure of loss after drying of substances appearing to contain water as the only volatile constituent.

The powdered stem bark of *A. scholaris* was accurately weighed, placed (without preliminary drying) in a tared evaporating dish, dried at 105˚C for 5 hours, and weighed again. The percentage moisture content was calculated with reference to the initial weight. The moisture content was calculated using the following formula:-

$$\text{Moisture content} = \frac{\text{Pw-Fw x 100}}{\text{W}}$$

Where Pw = Preweighed sample
Fw = Final weight of the dried sample
W = total weight of the sample

Ash Values:
The ash values including total, and acid insoluble ash contents were determined so as to estimate the total amount of the inorganic salts present in the drug. The ash remaining following ignition of plant materials was determined by two different methods to measure total and acid insoluble ash contents.

Total Ash:
The method measures the total amount of material remaining after ignition including both 'physiological ash' derived from the plant tissue itself and 'non-physiological ash', which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface.

Procedure:
Two grams of ground air-dried material of saptaparna was accurately weighed in a previously ignited and tared crucible. The material was spread

as an even layer and ignited by gradually increasing the temperature up to 500-600˚C until it became white, indicating the absence of carbon. The crucible was cooled and weighed. The percentage of total ash content was calculated according to the following formula.

$$\text{Total ash content} = \frac{\text{Pw-Fw x 100}}{\text{W}}$$

Where
Pw= Pre weighed crucible
Fw= Final weight of the crucible containing ash
W= Total weight of powdered plant material

Acid Insoluble Ash:
The residue obtained after boiling the total ash was diluted with hydrochloric acid, and ignited. The insoluble matter remaining after ignition was measured as the presence of silica.

Procedure;
Twenty five ml of hydrochloric acid was added to the crucible containing total ash, covered with a watch glass and boiled gently for 5 minutes followed by the addition of 5 ml of hot water to the crucible. The insoluble matter was collected on an ashless filter paper and washed with hot water until the filtrate became neutral. The filter paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited. The crucible was allowed to cool in a suitable desiccator for 30 minutes, and the residue was weighed immediately. The amount of acid insoluble ash was calculated in mg/g of air dried material according to the formula:-

$$\text{Acid insoluble ash contents} = \frac{\text{Pwb-Fwa x 100}}{\text{W}}$$

Where Pwb = Final weight of the crucible with total ash
Fwa = Final weight of the crucible with acid insoluble ash
W = Total weight of powdered plant material.

Extractive values:
These are used to determine the amount of the matter which is soluble in the solvents used including alcohol and water. The percentage of alcohol and water-soluble extractives were calculated and used as standards.

Determination of alcohol-soluble extractive:
Five grams of air dried coarsely powdered material was macerated in 100 ml of alcohol in a closed conical flask for twenty four hours, with frequent shaking during first six hours and allowed to stand for next eighteen hours thereafter it was filtered rapidly with caution to avoid loss of solvent. The 25 ml of the filtrate was evaporated to dryness at 105˚C in a tared flat-bottomed shallow dish and weighed. The percentage of alcohol-soluble extractive was calculated with reference to the air-dried material.

Determination of water-soluble extractive:
Five grams of coarsely powdered air dried material was macerated in 100 ml of chloroform-water (0.1%) in a closed flask for 24 h, shaking frequently until six hours and allowed to stand for another eighteen hours. Thereafter it was filtered rapidly, with precautions to avoid loss of solvent by evaporation. The 25 ml of the filtrate was evoporated to dryness at 105˚C in a tared flat-bottomed shallow dish and weighed. The percentage of water soluble extractive was calculated with reference to the air dried material. All the tests were done in triplicate.

Isolation of chemical constituents:
The alcoholic extract (750 g) of *A. scholaris* was suspended in 1500 ml distilled water and subsequently extracted with hexane (8 X 500 ml), chloroform (8 X 500 ml) ethyl acetate (8 X 500 ml) and n-butanol (8 X 500 ml). The solvent was removed from all four fractions using a rotary vacuum evaporator under reduced pressure. This has yielded 85 g hexane, 115 g chloroform, 140 g ethyl acetate and 150 g n-butanol soluble fractions.

In our earlier studies fractionation guided extraction of *A. scholaris* has shown that chloroform extract had a greater cytotoxic effects than the other extracts[11], the chloroform fraction was used for isolation of chemical constituents using column chromatography. The chloroform crude extract was partitioned between 3% HCl and ethanol, and the aqueous layer was basified to pH 10 by addition of NaOH and extracted once again

with $CHCl_3$ that yielded 2.0 g of crude yellow coloured residue.

Column Chromatography:

The 15 g yellow residue was dissolved in 20 ml $CHCl_3$ and adsorbed onto neutral alumina (20 g). After evaporation of the solvent it was loaded onto a neutral alumina column (150 g) prepared in hexane. The column was eluted sequentially with hexane, hexane: toluene graded mixtures (95:5, 90:10, 80:20 and 50:50), toluene, graded mixtures of toluene: chloroform (95:5, 90:10, 80:20 plus 50:50), chloroform and finally chloroform: methanol (95:5, 90:10, 80:20 and 50:50). The elution was monitored by TLC (Silica gel G; visualization: vanillin-sulphuric acid reagent heated at 110°C). Each time 5 ml fraction was collected and identical eluates (TLC monitored) were combined and concentrated under reduced pressure so as to get a final volume of 5 ml and stored in a refrigerator until further analysis. Elutions carried out with chloroform: methanol graded mixture (80:20) resulted in a mixture of two compounds. The removal of solvent yielded a residue of 150 mg, which was subjected to preparative TLC in the solvent system of hexane and toluene (80:20), that has led to the isolation of two pure compounds. These were designated as compound I (40 mg) and compound II (43 mg) for convenience. The other eluates gave brown resinous masses and were not analysed further.

HPTLC Analysis of Chloroform Fractions:

The samples on aluminum backed HPTLC were applied with the help of an automatic applicator (Linomat IV), where a known quantity of sample was dissolved in a known volume of solvent before application to precoated Silica gel 60 F254 plates of 0.2 mm thickness (E. Merck, Mumbai, India). The samples were allowed to run using methanol as mobile phase. The solvent of the applied sample was completely evaporated and the chromatogram developed in rectangular glass chambers or twin trough chambers. The plates were visualized under UV 254 and 366 nm. The R_f values were recorded.

The yellow residue was dissolved in methanol at a concentration of 1 mg/ml and the samples were applied onto a TLC plate. The TLC plates were developed in the solvent system in twin trough chamber to a height of 8 cm, dried and scanned in a densitometer (CAMAG, Switzerland) at 300 nm and the calibration curve was prepared by plotting peak area against concentration applied. A Camag HPTLC system equipped with an automatic TLC sampler (ATS4), TLC scanner 3 and integrated software WINCATS version 1.4.1 was used for the analysis. Sample from HPLC and sample from HPTLC were applied to the plates as 8 mm long bands, 8 mm apart by use of a Camag Linomat (V) sample applicator equipped with a 100 µl microsyringe and an automatic TLC sampler (ATS4) under a flow of N_2 gas.

High Performance Liquid Chromatography (HPLC):

HPLC is one of the important analytical techniques, essential for both quantification and standardization of the herbal materials. The principle advantages of HPLC when compared to classical column chromatography are improved resolution of the separated substances, faster separation times and the increased accuracy, precision and sensitivity with which the separated components may be easily quantified. The HPLC consisting of Waters model (Waters Corp, Milford, USA), a binary pump (Waters 515) equipped with a online degaser, Spherisor ODS2 column RP – 18 (250 x 4.6 mm, i.d., 5 µm pore size), guard column of same chemistry, a Waters PCM and Rheodyne injector with a 25 µl loop was used. Detection was done using 2996 Photodiode Array detector. The chromatograms were recorded using Waters Millenium software.

Fifty milligrams of chloroform extract was dissolved in 2 ml of methanol and subjected to HPLC analysis, where it was eluted using a binary gradient of H_2O/CH_3CN (0%-80%) at a flow rate of 3 ml/min. The alkaloids were detected at λ 250 nm. The alkaloids were collected and compared with the alkaloids separated by HPTLC.

Chemical analysis of isolated components:

The UV & IR spectra of isolates were also recorded, using UV-VIS and IR spectrophotometer, respectively. The isolated compounds were subjected to Mass and NMR spectral analysis for further identification.

Cytotoxicity:
The cytotoxic effect of isolated compounds was evaluated *in vitro* in various neoplastic cell lines of different tissue origin.

Cell line and culture:
The HeLa, HepG2, KB, MCF-7 and U373MG cells (National Centre for all Sciences, Pune) of human origin were routinely grown in 75 cm^2 flasks (Falcon, Becton Dickinson, USA) with loosened caps, containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 µg ml^{-1} gentamycin sulfate in a humidified atmosphere of 5 % CO_2 in air at 37 °C in a CO_2 incubator (NuAire Inc., Plymouth, MN, USA).

MTT assay:
The cytotoxicity of the isolated compounds was determined using MTT assay as described earlier (Mosmann, 1983). The MTT assay was carried out by inoculating $5X10^3$ exponentially growing cells into several individual microwells containing 100 µl MEM in the presence or absence of 1, 5, 10, 20, 30, 40, 50 or 100 µg of isolated compounds from *A. scholaris* in 96 well microplates. The microplates were incubated at 37° C in a CO_2 incubator for 1 h. Thereafter, the drug containing medium was removed, 20 µl of MTT (5 mg/ml) added into each well and the cells were incubated at 37°C for another 4 h. The microplates were removed and the formazan crystals thus formed were dissolved into the lysis buffer containing 12% SDS, 5% isobutanol and 12 mM HCl and the absorbance was recorded at 570 nm using a microplate reader (Bio-Rad Laboratories Inc., India). Eight wells were used for each concentration and the experiment was repeated thrice and the data of all three experiments were combined. The test of homogenecity was applied to determine the statistical difference, if any among all repeat experiments. Since the differences among all repeat experiments were statistically non-significant, the data of all experiments were combined and the concentration of the compound that inhibited cell growth by 50% (IC_{50}) was determined from cell survival plots.

RESULTS:
The results are presented in **Table 1-9** and **Fig. 1-9**.

Extraction and Phytochemical analysis:
The drying of 23 kg of *Alstonia scholaris* stem bark yielded 20 kg of dried bark, and this reduction in weight was due to ≈ 13% loss in its water contents (**Table 1**). The analysis of dried stem bark of *Alstonia scholaris* showed presence of 2% foreign matter, 3% moisture; 4% total ash, 0.3% acid-insoluble ash; 6.5% ethanol-soluble extractives and 8.5% water-soluble extractives (**Table 2)**.

The extraction of 20 kg of dried stem bark powder in 85 % ethanol yielded 3 kg of ethanol extract, which was equal to 15% of the initial weight (**Table 3).** The subsequent extraction of this ethanol fraction in hexane (8 X 500 ml), chloroform (8 X 500 ml) ethyl acetate (8 X 500 ml) or n-butanol (8 X 500 ml) yielded 340 g of hexane, 460 g of chloroform and 560 g of ethanol and 600 g n-butanol soluble fractions after evaporation of each solvent (**Table 4**).

The preliminary phytochemical screenings of chloroform extract of *Alstonia scholaris* showed that it contained alkaloids as chemical entities, which was indicated by employing Dragendorff's, Hager's, Wagner's and Mayer's tests **(Table 5**).

HPTLC of *Alstonia scholaris:*
The alkaloids were further isolated using HPTLC and HPLC techniques. The HPTLC of the alkaloid components revealed presence of two compounds (**Fig. 1**) that was confirmed on the basis of the Rf values (**Table 6**) and densitometry (**Fig. 2**) of the individual spots.

HPLC of *Alstonia scholaris:*
The presence of two alkaloid were further confirmed by subjecting the chloroform extracts to HPLC analysis, where it showed a total of 6 peaks at 250 nm with the major peaks at 80.46 and 41.32 retention time (RT), respectively (**Table 7**). These peaks were identical with the two alkaloids isolated by HPTLC (**Fig. 3**).

MTT Assay of alkaloid I &II:
The cytotoxic effects of alkaloids I and II were evaluated by MTT assay. Treatment of HeLa, HepG2, KB, MCF and U373MG with different concentrations of alkaloid I resulted in a concentration dependent increase in its cytotoxic

effect on all the cell lines evaluated (**Table 8**). The cytotoxic effect of this alkaloid was mild as it reduced the cell survival only by 18% (**Table 8**). Therefore, its chemical analysis was not further undertaken.

Inoculation of various concentrations of alkaloid II in the microwells of 96 well plates containing HeLa, HepG2, KB, MCF-7and U373MG showed a concentration dependent increase in its cytotoxic effect in all the cell lines evaluated (**Table 9**). The cytotoxic effect of alkaloid II increased with increasing concentrations in all cell lines and a maximum cell killing effect was observed for 100 µg/ml. HeLa, KB and U373MG cells were more sensitive to its cytotoxic effects when compared to HepG2 and MCF-7 cells (**Table 9**). Therefore, this alkaloid was subjected to further chemical analyses and identification.

Identification of the Compound:

The compound II was a yellow amorphous powder and it was subjected to UV, NMR, IR, and MASS spectroscopies. The UV spectroscopy (Shimadzu corporation, Tokyo, Japan) of this compound's showed λ_{max} of 346 nm (**Fig. 5**). The Infrared spectra (Model 397, Perkin Elmer Massachusetts 02451, USA) did reveal different stretchings, where 3375 indicated presence of OH stretching groups, 3325 indicate NH stretching, 3168 showed aromatic CH stretching, 1979 showed N-Oxide stretching, 1368 indicated C stretching in COC linkage, 1289 revealed CN stretching in C-N-C linkage and 1689 showed C=O linkage (Figure 6). The chemical shifts are a very sensitive indicators of the chemical environment of the resonating nuclei and they were measured in high-resolution using JEOL-FX–90 Q NMR spectrometer (JEOL Ltd., Tokyo, Japan). NMR frequencies of 60 to 800 MHz indicated the presence of hydrogen, compared to the range of about 15 to 80 MHz for medical magnetic resonance imaging (MRI) (**Fig. 7**).

The molecular weight of this compound was evaluated using a JEOL–JMS-DX 303 mass spectrometer equipped with a JMA-DA 5000 data station (**JEOL Ltd.,** Tokyo, Japan). The mass spectroscopy data showed that this compound has a molecular mass of 519.2342 with a molecular formula of C26H35N2O9 (**Fig.8**). The interpretion of all spectroscopic results showed this compound to be Echitamidine-N-Oxide-19-O-β-D-Glucopyranoside (EOG) whose structural formula is given in **Fig. 9** below.

TABLE 1: THE LOSS OF WEIGHT ON DRYING THE FRESH STEM BARK OF *ALSTONIA SCHOLARIS*

Weight before drying (kg)	Weight after drying (kg)	Loss after drying (%)
23.00	20.0	13.04

TABLE 2: PHYSICOCHEMICAL PARAMETERS OF DRIED STEM BARK POWDER OF ALSTONIA SCHOLARIS

Foreign matter	Moisture content	Total ash	Acid-insoluble ash	Ethanol-soluble extract	Water-soluble extract
2%	3%	4%	0.3%	6.5%	8.5%

TABLE 3: YIELD OF 85 % ETHANOLIC EXTRACTS FROM STEM BARK POWDER OF ALSTONIA SCHOLARIS

Extracted weight (kg)	Weight of extract (kg)	Extract yield (%)
20.0 kg	3 kg	15.0 %

TABLE 4: YIELD OF VARIOUS FRACTIONS OF 85% ETHANOLIC EXTRACT OF *ALSTONIA SCHOLARIS*

Hexane Fraction	chloroform fraction	ethyl acetate fraction	n-butanol fraction
340 g	460 g	560 g	600 g

TABLE 5: QUALITATIVE ANALYSIS OF 85 % ETHANOLIC EXTRACT OF ALSTONIA SCHOLARIS FOR THE PRESENCE OF ALKALOIDS

Tests	Inference
a) Dragendorff's test	+ve
b) Hager's test	+ve
c) Wagner's test	+ve
d) Mayer's test	+ve

TABLE 6 CHARACTERISTICS OF COMPOUNDS ISOLATED FROM CHLOROFORM FRACTION OF ETHANOLIC EXTRACT OF ALSTONIA SCHOLARIS

Compound	Physical state	Solvent system	Spot Colour	Spraying reagent	R_f value
I	White amorphous powder	Petroleum ether: benzene (80:20)	Pink	Vanillin-sulphuric acid	0.45
II	Light Yellow amorphous powder	Petroleum ether: benzene (80:20)	Pink	Vanillin-sulphuric acid	0.67

TABLE 7: RETENTION TIME AND RELATIVE PERCENTAGE OF DIFFERENT COMPONENTS OF CHLOROFORM EXTRACT OF ALSTONIA SCHOLARIS BY HPLC.

Retention time (RT)	Amount in each peak (%)
13.34	2.33
41.32	23.69
80.46	56.27
100.73	8.38
125.83	7.34
152.24	3.11

TABLE 8 CYTOTOXIC EFFECT OF VARIOUS CONCENTRATIONS OF COMPOUND I IN DIFFERENT CELL LINES BY MTT ASSAY

Concentration µg/ml	Cell viability(%viability)				
	HeLa	HepG2	KB	MCF-7	U373MG
0	100.11±1.21	100±0.84	100±0.11	100±0.21	100±0.23
1	98.66±1.33	98.21±0.66	97.45±0.94	97.98±0.65	96.45±0.76
5	98.21±1.54	97.32±0.65	96.54±0.94	96.94±0.43	97.44±0.65
10	95.43±0.54	96.34±0.43	95.30±0.54	96.43±0.54	96.43±0.54
20	92.54±0.65	93.22±0.94	93.54±0.84	92.54±0.85	94.32±0.54
30	90.43±0.53	91.22±0.32	90.32±0.74	91.43±0.73	91.43±0.66
40	89.49±0.43	88.43±0.32	87.95±0.04	88.93±0.83	89.43±0.77
50	85.39±0.65[a]	84.39±0.42[a]	85.39±0.45[a]	86.42±0.59[a]	85.32±0.34[a]
100	84.49±0.34[a]	84.39±0.23[a]	83.54±0.32[a]	82.35±0.74[a]	83.33±0.43[a]

p < a = 0.05, b = 0.01, c = 0.001, no symbols = not significant when compared with the concurrent non-drug treated group. SEM= standard error of the mean

TABLE 9 CYTOTOXIC EFFECT OF VARIOUS CONCENTRATIONS OF COMPOUND II IN DIFFERENT CELL LINES BY MTT ASSAY

Concentration µg/ml	Cell viability(%viability)				
	HeLa	HepG2	KB	MCF-7	U373MG
0	100.13±1.44	100±0.21	100.10±0.04	100.06±0.13	100.01±0.11
1	96.33±2.49	89.32±0.41	91.75±1.10	87.18±0.35	93.28±0.24
5	74.25±1.07[a]	85.02±0.24	81.35±0.26	78.27±0.15[a]	78.52±0.36[a]
10	64.47±0.32[a]	71.04±0.16[a]	67.25±0.26[a]	72.36±0.30[a]	66.79±0.26[a]
20	54.62±0.21[a]	59.64±0.30[a]	63.23±0.20[a]	62.93±0.50[a]	63.91±0.37[a]
30	41.27±0.32[a]	51.96±0.24[a]	60.02±0.30[a]	60.01±0.45[a]	52.25±0.27[a]
40	32.74±0.21[a]	44.05±0.28[a]	37.19±0.21[a]	48.41±0.24[a]	38.48±0.38[a]
50	28.07±0.32[a]	37.62±0.37[a]	30.53±0.21[a]	44.10±0.35[a]	31.19±0.29[a]
100	28.77±0.27[a]	36.67±0.58[a]	29.16±0.71[a]	32.67±0.21[a]	29.08±0.35[a]

p < a = 0.05, b = 0.01, c = 0.001, no symbols = not significant when compared with the concurrent non-drug treated group. SEM= standard error of the mean

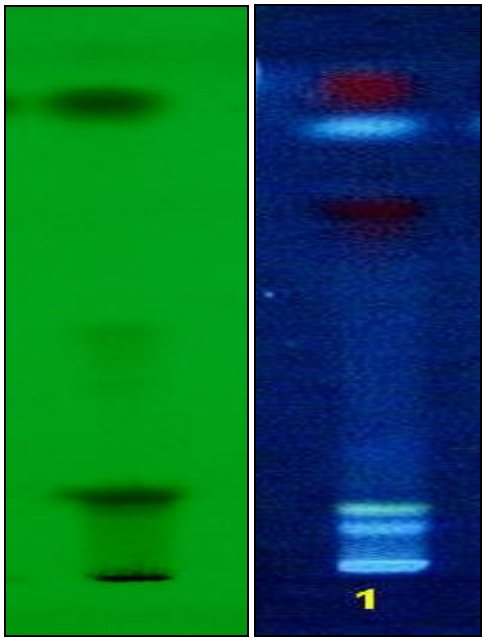

FIG. 1: HPTLC ANALYSIS OF CHLOROFORM FRACTION OF 85 % ETHANOLIC EXTRACT OF ALSTONIA SCHOLARIS. SOLVENT SYSTEM TOLUENE: ETHYL ACETATE (70:30)

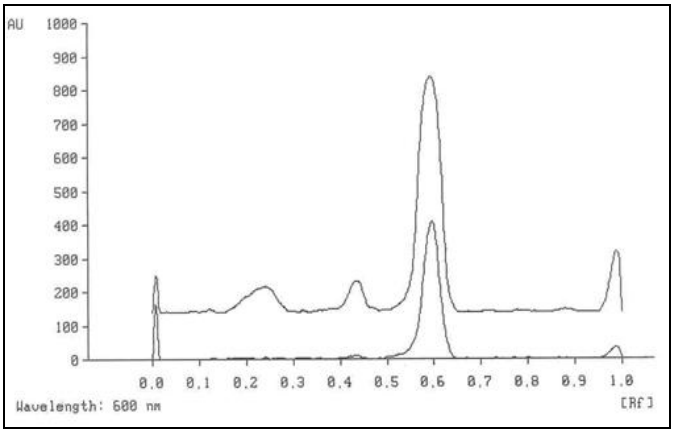

FIG.2: TLC DENSITOMETRIC SCAN AT 600 NM OF CHLOROFORM FRACTION OF ETHANOLIC EXTRACT OF *ALSTONIA SCHOLARIS*, COMPARISION OF COMPOUND OBTAINED FROM THE COLUMN CHROMATOGRAPHY AND COMPOUND OBTAINED FROM THE HPLC.

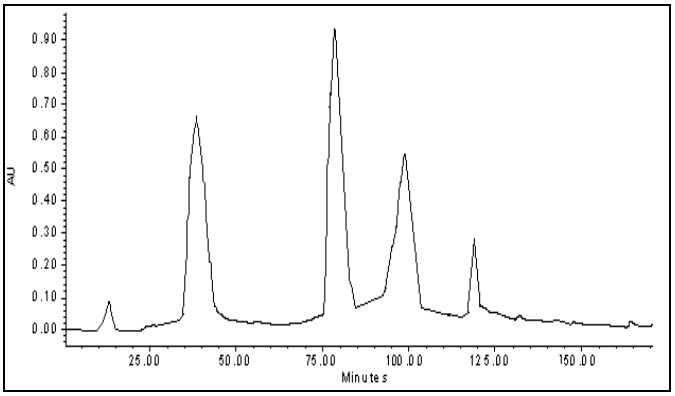

FIG. 3: HPLC CHROMATOGRAM OF ETHANLOIC EXTRACT OF *ALSTONIA SCHOLARIS* RECORDED AT 250nm.

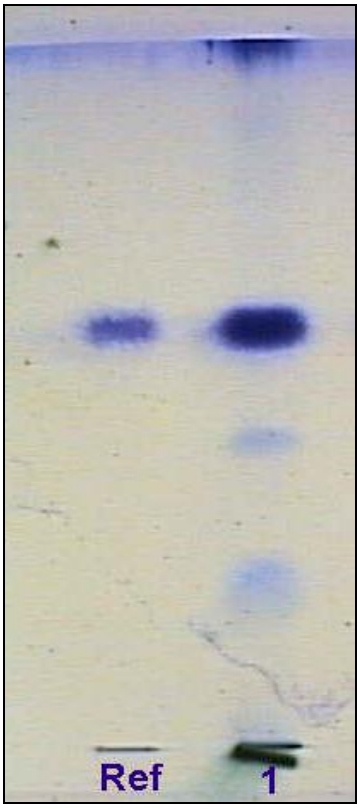

FIG. 4: HPTLC ANALYSIS OF COMPOUND ISOLATED FROM CHLOROFORM FRACTION OF ALSTONIA SCHOLARIS THE PERCENTAGE OF COMPOUND IS FOUND TO BE NOT LESS THAN 0.30 % (W/W). COMPARISION OF COMPOUND OBTAINED FROM THE COLUMN CHROMATOGRAPHY AND COMPOUND OBTAINED FROM THE HPLC

UV Spectral Characteristics of Compound II:

UV (Methanol); λ_{max} (logε) 346 (3.35), 293(3.56), 327 (3.57) nm

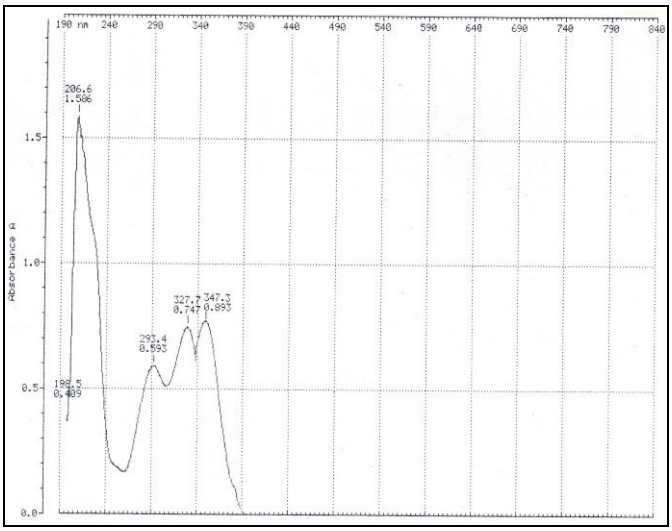

FIG. 5: UV SPECTRA OF COMPOUND II

IR Spectral characteristics of Compound II:

3478cm-1,3325cm-1,3168cm-1;1979cm-1; 1689cm-1;1368 cm-1;1289 cm-1;1160 cm-1.

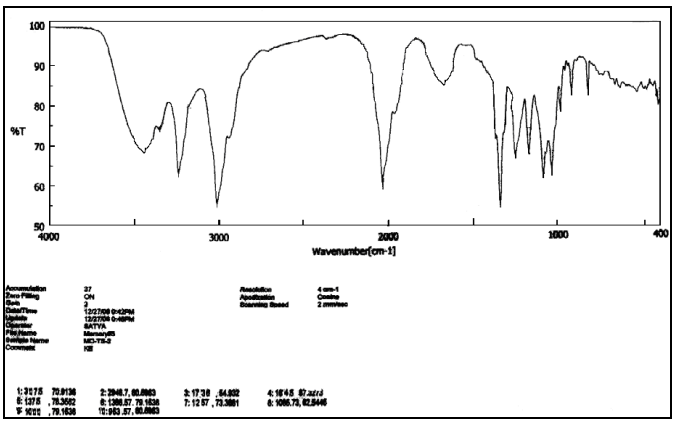

FIG. 6: IR SPECTRA OF COMPOUND II

1HNMR ($CDCl_3$) spectral characteristics of Compound II:

1H NMR (MeOH- d4,500 MHz)
δ 7.50 (1H, dd, J = 7.4, 1.0 Hz, H-9), δ 7.21(1H, dd, J=7.6, 1.0 Hz, H-12), δ 4.35 (1H, dd, J=3.3, 2.8 Hz, H-3), δ 4.30 (1H, d, J= 7.8 Hz, H-1′), δ 3.91 (1H, dq, J= 6.5, 2. 6Hz , H-19), δ 3.86(1H, dd, J= 12.2, 2.2 Hz, H- 6a′), δ 3.84(1H, d, J = 10Hz, H- 5β, partly overlap with δ H-6a′), δ 3.79 (3H, s, OCH3), δ 3.75(1H, dd, J= 13.5, 13.5 Hz, H-21 β, partly overlap with H - 5α) , δ 3.73 (1H , dd, J= 10 Hz , H-5α , partly overlap with H – 21 β), δ 3.68 (1H, dd, J = 12.2, 5.7 Hz , H-6b′), δ 3.65 (1H, dd, J = 13.5 , 5.7 Hz , H- 21α), δ 3.46 (1H ,dd ,J = 3.3, 2.8 Hz, H-15), δ 3.36 (1H, t, J=9.1Hz, H-3′), δ 3.31 (1H, t,H-4′,overlap with MeOH - d4 peak), δ 3.24(1H, ddd, J=9.4, 5.7, 2.2 Hz, H-5′), δ 3.18 (1H, dd, J=9.1, 7.8 Hz, H-2′), δ 2.60 (1H, ddd, J=14.6, 3.3, 3.3 Hz, H-14 a), δ 2.42 (1H, ddd, J=13.9, 10.0, 6.7 Hz, H-6β), δ 2.27 (1H, ddd, J=13.9, 7.2, 5.7 Hz ,H3-18), δ 1.30 (1H, ddd, J=14.6, 2.8, 2.8 Hz, H-14 b);

The signals due to methylene and methine protons overlapped with each other

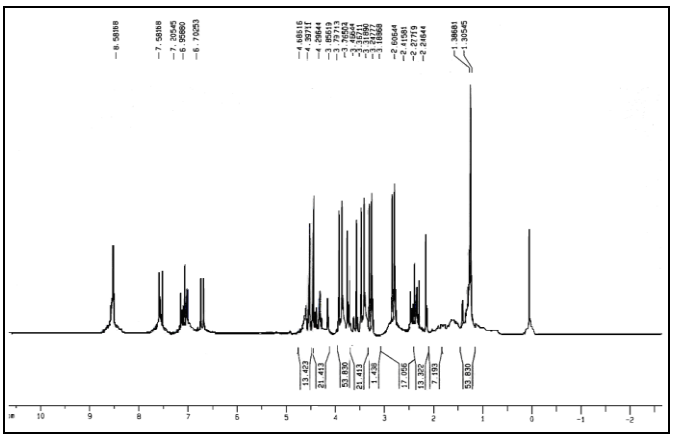

FIG.7: NMR SPECTA OF COMPOUND II

Mass spectral characteristics of Compound II:

The Molecular weight of the compound II was TOF MS ES+ 519

Molecular formula: C26H35N2O9
Molecular Weight: 519.2342

EIMS (m/z): [M + H]+ 519.2354, 372, N-Oxide O- (356.62), 340(-CH3), 296 (-COO), 281 (-CH3), 241 (C2H2 O-), 225 (CH3-), 197 (N-CH2), 180 (-CH2).

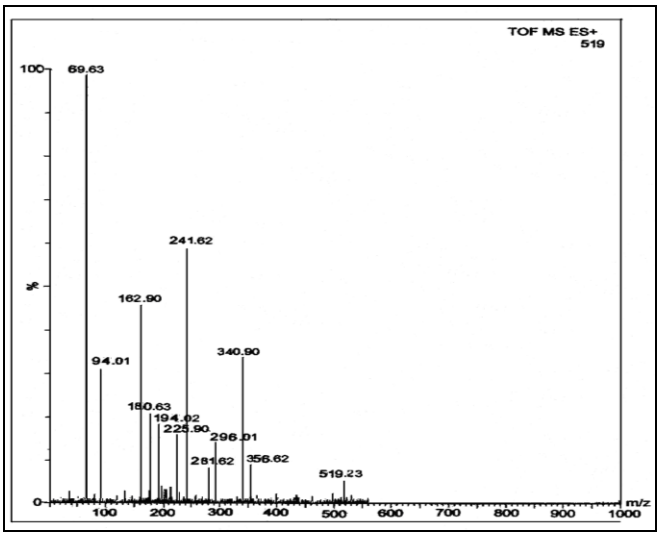

FIG. 8: MASS SPECTRA OF COMPOUND II

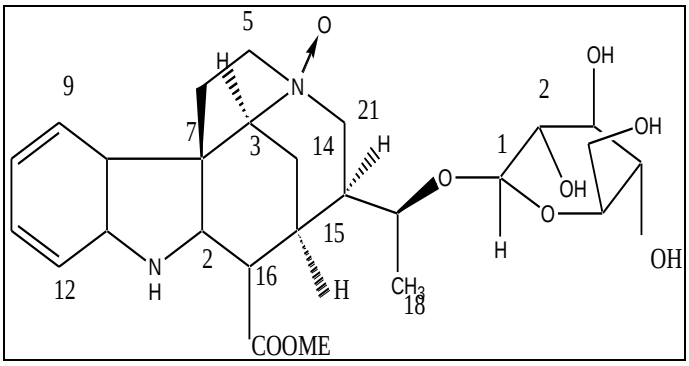

FIG. 9: MOLECULAR STRUCTURE OF ECHITAMIDINE-N-OXIDE-19-O-β-D-GLUCOPYRANOSIDE (EOG).

DISCUSSION: Phytochemical analysis is an important tool to identify various bioactive chemicals in the plants, which may or may not be of use for human health care. An attempt has been made to isolate and characterize an indole alkaloid from the chloroform fraction of 85% ethanolic extract of *Alstonia scholaris* that as shown some promise as an antineoplastic agent in earlier study[21].

Various phytochemical constituents including indole alkaloids have been isolated from *Alstonia scholaris* in India, Pakistan, Thailand, Philippines, Malaysia and Indonesia bearing different structural properties. Samples from continental countries (India, Pakistan and Thailand) have been found to contain picrinine-type indole alkaloids, whereas those from Indonesia and the Philippines mostly contained alkaloids bearing the angustilobine skeleton [22-24]. Echitamine chloride was isolated from the root and root bark of *Alstonia scholaris* as early as 1967 [19-20]. A yellow amorphous powder was isolated from the chloroform fraction of 85% ethanol extract of *A. scholaris* and identified as an indole alkaloid in the present study.

The UV, IR, NMR and mass spectroscopic analysis of this alkaloid showed that it contains 26 carbon, 35 hydrogen, 2 nitrogen and 9 oxygen atoms with a molecular weight of 519.2342. It was identified as echitamidine-N-oxide-19-O-β-D-glucopyranoside. Similarly, [17] had also isolated echitamidine-N-oxide-19-O-β-D-glucopyranoside isolated from stem bark of *A. scholaris* from the Australian region earlier [18].

However, they used HPLC technique only to isolate this compound along with other indole alkaloids, unlike the methodologies employed in the present study. In the present investigation echitamidine-N-oxide-19-O-β-D-glucopyranoside was isolated using column chromatography, HPTLC in addition to HPLC and it was compared with the compound obtained by these investigators. An another species *Alstonia glaucescens* have been reported to contain indole alkaloids like 17-O-acetyl- Nb- demethyl echitamine, echitamidine-N-Oxide, echitaminic acid, sweroside, echitamidine, Nb- demethylechitamine, 20-epi-19-echitamidine, N-b- demethylechitamine-N- oxide and echitamine[17]. Recent phytochemical analysis has shown the presence of seco-uleine alkaloids such as manilamine, N4-methyl angustilobine B, vallesamine, angustilbine B N4-oxide, 20 (s) tubotaiwine and 6,7- seco angustilobine B in the leaves of Philippine *Alstonia scholaris* from Philippine [24].

The leaves and bark of *Alstonia sholaris* have also been reported to contain alkaloids like L-agumamine (19-hydroxytubotaiwine), angustilobine B acid, losbanine (6,7-seco-6-nor-angustilobine B), tubotaiwine, its oxide, 6,7-secoangustilobine B, 17-0-Acetyl echitamine and echitamine [16] . It has also been reported to possess other alkaloids including 19-epischolaricine, Nb-methylscholarcine, Na-methylburnamine and vallesamine Nb-oxide, picrinine, nareline, and alschomine [14].

The minor alkaloids including akuammicine, akuammicine-Nb-methiodide, akuammicine-Nb-oxide, akuammigine, Nb-demethyl ehitamine, tubotaiwine, have been also reported from the *Alstonia scholaris* [20]. Venoterpine a monoterpenoid alkaloid has also been isolated from the fruits of *Alstonia venenat* [25]. The neutral constituents including α-amyrin, lupeol acetate, stigmasterol, β-sitosterol, campesterol have also been reported from the *Alstonia scholaris* root and bark [26-27].

The phytochemical analyses of chloroform faction of 85% ethanolic extract of *A. scholaris* showed the presence of echitamidine-N-oxide-19-O-β-D-glucopyranoside, an indole alkaloid. IR, NMR and Mass spectra showed that it contains 26 carbon, 35 hydrogen, 2 nitrogen and 9 oxygen atoms and its molecular weight is 519.3442[18]. The MTT assay revealed that it was highly effective in killing all neoplastic cell lines studied in a concentration dependent manner and it may act as a novel antineoplastic agent.

CONCLUSION: As far as authors are aware the cytotoxic effects of echitamidine-N-oxide-19-O-β-D-glucopyranoside have not been evaluated. Therefore, an attempt was made to study the cell killing effect of echitamidine-N-oxide-19-O-β-D-glucopyranoside in various human cancerous cell lines of different tissue origin. Exposure of HeLa, HepG2, KB, MCF and U373MG to various concentrations of EOG caused a concentration dependent rise in its cell killing and maximum effect was observed at 100µg/ml EOG indicating its cytotoxic nature and its application as antineoplastic agent. The cell killing effect in all cell lines screened was more or less equal indicating that EOG may act as a novel antineoplastic agent. These observations are in agreement with our earlier findings where a similar

effect has been reported with the crude chloroform fraction of 85% ethanol of *A. scholaris* stem bark[21]. An identical effect has been observed with another alkaloid echitamine chloride *in vitro* and *in vivo* [12]. Since the isolated compound echitamidine-N-oxide-19-O-β-D-glucopyranoside showed a good cytotoxic effect with different cancer cell lines, the mechanism of action need to be understood.

ACKNOWLEDGEMENTS: The authors are grateful to Indian Council of Medical Research, Government of India, New Delhi, for providing financial assistance in the form of senior Research Fellowship to carry out the work.

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How to cite this article:
Reddy DS: Phytochemical Analysis of Active Constituents of *Alstonia Scholaris* and their Cytotoxicity *in vitro*. Int J Pharm Sci Res 2016; 7(8): 3262-73.doi: 10.13040/IJPSR.0975-8232.7(8).3262-73.