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A SIMPLE METHOD FOR THE ISOLATION AND ESTIMATION OF URSOLIC ACID IN *ALSTONIA SCHOLARIS* R. BR.

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ABSTRACT: *Alstonia scholaris* R. Br. (Fam: Apocynaceae) is widely used plant in Indian medicine for the treatment of various ailments. Among the complex constituents of this plant, ursolic acid, a triterpene acid is reported in the leaves of *A. scholaris*. Ursolic acid was isolated from the methanolic extracts of the leaves of *A. scholaris*. The isolated marker ursolic acid was authenticated by UV, IR, Mass and NMR spectroscopy. A simple sensitive and HPTLC method was developed for the quantitative analysis of ursolic acid in leaves of *A. scholaris*. The method was found to be precise, with relative standard deviation (RSD) values of intraday and interday analysis in the range of 1.28-3.41% and 2.43-3.07% respectively. Accuracy of the method was checked by conducting recovery study at three different levels (50, 100 and 125%) and the average recovery was found to be 100.57%. The content of ursolic acid was found to be 3.10 ± 0.01gms/100gms of powdered leaves of *Alstonia scholaris*. The proposed method is rapid, simple and accurate and can be used for the routine quality control of ursolic acid.

INTRODUCTION: *Alstonia scholaris* R.Br (Apocynaceae), a traditional plant commonly known as Saptaparni has attracted considerable attention due to the presence of pharmacologically and phytochemically important alkaloids [1-3]. In contrast to alkaloids, there is scanty data on ursolic acid in this plant.

Ursolic acid has tremendous pharmacological actions such as anti-inflammatory [4] hepatoprotective [5] hair growth stimulant [6] and anti-tumor activity [7]. Banerji *et al*., reported the presence of pentacyclic triterpenoids like ursolic acid in *Alstonia scholaris* [8].

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In the present paper, we report a simple procedure for the isolation of ursolic acid from the leaves of *A. scholaris*. The isolated ursolic acid from the leaves of *Alstonia scholaris* was authenticated by spectroscopic methods. The isolated ursolic acid was considered as standard marker. Several chromatographic methods are reported for the quantification of ursolic acid in plants [9-12].

One of the literature surveys also reveals High performance thin layer chromatography (HPTLC) method for the quality control of ursolic acid in *A. scholaris* but the authors have not reported the percentage content of ursolic acid in the leaves of *A. scholaris* [13].

So the objective of the present study is also to develop a simple, sensitive and reproducible HPTLC method for the estimation of ursolic acid in the leaves of *A. scholaris.*

MATERIALS AND METHODS:

Plant material: Leaves of *A. scholaris* was collected from the botanical garden of L.M. College of Pharmacy, Ahmedabad in the month of mid-September 2007 and was identified by Prof. K.N. Patel of Department of Pharmacognosy, L.M. College of Pharmacy, Ahmedabad. A voucher specimen of the same is deposited at the herbarium of the Department of Pharmacognosy, L.M. College of Pharmacy, Ahmedabad.

Extraction and Isolation: The air-dried and pulverized leaves of *A. scholaris* (500 g) were defatted with petrol ether (60º-80ºC) in a Soxhlet apparatus for 6 hrs. The petrol ether layer was discarded and the marc was dried and extracted with methanol for 4 hrs in a Soxhlet apparatus. The methanolic extract was filtered and concentrated *in vaccuo* to yield a residue (50 g). The solid residue was then extracted with diethyl ether in a Soxhlet apparatus for 2 hrs. The ethereal solution was decolorised using activated charcoal. The ether layer was evaporated to dryness and the residue left was crystallized several times with methanol. A white amorphous solid was separated (M.P. 260-265ºC) [14]. Identity of this compound was further confirmed by spectral (UV, IR, ^{1}H NMR and Mass) data.

Quantitative estimation of marker compound by HPTLC:

- **HPTLC conditions**: The HPTLC system consisting of a LINOMATE-IV applicator and CAMAG TLC SCANNER-III with CATS 4 software: stationary phase: precoated silica gel F_{254} aluminium plate (10×20cm, E-Merck grade); mobile phase: Toluene: acetone: acetic acid (10:2:0.07) development: vertical in saturated twin trough chamber ; detection : 10% methanolic sulphuric acid reagent, pink color.

- **Preparation of standard solution and calibration curve for ursolic acid**: A stock solution of ursolic acid (1 mg/mL) was prepared by dissolving 10 mg of accurately weighed ursolic acid in methanol and making the volume up to 10 mL with methanol. From this stock solution standard solutions of 40-120 µg/mL were prepared by transferring different aliquots (0.4-1.2mL) of stock solution to 10 mL volumetric flask and adjusting the volume with methanol. 10 µL each of the standard solutions (400 to 1200 ng/spot) were applied in triplicate on TLC plate. The plate was developed in the solvent system up to a distance of 8 cm. After development the plates were dried and sprayed with the 10% methanolic sulphuric acid reagent followed by heating at 105º C for 5 minutes. The plates were scanned densitometrically at 550 nm. The peak areas were recorded. Calibration curve of ursolic acid was prepared by plotting peak area Vs concentration of ursolic acid applied.

- **Preparation of test sample solution**: The air dried leaves of *A. scholaris* (1 g) was collected and separately pulverized and refluxed thrice with methanol (25, 20 and 20 mL, each 1 hr) at 80º C. The extracts were combined and filtered while hot. The filtrate was concentrated to a tarry lump, which was dissolved in 50 mL of methanol. An aliquot of 5 mL was diluted up to the mark in a volumetric flask (25 mL). The test solutions (2, 5, 7 µL) were spotted thrice along with the standard solution.

Validation of HPTLC method: The method was validated according to the ICH guidelines on the validation of analytical methods [15]. All results were expressed as percentages, where n represents the number of values. For statistical analysis Excel 2000 (Microsoft Office) was used. A 5% level of significance was selected.

1. **Linearity**: The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical trans-formation, proportional to the concentration of an analyte in sample within a given range. The range of an analytical method is the interval between the upper limit and lower limit of an analyte

2. **Precision**: Instrument precision was checked by repeated scanning (n=7) of the same spot of ursolic acid standard solution (400ng/spot) and expressed as relative standard deviation (RSD). The repeatability of the method was confirmed by analyzing 400 ng/spot of ursolic acid on the HPTLC plates (n=5) and was expressed as RSD. Variability of the method was studied by analyzing aliquots of standard solution containing 200, 400 and 600 ng spot of ursolic

acid on the same day (intraday) and on different days (inter-day), and the results were expressed as RSD.

3. **Accuracy**: The accuracy of the method was assessed by performing recovery studies at 3 different levels (approximately 50, 100 and 125% addition of ursolic acid). The recoveries and average recoveries were calculated.

4. **Specificity**: It is an ability of the developed analytical method to detect the analyte quantitatively in presence of other compounds, which are expected to be present in the same matrix. The proposed method has been developed for estimating quantitatively ursolic acid in sample of *A. scholaris*. During analysis the other components present in sample solution did not interfere.

5. **Sensitivity**: The sensitivity of the method was determined with respect to Limit of detection (LOD) and Limit of quantification (LOQ). The LOD and LOQ were evaluated by applying different dilutions of the standard solutions of ursolic acid along with the blank (methanol).

RESULTS AND DISCUSSION: Ursolic acid, the standard marker was isolated by simple procedure avoiding the tedious column chromatographic technique. The standard marker was thoroughly characterized by spectroscopic analyses. UV (λ_{max} (methanol): 212.4 nm, IR spectrum shows peak of –OH(alcohol) at 3427cm^{-1} and of C=O(carbonyl group) at 1689.53 cm^{-1}, other peaks were obtained at 2650 cm^{-1} and 2358.7 cm^{-1}.

Mass spectrum gave M+ peak at the molecular weight 455; other peaks of different fragments were obtained at 439, 248, 203, 189 and 119. 1H NMR (300 MHz, $CDCl_3$): δ5.24(m, 1H, H-12), δ3.4(d,1H,OH-3), δ3.2(m,1H,H-3), δ2.2(d, 1H, H-18), δ2.02-1.15(m,22H), δ2.0(1H, COOH-28), δ0.76-1.01(m, 21H, 7Me). The data was compared with the reported value [16-17].

The studies at the preliminary level revealed that toluene: acetone: acetic acid (10:2:0.07) was ideal as mobile phase and gave a sharp band at Rf (0.45) of ursolic acid. The solvent system was found suitable for test samples.

The spot of the chromatogram was visualized (pink) by spraying with 10% methanolic acid reagent followed by heating at 105º C for 5 minutes.

Chromatograms obtained from ursolic acid standard and from methanolic extract of leaves of *Alstonia scholaris* are shown in **Figure 1 and 2** respectively. The HPTLC method was validated for precision, accuracy and repeatability. The validation parameters are stated in **Table 1**.

The method is specific for ursolic acid because it resolved the compound well from other components of *Alstonia scholaris* leaves (Figure 2). The standard compound was found to be present in test samples at the same R_f.

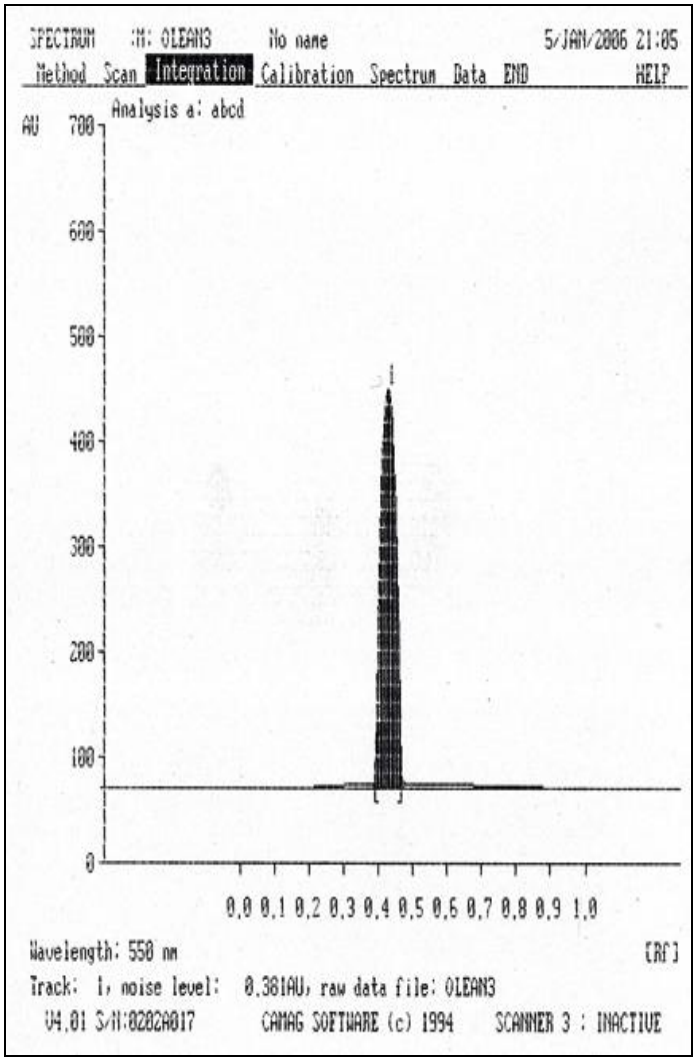

FIGURE 1: HPTLC CHROMATOGRAM FOR STANDARD URSOLIC ACID

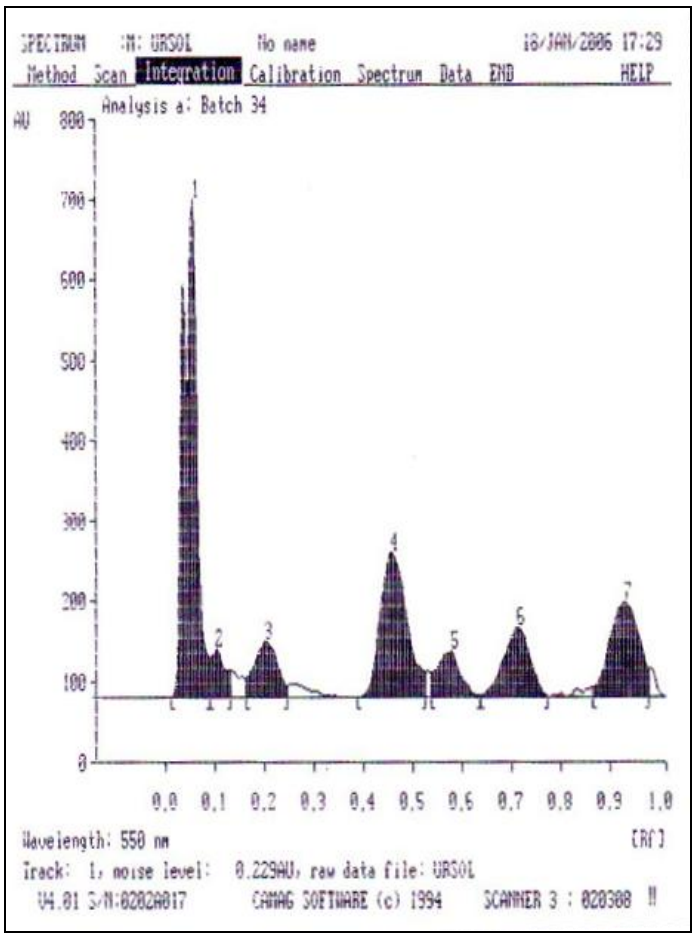

FIGURE 2: CHROMATOGRAM OBTAINED FROM METHANOLIC EXTRACT OF *ALSTONIA SCHOLARIS* R.BR LEAVES. PEAKS 1-3 AND 5-7 ARE FROM UNKNOWN COMPOUNDS. PEAK 4 IS THAT FOR URSOLIC ACID

TABLE 1: VALIDATION PARAMETERS FOR HPTLC METHOD DEVELOPMENT OF URSOLIC ACID

Sr. No.	Parameters	Results
1	Range, ng/spot	400-1200
2	Instrumental Precision {RSD(%), n=7}	0.49
3	Repeatability {RSD(%),n =7}	1.15
4	Intraday precision {%CV, n=3}	1.28-3.41
5	Interday precision {%CV, n=5}	2.43-3.07
6	Accuracy (average recovery, %)	100.57
7	Limit of detection, ng	50
8	Limit of quantitation, ng	100
9	Specificity	Specific
10	Linearity (r)	0.998

The peak purity was confirmed by comparing the standard and test sample spectrum. A polynomial regression was obtained between response (peak area) and ursolic acid concentration in the range of 400-1200 ng/spot (Table 1). The calibration spots were represented by the polynomial equation Y= -0.00x2 + 12.47x + 2233.63, where y is the peak response and x= the amount of ursolic acid (ng); the co-relation co-efficient was 0.9998 (**Figure 3**).

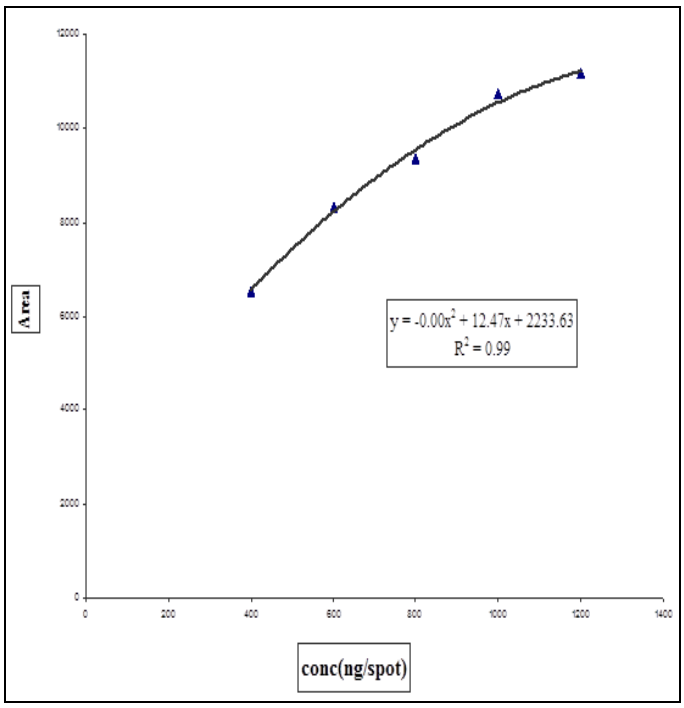

FIGURE 3: CALIBRATION CURVE OF URSOLIC ACID

Instrument precision was studied by scanning the same spot of ursolic acid seven times (%CV=0.49). The repeatability of the method was tested by analyzing seven application of the same standard solution %CV=1.15. The intraday and interday RSD was found to be in range of 1.28-3.41% and 2.43-3.07% respectively.

The accuracy of the method was determined at three different levels (50, 100,125%) by adding known amounts of standard solution of ursolic acid to test solutions of ursolic acid. The average recovery at the three different levels was found to be 100.57. The LOD and LOQ for ursolic acid were found to be 50 and 100 ng respectively.

The percentage of ursolic acid in the alcoholic extract of leaf samples were calculated using peak area parameters. The ursolic acid content in the leaves of *A. scholaris* was computed from the calibration curve. It was found to be 3.10±0.01 gm/100 gm of powdered leaf sample of *Alstonia scholaris* (**Table 2**).

TABLE 2: HPTLC DATA FOR QUANTITATIVE ANALYSIS OF URSOLIC ACID IN THE LEAVES OF *A. SCHOLARIS* R. Br. (result for 5µL of test sample spotted)

Ursolic acid content	Area (Mean ± S.D), n=3
Amount /spot	622.40 ± 3.45 ng
Amount /1gm	31.06 ± 0.15 mg
Amount/100 gm	3.10 ± 0.01gm

CONCLUSION: A simple method was developed for the isolation of ursolic acid from the leaves of *A. scholaris*. The isolated compound was identified by different spectroscopic methods and it was considered as standard compound for the estimation of ursolic acid. The proposed HPTLC method was successfully applied for the estimation of ursolic acid from the leaves of *A. scholaris*.

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