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Original Article

Pharmacognostic evaluation of medicinally important mangroves *Avicennia marina* and *Sonneratia apetala*

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ABSTRACT

Avicennia marina Forssk. (Avicenniaceae) and *Sonneratia apetala* Buch.-Ham. (Lythraceae) are two mangrove trees, found in intermediate estuarine zones. Leaves of *A. marina* have been reported to possess antimicrobial, antioxidant, cytotoxic activities whereas leaves of *S. apetala* are reported to possess antimicrobial and antifungal activities. Though flavonoids, glucosides, and triterpenoids like betulin, lupeol, avicenol etc are reported to be present, no chromatographic method exists till date for their quality evaluation. In the present study, macroscopic and microscopic, physicochemical, phytochemical and safety profile of their leaves has been evaluated and discussed. Findings of this study can be useful as reference information in order to evaluate quality of *A. marina* and *S. apetala* leaves. Chromatographic separation was achieved on TLC plates using toluene: methanol (8:1, v/v). A compact spot of ursolic acid at R_f value of 0.30 ± 0.02, β-sitosterol at 0.46 ± 0.02 and lupeol at 0.59 ± 0.02 was observed in the methanolic extracts at 366 nm. Methanolic sulphuric acid reagent (10%) was used as the derivatizing reagent. The method has been validated as per ICH guidelines and can be a useful as an analytical tool for quality evaluation of plants rich in these three triterpenoids.

Keywords: Mangroves, HPTLC, Simultaneous estimation, β-sitosterol, lupeol and ursolic acid

INTRODUCTION

Although early workers regarded mangrove forests as unimportant, transitional communities with a low productivity, most ecologists today view them as highly productive, ecologically important systems. Mangrove habitats are one of the most hostile environments with its fluctuating tidal and saline regime. Only limited plant species can survive under such condition. Nevertheless, these plants are a valuable resource and provide economical and ecological benefits to the coastal people. Mangrove forests have been utilized for many functions including wood production, firewood and charcoal [10]. However, wood-related activities or industries are very destructive and the rates of mangrove renewal do not match this at all [4]. Recently, it has been strongly recommended that mangroves should be considered as a valuable source for chemical constituents with potential medicinal use. Although the chemical constituents of most mangrove plants have not been studied extensively, investigations so far have led to the discovery of several novel compounds with prospective medicinal value for the discovery of new chemotherapeutic agents [5].

Avicennia marina Forssk. (Avicenniaceae) commonly called as *Tivar* is a cosmopolitan small mangrove tree widely distributed along tropical and subtropical coastlines. The barks, leaves, and fruits of this species have been used as traditional medicine in Egypt to treat skin diseases [1]. *A. marina* contains abundant chemical components which include triterpenoids like Betulin, β-sitosterol, lupeol, naphthalene derivatives like Avicequinone A-G etc [13]. Leaves of *A. marina* have been reported to possess antimicrobial, antioxidant, anticandidal and cytotoxic activities

Sonneratia apetala Buch-.Ham (Lythraceae) commonly called as mangrove apple or *Kandal* is another small tree found along south asian coasts. This plant has been traditionally used to treat hepatitis [12]. The literature also reports that the leaf part of the plant is widely used for dysentery, sprains and bruises, in eye troubles and for open soresin children ears. They are also reported to be used in heart troubles [11]. Ethanolic extract of this plant has also been reported to possess antimicrobial activity [7]. However very little scientific data is available on these plants regarding their phytochemical constituency. This plant have

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been reported to possess flavones, flavanoids, terpenoids and phytochemicals like betulin, β-sitosterol [3] etc, but no chromatographic method exists till date for their quality evaluation. Also, no scientific baseline data on the quality control parameters for these plants is available in any of the pharmacopoeias.

Thus in the present work, limits for quality parameters through the analysis of physicochemical evaluation and phytochemical analysis have been established along with the microscopic analysis of the leaves. Further, the quality of the leaves of these two mangroves has been evaluated using HPTLC technique. Simultaneous estimation of three triterpenoids namely β-sitosterol, lupeol and ursolic acid has been carried out using a single mobile phase from the leaves of *A. marina* and *S. apetala*.

MATERIALS AND METHODS

Plant material: Fresh leaves of *Avicennia marina* and *Sonneratia apetala* were collected from Airoli, Mumbai and the herbaria were authenticated from Agharkar Reasearch Institute, Pune (Auth. 13-017 and Auth 13-013 respectively). Samples were carefully segregated, cleaned, shade dried for a week and oven dried at 37° C to constant weight. Further, the samples were powdered, sieved (BSS 85) and stored in airtight bottles at room temperature.

Drugs and chemicals: Chemicals of analytical grade were purchased from Merck Specialities Pvt. Ltd, Mumbai. The reference standards ursolic acid (98.5% purity), β- sitosterol (98.0 % purity) and lupeol (97.0% purity) were procured from Sigma Aldrich Chemical Company, Steinheim, Germany. The Derivatizing reagent i.e 10% Methanolic sulphuric acid reagent was prepared according to the procedure described in Reich and Schibli [6].

Microscopy: Macroscopic characters of fresh leaves such as type of the leaf base, presence or absence of petiole, characters of lamina, venation, margin, apex, base, surface and texture were studied. Thin transverse sections of the leaf across the lamina and crossing the midrib were taken, stained with dilute safranin and observed under 45X magnification using light microscope equipped with a camera. Further, the powder of the dried leaves was also evaluated microscopically and distinctive characters were noted.

Physicochemical evaluation: The quality of the leaves was assessed by determining the proximate parameters like foreign organic matter, ash content, acid insoluble and water soluble ash content and loss on drying using standard pharmacopoeial methods [8].

Phytochemical analysis: The powder of dried leaves was then subjected to a phytochemical evaluation by successive soxhlet extraction with various organic solvents in order to analyze the percent extract of major class of compounds present in the raw materials using the method reported by Harborne [2]

Preparation of Standard and Sample Solutions: Stock solutions of pure compounds (1000 µg mL^{-1}) were prepared by dissolving 10 mg of accurately weighed standards in small amount of methanol and making the volume up to10 mL in a standard volumetric flask. The stock solutions were further diluted for the preparation of working solutions. Accurately weighed 100 mg of dried plant powder of *A. marina* and *S. apetala* was separately extracted in 10 mL methanol, vortexed and kept standing overnight. Next day, the extracts were filtered using Whatman filter paper no. 1 in dry stoppered test tubes and the filtrate (10 µL) was used for HPTLC analysis.

HPTLC - Instrumentation and operating conditions: Chromatographic separation was achieved on TLC plates pre‑coated with silica gel 60 F_{254}. Samples were spotted using the CAMAG Linomat 5 sample spotter (CAMAG Muttenz, Switzerland) equipped with syringe (Hamilton, 100 µL). Chromatograms were run to a solvent front of 85 mm in a glass twin trough chamber (CAMAG) pre-saturated for 20 mins with toluene: methanol (8:1, v/v) as the mobile phase. Post chromatographic derivatization was carried out by dipping the plate in 10% Methanolic sulphuric acid reagent. Densitometric scanning was performed using CAMAG TLC Scanner 4 equipped with winCATS software at 366nm and CAMAG ‑Reprostar 3 was used for photo documentation. Quantitative evaluation of the plate was carried out in the reflectance mode at 366 nm with slit width of 6 mm × 0.45 mm, scanning speed of 20 mm/s and data resolution set at 100 µm /step.

Method Validation: The developed HPTLC method for estimation of β – sitosterol, lupeol and Ursolic acid was validated as per ICH guidelines for the parameters like sensitivity, linearity, precision, recovery, specificity and ruggedness [9].

Specificity and sensitivity: Specificity of the method was confirmed by comparing the bands of the sample solutions with that of the respective reference standards in terms of Rf and color in fluorescence mode. Sensitivity of the method was determined with respect to limit of detection (LOD,

S/N of 3:1) and limit of quantification (LOQ, S/N of 10:1).

Preparation of calibration curve and quality control samples: For constructing the calibration curve, appropriate dilutions were prepared from the stock solutions. The working standards in the range of 5-100 μg mL^{-1}, 5-60 μg mL^{-1}, 5-75 μg mL^{-1} for ursolic acid, β-sitosterol and lupeol, respectively, were used to obtain a seven point linear calibration curve. Further, quality control samples were prepared and analyzed for precision, accuracy and ruggedness studies.

Repeatability and precision: The repeatability of the method was affirmed by analyzing 5 μg mL^{-1} of all the three markers on a HPTLC plate (n = 5) and expressed as % RSD. Precision were assessed by measurement of intra and inter-day variation. The result was expressed as % RSD.

Accuracy and ruggedness: The accuracy of the method was assessed by spiking the QC samples in plant matrix and calculating the percent recovery for each marker. Ruggedness was assessed by deliberately incorporating small variations like change of analyst, mobile phase and change in spotting volume like in the optimized chromatographic conditions. Response and R_f of QC samples was observed. Results were expressed in terms of percent mean difference.

Assay: The content of all the three markers from the leaves of *A. marina* and *S. apetala* was determined by applying the samples (10 μL) along with pure standards.

Estimation of the markers: The quantity of the markers was calculated using the regression equation obtained from the regression analysis of the calibration curve.

Statistical Analysis: The statistical analysis of the results obtained was done using Microsoft Excel 2007.

SAFETY EVALUATION

Safety study of the methanolic extract of the leaves of *A. marina* and *S. apetala* was conducted in mice as per OECD guidelines (No. 420, fixed dose procedure). The mice were fasted overnight for 10-14 hours and administered with the extract (2.0 g/kg) orally. The animals were observed individually during the first 30 min for all reflexes, periodically during the first 48 hours with special attention given during the first 4 hours (short-term toxicity) and daily thereafter for a total of 14 days (long-term toxicity) for alteration from general behavior and clinical symptoms like alteration of skin and fur texture, ptosis, excessive salivation, breathing problems, diarrhea etc. Daily body weight, food and water intake record was also maintained. The results were compared with control group (orally administered with DW)

RESULT AND DISCUSSION

Macroscopic characters: *Avicennia marina* grows as a shrub or tree to a height of three to ten meters. The leaves are thick, five to eight centimeters long. They are bright and glossy green on the upper surface, and silvery-white, or grey, with a layer of very small matted hairs on the surface below. *Sonneratia apetala* is a small tree growing in salt swamps. The leaves of are opposite, oblong, obtuse and lanceolate in shape. They are entire, evergreen and coriaceous. The base of the leaves is narrowed into a petiole (Fig. 1)

Microscopy: The transverse section of the leaf of *Avicennia marina* showed single upper epidermis followed by a multilayered palisade and 2-3 layered spongy tissue in the mesophyll region. This was followed by a single layer of lower epidermal cells. The distinct feature is the presence of T-shaped salt excretory glands on the surface of the leaf. The transverse section (Fig. 2) also shows the presence of sunken stomata symbolizing reduced transpiration rates found in the plant. Sclerenchyma cells were found present on the lower surface of the leaf midrib. The leaf section of *S. apetala* also showed a similar vascular bundle structure as in *A, marina.* The leaf T. S shows single epidermal layers on both the surfaces of the leaf. In the mesophyll region, the plant shows the presence of palisade tissue on both surfaces followed inwards by the spongy tissue layers. The leaf shows presence of air pockets near the surface.

Powder microscopic evaluation (Fig. 3) supports light microscopy by the presence of glandular cells in the powder of *A. marina* along with spiral xylem vessels, fibres and a mass of sclerenchymatous cells. This data correlation emphasizes on powder microscopy to be a potent quality evaluation tool. Leaf powder of *S. apelata* showed the presence of spiral xylem, fibre and sclerenchyamatous cells. Treatment with Lugol's reagent confirmed the presence of starch grains in the sample.

Physicochemical and phytochemical analysis: The proximate parameters such as foreign organic matter, ash content (Total, acid insoluble and water soluble), loss on drying and the extractive values in various solvents have been summarized in the tables 1 and 2. As no monograph is available for both of the plants, limits have been prescribed for the same. For both the samples, percent extractive

value in 50:50 hydroalcohol was found to be highest (*A. marina:*18.62% , *S. apetala:*19.13%), followed by methanol in the category of organic solvents. Amongst all the phytochemicals fractions extracted, leaves of *A. marina* and *S. apetala* were found to be rich in alkaloids (14.57 % and 17.68 % respectively), whereas the fractions of fats and waxes were found to be least in both the plants (0.47 % and 0.66 % respectively) (table 3). The leaves were also found to be source of terpenoids and phenolics, hence were further subjected to chromatographic characterization using validated HPTLC technique.

Method development: The plants, *A. marina* and *S. apetala* have been reported to contain triterpenoids and phenolics [13] [3] but, no report is available of chromatographic separation of individual phytochemical constituents form these plants. In the current research work, chromatographic characterization was carried out by developing a validated HPTLC method for the estimation of ursolic acid, lupeol and β-sitosterol.

In order to obtain good separation amongst three triterpenoids viz. ursolic acid, β-sitosterol and lupeol, various solvent systems were tried on normal phase HPTLC, out of which mobile phase composition of toluene: methanol (8:1, v/v) showed good resolution for ursolic acid, β – sitosterol and lupeol from other phytoconstituents simultaneously (Fig. 6). The R_f values of ursolic acid, β- sitosterol and lupeol were found to be 0.31 ± 0.02, 0.48 ± 0.02 and 0.59 ± 0.02 respectively. The method developed was validated as per ICH guidelines.

Visualization of spot of these markers directly under UV or visible radiation is not possible as none of them have chromophoric groups. Therefore, developed TLC plate was derivatized by dipping the plate in 10% methanolic sulphuric acid reagent. Reproducible results were obtained with dipping technique in a glass chamber filled with reagent instead of spraying. The derivatized plate was air dried and kept in oven for 5-7 min at 100°C before densitometrically scanning the plate at 366 nm. The method was found to be selective and had a good resolution.

Method validation: The objective of method validation was to confirm that the present method was suitable for its intended purpose as described in the ICH guidelines. The described method has been extensively validated in terms of specificity, linearity, repeatability, precision, accuracy, recovery and ruggedness. The validation results are summarized in table 4. The high recovery values from the mixture of compounds and the high repeatability indicated a satisfactory accuracy in the proposed method. The ruggedness of the method was also assessed. Minor modifications in the initial mobile phase composition had no major effect on the peak resolution of the compounds, i.e., all the compounds were well resolved with no merged peaks though there was minimal shift in the retardation factor (RF). Therefore, the HPTLC method for the separation of compounds can be regarded as selective, accurate, precise, and robust and has a wide scope in the area of natural product separation, characterization, drug development, and their quality control/standardization. All parameters enlisted in the above-mentioned validated experiments lie within the permissible limits recommended by the ICH guidelines.

Detection and quantitation: The method was further applied in the detection and quantitation of the biomarkers simultaneously from the methanolic extract of the leaves of *A. marina* and *S. apetala*. Identification of these three phytoconstituents in the leaves extract was confirmed by comparing the R_f, overlay and color of band with that of the standard. The content of ursolic acid, β- sitosterol and lupeol in *A. marina* was found to be 1.4286 ± 0.0064, 0.0934 ± 0.0010 and 0.7575 ± 0.0180 mg/ g of the sample respectively. In *S. apetala*, the content was found to be 2.1582 ± 0.0268 and 0.0832 ± 0.0032 mg/g of ursolic acid and β-sitosterol respectively (Table 6). Lupeol was not detected in the leaves of *S. apetala.*

Safety evaluation: The safety of both the drug was established by acute oral toxicity study carried out on mice at 2.0 g / kg body weight. The methanolic extracts of both the plants were found to be safe as it showed no abnormal fluctuation in body weights and food and water intake of the animals. Clinical symptoms of toxicity were also found to be absent during the period of the study and no mortality was recorded. The safety study of the mangroves revealed that the in form of methanolic extract of the leaves, they can be considered safe with a wide margin for oral use.

CONCLUSION

The evaluation of pharmacognostical parameters may ensure the identity and authenticity of *A. marina* and *S. apetala* leaves. The plant was found to be a good source of pharmacologically active markers ursolic acid, β-sitosterol and lupeol by HPTLC analysis. Both the plants were also found to be rich sources of alkaloids. Data generated from the acute oral toxicity study also ensures an adequate safety margin for their intended use. As no monographs are available for these plants, the present research work can be used as baseline database for the compilation of a monograph and

can be used for further research and evaluation of the therapeutic potential of these plants. The developed HPTLC method can be used as a quality control tool for these plants as well as in plants reported to contain these markers.

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HABITAT:

Fig1 (a)

Fig 1 (b)

Figure 1: a) Habitat and twig of *Avicennia marina*, b) A Habitat and a twig of *Sonneratia apetala*

Microscopy

Fig 2 (a)

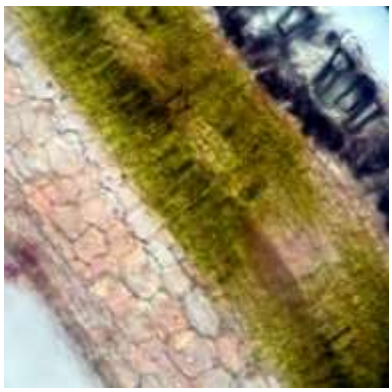

Fig 2 (b)

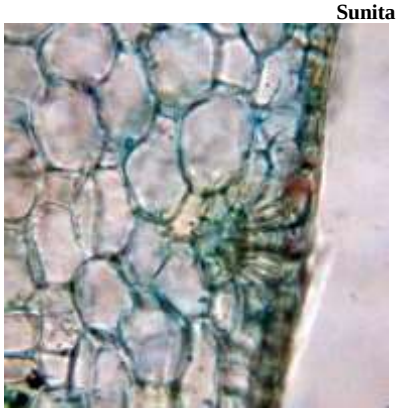

Fig 2 (c)

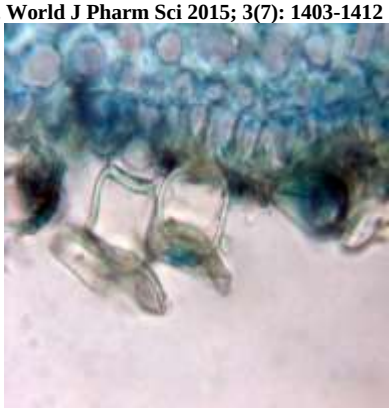

Fig 2 (d)

Figure 2: T.S of leaf of *Avicennia marina* a)T.S of midrib, **b)** T. S of lamina, **c)** Sunken stomata **d)** T shaped cells on lower leaf surface.

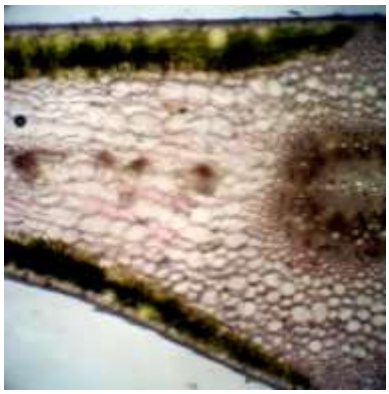

Fig 3 (a)

Fig 3 (b)

Fig 3 (c)

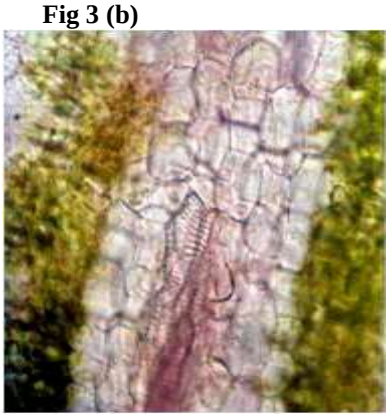

Fig 3 (d)

Figure 3: T.S of leaf of *Sonneratia apetala*
a) T.S of lamina, **b)** T. S of midrib, **c)** T. S sowing sunken stomata **d)** vascular bundle

Powder microscopy

Fig 4 (a)

Fig 4 (b)

Fig 4 (c)

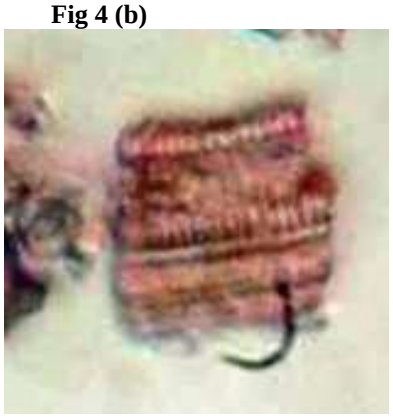

Fig 4 (d)

Figure 4: Powder microscopy of *Avicennia marina*
a) fibre, **b)** sclerenchyamatous tissue, **c)** T shaped cells **d)** xylem vessel

Fig 5 (a)

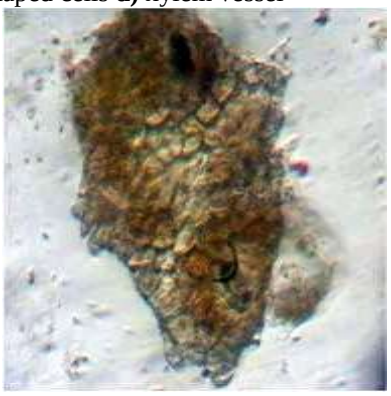

Fig 5 (b)

Fig 5 (c)

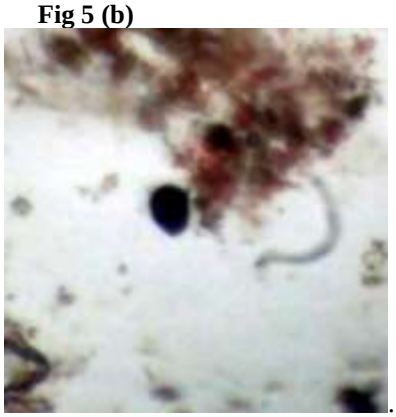

Fig 5 (d)

Figure 5: Powder microscopy of *Sonneratia apetala*
a) fibre, **b)** sclerenchyamatous tissue, **c)** Xylem vessel, **d)** Starch grain

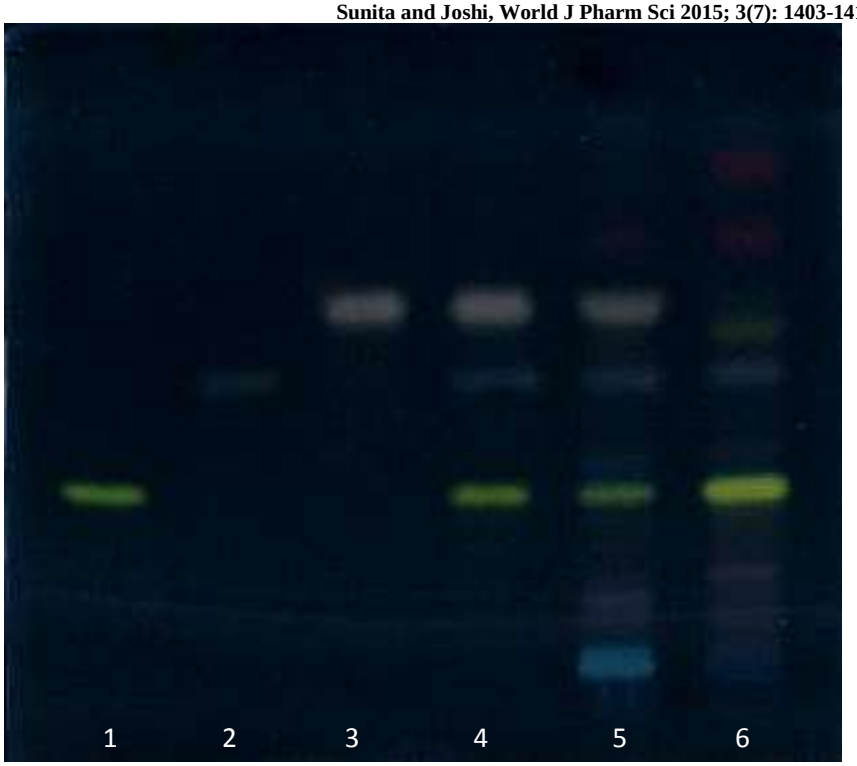

Figure 6 (a): Track details:
Track 1: Ursolic acid**, Track 2:** β-sitosterol**, Track 3:** Lupeol**, Track 4:** Simultaneous Ursolic acid, β-sitosterol and Lupeol**, Track 5:** *Avicennia marina***, Track 6:** *Sonneratia apetala*

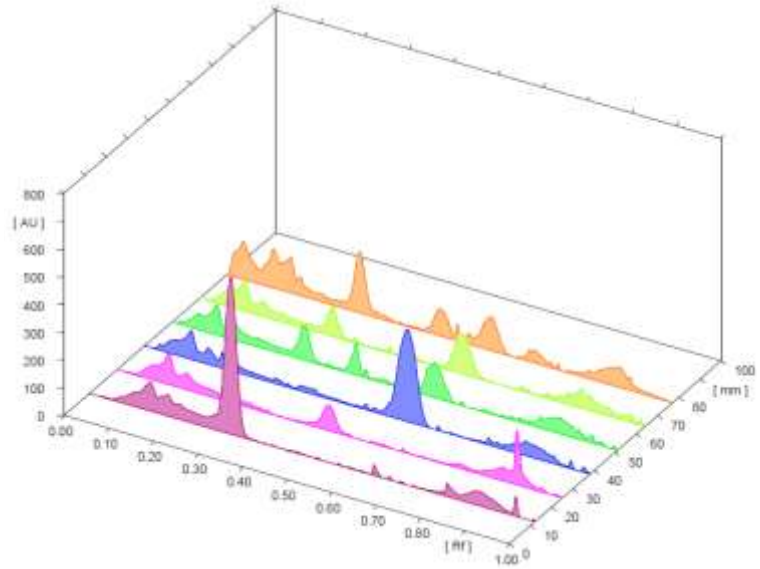

Figure 6 (b): Overlay of tracks at 366nm after derivatization by 10% methanolic sulphuric acid

Table 1: Preliminary analysis

Parameters	**Observed values (%)**		**Suggested limits (%)**	
	A. marina	***S. apetala***	***A. marina***	***S. apetala***
Foreign organic matter	0.41 ± 0.0112	0.43 ± 0.0152	0.378 - 0.444	0.393 – 0.485
Total ash	17.27 ± 0.2001	11.78 ± 0.0660	16.669 - 17.871	11.589 – 11.985
Acid insoluble ash	0.90 ± 0.0013	0.65 ± 0.0596	0.896 - 0.904	0.478 – 0.836
Water soluble ash	4.30 ± 0.1373	4.88 ± 0.5672	3.889 - 4.711	3.180 – 6.583
Loss on drying	9.67 ± 0.4588	8.89± 0.1800	8.297 - 11.050	8.356 – 9.437

Table 2: Extraction in different solvents

Solvents	Extraction (%)	
	A. marina	*S. apetala*
Ethanol	**8.96**	**10.52**
Methanol	**10.24**	**11.96**
Toluene	**6.16**	**5.00**
DW	**16.32**	**17.64**
Hydroalcohol (50-50%)	**18.92**	**19.13**
Ethyl acetate	**3.04**	**3.70**
Acetonitrile	**0.26**	**0.64**
Cyclohexane	**0.10**	**0.04**

Table 3: Phytochemical analysis

Parameter	Percentage (%)	
	A. marina	*S. apetala*
Fats and waxes	0.47	0.66
Fibres	72.69	71.58
Terpenoids and Phenolics	3.511	2.957
Quaternary Alkaloids and N-Oxides	2.961	3.05
Alkaloids	14.57	17.68
TOTAL	**94.204**	**95.927**

Table 4: Results of method validation experiment for simultaneous estimation of triterpenoids (ursolic acid, ß-sitosterol and lupeol)

Parameters	Ursolic acid	β- sitosterol	Lupeol
R_f	0.31	0.48	0.59
LOD and LOQ (µg mL^{-1})	1 and 5	5 and 15	15 and 45
Linear Range (µg mL^{-1})	5-150	15-35	45-105
System Suitability (% CV)	1.8243	1.6344	1.5781
Intraday Precision (% CV)	1.7021	0.50	0.89
Interday Precision (% CV)	1.99	0.93	1.15
Recovery	98.80%	99.32%	98.62%

Table 5: Optimized chromatographic conditions

Parameters	Specifications
Stationary Phase	Merck silica gel 60 F_{254} HPTLC pre-coated plates
Sample Applicator	Camag Linomat 5
Development distance	85 mm
Derivatization	10% Methanolic sulphuric acid reagent
Densitometric scanner	Camag scanner 4
Software	winCATS planar chromatography manager software version 1.4.7
Lamp, wavelength	Mercury, 366 nm
Photodocumentation	Camag Reprostar 3

Table 6: content of biomarkers

Plant	Ursolic acid	β-sitosterol	Lupeol
	Concentration (mg/g) Mean ± SD, n=3		
A. marina	1.4826 ± 0.0064	0.0934 ± 0.0010	0.7575 ± 0.0180
S. apetala	2.1582 ± 0.0268	0.0832 ± 0.0032	NOT DETECTED

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