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Original Article

CHROMATOGRAPHIC ANALYSIS FOR "DIOSGENIN" CONTENT IN *ABUTILON INDICUM* (L) SWEET

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ABSTRACT

Objective: Chromatographic evaluation of steroidal saponin 'diosgenin' from leaves of the medicinal plant by HPTLC.

Methods: HPTLC analysis was performed with methanolic (MeOH) and ethanolic (EtOH) leaf extract of *Abutilon indicum*. HPTLC analysis was carried out with Toluene: ethyl acetate: formic acid [6:5:1, v/v/v] mobile phase and anisaldehyde-sulphuric acid reagent as spraying reagent for derivatization. Quantitative evaluation of diosgenin content in extracts was estimated using diosgenin standard curve (500-3000 ng/spot).

Results: HPTLC plate scanned at 366 nm showed good resolution and band color after derivatization. The appearance of brown color spot on chromatograms confirmed the diosgenin content in samples and it was observed to be 0.4 and 0.11 % (w/w) in MeOH and EtOH extracts, respectively.

Conclusions: *A. indicum* plant leaves are a good source of steroidal saponin diosgenin and can be used as an alternate natural source to synthesize herbal drugs to control human population, various tumor; oral contraceptives, sex hormones and other steroids. Further, the proposed HPTLC method is a faster, precise, accurate and cost effective, thus, help to the pharmaceutical companies for quantity, quality control and standardization of herbal formulations for routine analysis of diosgenin.

Keywords: Diosgenin; HPTLC, Steroidal saponin, *Abutilon indicum.*

INTRODUCTION

Nature has blessed us with an immense biodiversity of plants. These have been exploited for the various traditional medicinal system in India since past because they are known to contain various secondary metabolites which make them useful against various ailment and diseases.

Abutilon indicum (Linn.) a member of Malvaceae family commonly called as 'Country mallow' (English), 'Kanghi' (Hindi) and 'Atibala' (Sanskrit) is a perennial shrub, softly tomentose and up to 3 m in height. This plant is found as a weed throughout the hotter parts of India. It has enormous importance and traditionally used for their varied pharmacological and medicinal activities for the treatment of various ailments. They contain specific phyto-constituents responsible for their biological activity. The plants are widely used for their hepatoprotective, anti-inflammatory, lipid lowering, antioxidative, hypoglycemic, antifungal, wounds healing, antibacterial and larvicidal properties [1-5]. The leaves are effective in the ulcer, for the treatment of diabetes, diuretic infection and gingivitis [6-8]. In Ayurveda, Unani and Siddha system of medicine, it used as a remedy for jaundice, piles, ulcer and leprosy [9].

A saponin molecule consists of an aglycone (or sapogenin) and one or two sugar moieties. According to the structures of the aglycone, saponins can be classified into two types: triterpenoid and steroidal [10, 11]. They have been reported to have a variety of beneficial health effects. The therapeutic effects of a large number of folk medicines are thought to be associated with their saponin content [12].

Diosgenin is a bioactive steroidal sapogenin of great interest to the pharmaceutical industry [13]. It serves as an important starting material for the production of steroidal drugs and hormones such as testosterone, glucocorticoids, progesterone, corticosteroids, and oral contraceptives [14, 15] and possesses estrogenin and antitumour properties [16]. Due to innumerable health benefits of this compound, currently, great attention is being given to determine quality, efficacy, and standards of the herbal raw material. To date, diosgenin and related steroidal saponins were commercially obtained from only few plants like *Dioscorea*, *Yucca* and *Trigonella* [13, 17] and thus, there is an anthropogenic pressure on these plants. Therefore, it is essential to discover a new and alternate source of these compounds due to decreasing plant resources as well as increasing demand. There is an urgent need to develop a simple method for simultaneous estimation of diosgenin using sophisticated instruments like high-performance liquid chromatography (HPLC) and (HPTLC). There are ample of studies on analysis of diosgenin content in different plants like fenugreek, *Dioscorea, Yucca,* etc but as per our knowledge till date no study has been done to estimate diosgenin content in *A. indicum*. Hence, the objective of this work, therefore, was to evaluate *A. indicum* plants leaves for saponin content. If it is found in high concentration would present as rich sources of it, which can be exploited for commercial uses and in ethnomedicine as drugs.

Till date, several analytical methods including colorimetry, spectrophotometry, HPLC, HPTLC, and TLC have been reported for analysis of diosgenin individually [18-23]. However, there is no any HPTLC technique available anywhere else for simultaneous estimation of diosgenin in *A. indicum*. Thus, an attempt has been made to develop a simple, fast, accurate, precise and cost-effective HPTLC method for estimation of diosgenin content in medicinal plant *A. indicum.*

MATERIALS AND METHODS

Plant material

The leaves of *Abutilon indicum* (L) Sweet plant were used as an experimental material and collected from Greenhouse of The Institute of Science, Mumbai. The plant was identified, authenticated and deposited in the Blatter Herbarium, Department of Botany, St. Xavier's College, Mumbai (Voucher specimen no-K. V. S. 1888 of K. V. Shenoy). The leaves were shade dried and grounded to the fine powder.

Qualitative determination of saponins

The homogenous sample of leaves of experimented plant was subjected to phytochemicals analysis for qualitative determination of saponin according to the method described by Nyam *et al.* [24]. The performed qualitative tests were briefly described.

In a test tube, 0.5g of the extract was shaken with water. A stable frothing was taken as evidence for the presence of saponin.

HPTLC analysis of diosgenin

Preparation of plant extracts

The extract from dried leaf powder for HPTLC analysis was prepared using ultrasonication extraction technique [25]. In the present study ethanol and methanol extract were analyzed independently. For sonication 1 gm of leaf powder was mixed in 50 ml of solvents separately and sonicated for 20 min (Sonics-Vibra Cell, VCX-130). After sonication extracts were filtered using Whatman filter paper No.1. The sample extracts were evaporated to dry and made a final concentration of 10 mg/ml of stock solution used for the analysis. For HPTLC analysis, 5 µl from each stock solution was used.

Preparation of standard solution

The standard stock solution for diosgenin was prepared by dissolving 5 mg in 5 ml of methanol by means of sonicated for 15 min. From this stock (1 mg/ml), six different concentrations (100-600 µg/ml) of diosgenin standard were prepared to prepare diosgenin standard calibration curve (fig. 1).

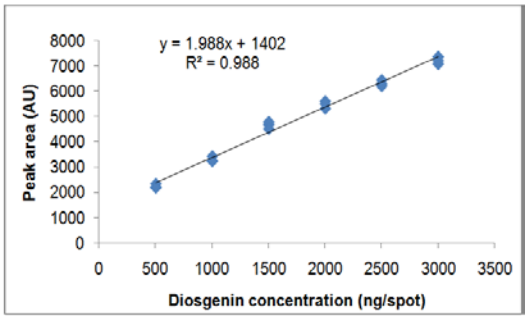


Fig. 1: HPTLC calibration curve for diosgenin standard

Chromatographic conditions

Chromatography was performed on pre-activated (at 1100 °C) silica gel 60 F_{254} HPTLC plates. Both, sample and standard (5 µl each) compounds were applied to the layer as 6.0 mm wide bands, positioned 8.0 mm from the bottom of the plate, using an automated CAMAG LINOMAT-5, TLC applicator instrument with nitrogen flow providing the delivery by 100 µl Hamilton syringe.

Chromatogram development

Sample loaded TLC plate was kept in glass twin-trough developing chambers (10 mm × 10 mm, with metal lid) previously saturated with solvent vapor with mobile phase, for 30 min, at room temperature 25±2 °C.

Mobile phase

Toluene: ethyl acetate: formic acid [6:5:1 v/v/v] [19].

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG Reprostar-3) and the images were captured at 254 nm and 366 nm.

Derivatization

The developed plate was sprayed with spraying reagent anisaldehyde-sulphuric acid reagent [13] and TLC plate was dried at 110 °C for 10 min in hot air oven. Immediately after drying, the plate was photo-documented in daylight, UV 254 nm and 366 nm mode using CAMAG-TLC Scanner-3 equipment.

Scanning, detection and quantification

After derivatization, the plate was fixed in scanner stage and scanning was done at 366 nm. The peak table, peak display, and peak densitogram were noted. The compounds were investigated according to their R_F values with the corresponding spot of standard diosgenin. The percentage of diosgenin present in MeOH and EtOH extract was calculated by diosgenin standard calibration curve.

Validation of method

Linearity

The linearity of the method was assessed by the analysis of standard solution (1 mg/ml) at six concentration levels, that is, 0.5–3.0 μg/spot of diosgenin. The linearity was evaluated using linear least-squares regression analysis for generation of calibration curve. The regression equation with slope, intercept, and coefficient of correlation (r) was calculated (table 1 and fig. 1).

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD was determined based on the lowest concentration detected by the instrument from diosgenin standard while the LOQ was determined based on the lowest concentration quantified in the samples (table 1).

Method precision (repeatability)

The repeatability of the method was assessed by spotting 500 and 1000 ng diosgenin standard 6 times each and was analyzed each accordingly by HPTLC method. The percentage relative standard deviations were expressed as RSD (%) (table 1).

Accuracy

Accuracy was determined as percent recovery by the standard addition method. The pre-analyzed samples (200 µg/spot) spiked with the standard and the mixtures were reanalyzed by the developed method. The spiking was done at two different concentration levels (500 and 1000 ng/spot) and average percent recovery and % RSD were calculated at each concentration level (table 2). The experiment was conducted in triplicate.

Robustness

Robustness was studied in triplicate at 1000 ng/band by making small changes to the volume of the mobile phase, the composition of mobile phase, and saturation time of development chamber. The effects on the results were examined by calculation of RSD (%) and R_F values (table 1).

Specificity

The specificity of the method was ascertained by analyzing standard and test samples. The diosgenin in the test samples was confirmed by comparing R_F and UV spectra of the spots with that of the standard (fig. 2-4 and photo plates 1 and 2).

Quantification of diosgenin

The methanolic and ethanolic extracts (20 µl) were applied in triplicate and densitograms were obtained under same conditions as that of the standard. Area under the peak corresponding to that of the standard was recorded and content of the same was calculated from the regression equation obtained from the calibration curve.

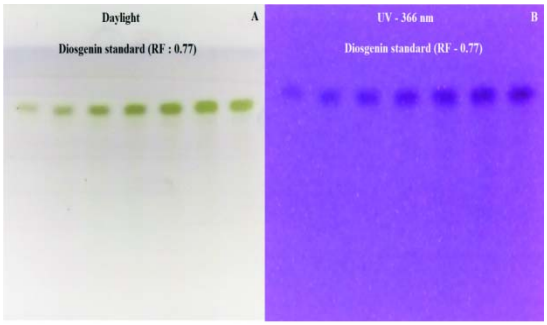
RESULTS AND DISCUSSION

Qualitative analysis

The qualitative froth test in methanolic, ethanolic and aqueous extracts of *A. indicum* leaves revealed the presence of saponin.

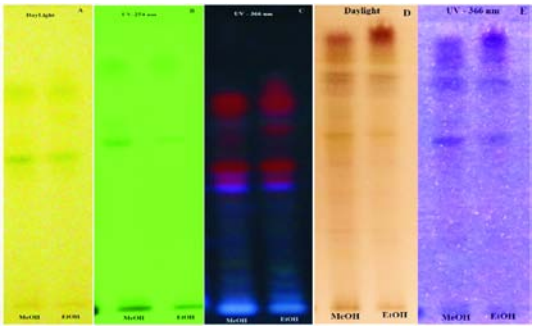
Chromatographic fingerprinting

HPTLC analysis of MeOH and EtOH leaf extract of experimented plant was carried out with toluene: ethyl acetate: formic acid [6:5:1, v/v/v] mobile phase which gives good separation of saponins. HPTLC chromatograms of MeOH and EtOH observed before and after derivatization at 254, 366 nm and daylight are shown in photo plates 1&2. HPTLC plate visualized at 366 nm and daylight showed best resolution and intense color spots after derivatization with the anisaldehyde-sulphuric acid reagent. Blue, brown, yellow and purple colored spots were observed at daylight mode, confirmed the presence of saponin in the given samples and standard. The appearance of brown color spot at R_F= 0.77 established the diosgenin in the samples.



Photoplate 1: HPTLC profile of standard diosgenin at 366 nm after derivatization

After derivatization at 366 nm, standard diosgenin showed a single peak with R_F value 0.77 and retention area 3047 (fig. 2). Densitograms of MeOH sample displayed the presence of 10 types of saponin and 6thpeak with R_F value 0.79 and retention area 519.2 was homologous to the standard diosgenin (fig.3). While in EtOH sample it displayed 8 types of saponins at 366 nm and no significant appearance of the peak (area after 4th peak in fig. 4) homologous to diosgenin was detected (fig. 4). However, an intense peak coincided to diosgenin with R_F value 0.79 was observed when the sample was spotted in high quantity (10-20 ul).



Photoplate 2: HPTLC profile of MeOH and EtOH extract of *A. indicum*, (A). Day light before derivatization, (B). UV-254 nm before derivatization, (C). 366 nm before derivatization, (D). Daylight after derivatization, (E). 366 nm after derivatization.

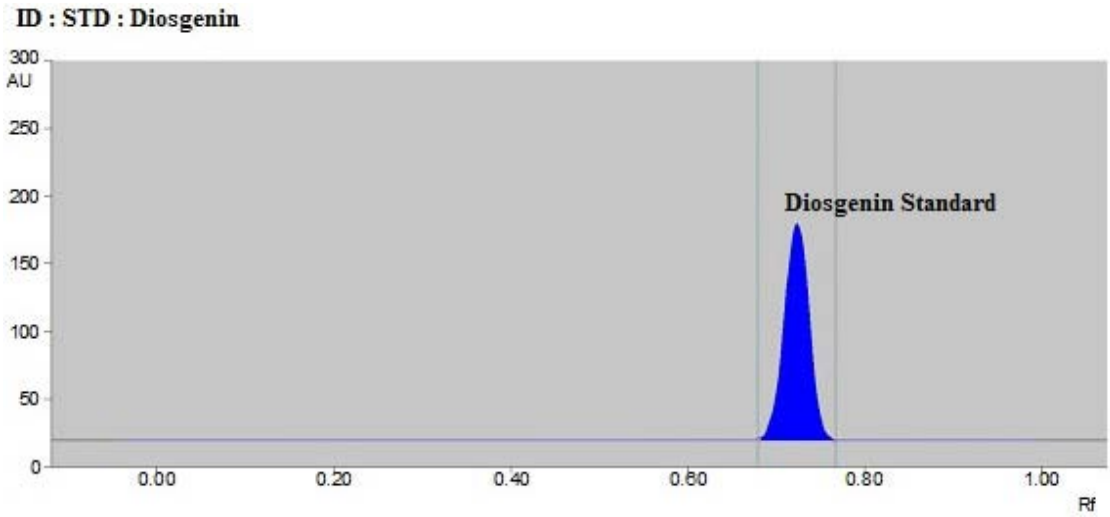


Fig. 2: HPTLC densitogram of diosgenin standard

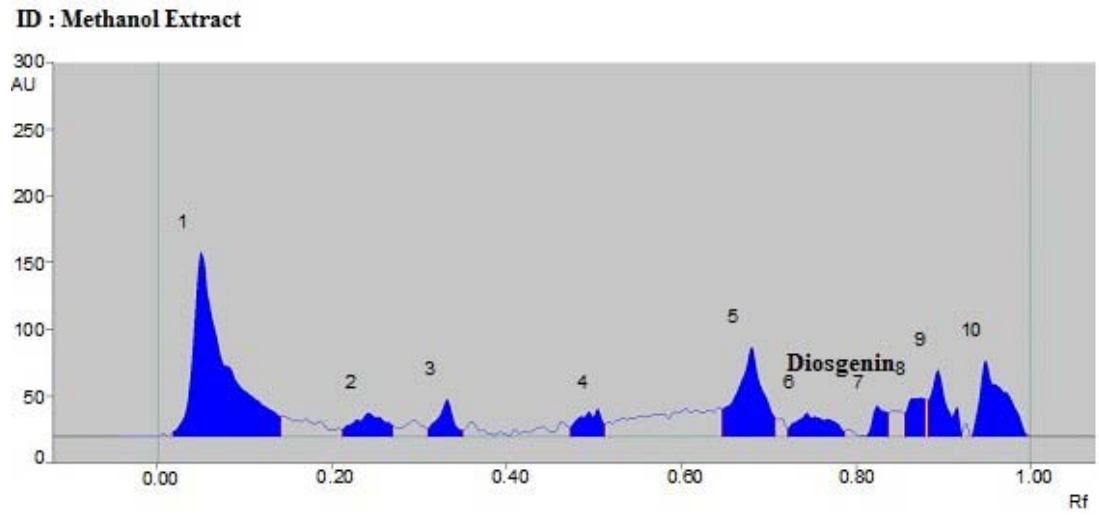


Fig. 3: HPTLC densitogram of methanolic extract of *A. indicum* showing diosgenin in sample

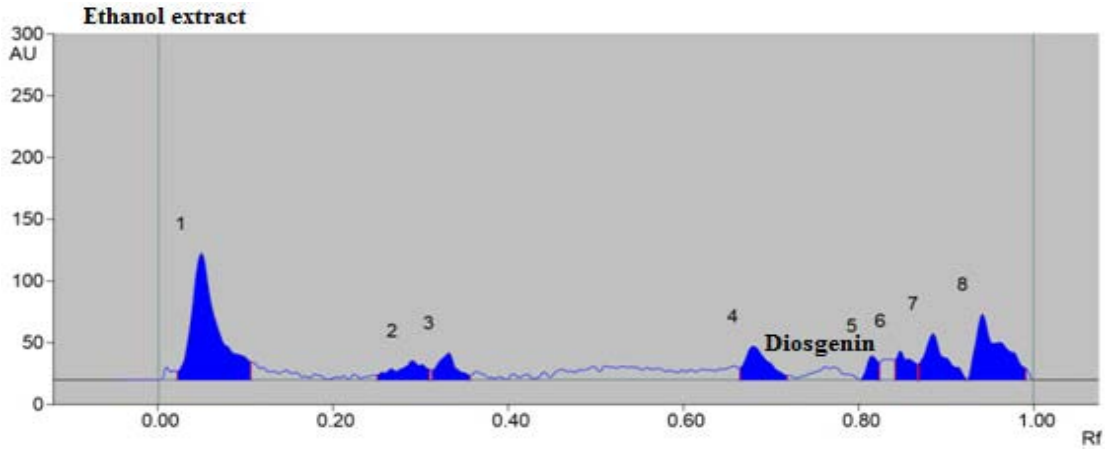


Fig. 4: HPTLC densitogram of ethanolic extract of *A. indicum* showing diosgenin in sample

A quantitative HPTLC method that gives dense and compact spots with significant R_F values for simultaneous determination ion of diosgenin in *A. indicum* leaves was developed. The six point linear calibration curves of diosgenin were linear in the range of 500–3000 ng for diosgenin, which indicates that the method has good sensitivity. Specificity of the proposed method was evaluated by comparing the R_F values acquired from standards and from test samples separated under identical conditions (fig. 2, 3 and 4).

As shown in table 1, the lower limits of detection (LOD) obtained for diosgenin was 176.55 ng/spot with good linearity, while the limit of quantification (LOQ) obtained was 535 ng/spot indicating the sensitivity of the method to be adequate. The instrumental precision and repeatability of the method were assessed by determination of the RSD (%) of peak area (table 1). the precision were studied by triplicate assay of two different concentrations of diosgenin (500 and 1000 ng per spot. RSD values obtained (1.6–1.87%) indicated that the method is precise (table 1). Good recovery was obtained by the methanolic sample at two different concentration levels of diosgenin standard. The percent recoveries obtained after sample processing and applying was found to be in the range of 98.4±0.24%. Results obtained for determination of robustness indicated that the method was able to withstand minor experimental changes (table 1). The developed method was validated as per ICH guidelines [26].

The HPTLC method developed here for the diosgenin in *A. indicum* is thus, simple, precise, specific, accurate, rapid and cost-effective and chromatograms developed with methanol and ethanol extract from *A. indicium* leaves may be treated as fingerprints and could be used efficiently for identification and quality assessment of the plants and plant's related drugs.

Quantification of diosgenin

Interestingly, while using the developed method, the amount of diosgenin in methanolic and ethanolic leaves extract of *A. indicum* was found to be 0.43% (w/w) and 0.10% (w/w), respectively (table 3). Our results are in good agreement with the results reported earlier for *Trigonella foenum-graceum* by Trivedi *et al.* [27] and Laila *et al.* [21], Similarly, Shah and Lele [13] validated HPTLC method for diosgenin and found 0.078, 0.133 and 0.048% diosgenin in fresh and dried tuber as well as callus of *Dioscorea alata Var purpurae,* respectively. Thus, this method is a valuable tool for accurate detection and simultaneous estimation of biological compound diosgenin.

Table 1: Validation parameters for diosgenin by HPTLC

S. No.	Parameters	Diosgenin
1.	Linearity range, n=3	500-3000 ng/spot
2.	Correlation coefficient (r^2), n=3	0.988
3.	Regression equation (Y= m x+c)	Y=1.988x+1402
5.	Precision repeatability (RSD %), n=6	1.6-1.87%
6.	Robustness (RSD), n=3	
	Mobile phase volume	1.26–1.9%
	Mobile phase composition	R_f = 0.73-0.80
	Saturation time	
7.	Specificity	Specific
8.	Limit of detection (LOD), n=3	176.6 ng
9.	Limit of quantification (LOQ), n=3	535.0 ng

Table 2: Results from study of recovery for diosgenin

MeOH sample (ug/spot)	Diosgenin in sample (ng)	Diosgenin added (ng)	Total diosgenin (ng)	Diosgenin found in mixture (ng)	*Recovery (%)	*Average recovery (%)
200	851.60	500	1351.6	1328±13.0	98.3±0.98	98.4±0.24
200	851.60	1000	1351.6	1825±12.8	98.6±0.70	

* mean±relative standard deviation (%), n=3

Table 3: Diosgenin content in methanolic and ethanolic leaf extract of *A. indicum* plant by proposed method

S. No.	Samples	Diosgenin content (%,W/W)
1.	MeOH	0.43±0.018
2.	EtOH	0.10±0.009

Mean±Standard deviation (SD), n=3

CONCLUSION

This work justifies the usage of this plant in ethnomedicine, and various clinical and therapeutics. It is clear from the present work that this plant has an appreciable amount of diosgenin in its leaves, thus, it can also be a natural alternate source for manufacturing of various health related hormones, steroids and oral contraceptives; and also releases pressure from some plants which are extensively exploited to produce diosgenin. In addition, plant leaves extract could also be potentially used in herbal drugs formulations as anti-cancer, antibiotics, antidiabetic, insecticide, antifungal, anti-inflammatory, and antioxidant, antiprotozoal, anti-ulcer and antiviral agents.

The developed HPTLC method is simple, accurate, precise and cost-effective and can be utilized for the routine analysis of the quantitative determination of diosgenin. The developed HPTLC fingerprints will help the drug's manufacturer for quality control and standardization of herbal formulations.

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CONFLICT OF INTERESTS

Declared none

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