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Quantitative Estimation of Piperine and Diosgenin in Ayurvedic Formulation

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Authors' contributions

This work was carried out in collaboration between both authors. Author SP designed the study, improved on the draft and made the final copy. Author SK did the laboratory work, managed the literature searches and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Goksuradi guggulu is a polyherbal formulation official in "Ayurvedic formulary of India" and used for dysuria, urinary obstruction, increased frequency and turbidity of urine, calculus, excessive vaginal discharge, gout. A simple, specific and precise high performance thin-layer chromatography (HPTLC) method has been developed for quantification of piperine and diosgenin in Goksuradi guggulu. We report the extraction and estimation of these compounds in a laboratory prepared sample of Goksuradi guggulu and two of its marketed formulations. The compounds were chromatographed on precoated silica gel G 60 254 plates in the mobile phase comprising of toluene: hexane: ethyl acetate (6.8:0.2:3). Under the optimized chromatographic conditions, the calibration plot was found to be linear in the range of 0.2-1µg $spot^{-1}$ for piperine and 1.0 -3.0 µg $spot^{-1}$ for diosgenin. The correlation coefficient (R2) was 0.9979 and 0.9915 for piperine and diosgenin respectively. Mean recovery (% w/w) for piperine in all the formulations was in the range of 97.61 - 98.90 and for diosgenin, it was 93.76 - 94.33.

Keywords: Piperine; diosgenin; HPTLC; quantitation.

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1. INTRODUCTION

The concept of standardization is relatively new for phytomedicines but is rapidly becoming essential as a means of ensuring a consistent supply of high-quality phytopharmaceutical products. Standardization can be defined as the establishment of reproducible pharmaceutical quality by comparing a product with established reference substances and by defining the minimum amounts of one or several compounds or a group of compounds [1]. A standardized product ensures safety and therapeutic efficacy and hence increases the level of trust people have in herbal drugs. A marker is a chemical entity in the plant material that may or may not be chemically defined and may serve to provide a characteristic fingerprint of the plant [1]. Guggulu is an oleoresin obtained from the plant *Commiphora weightii*. Ayurvedic preparation having this oleoresin as main effective ingredient is known as guggulu. This paper encompasses the study of a guggulu preparation viz. Goksuradi Guggulu; official in Ayurvedic formulary of India. It contains Goksura, Guggulu, Sunthi, Marica, Pippali, Haritaki, Bibhitaka, Amalaki, Musta. This preparation is prescribed for the treatment of dysuria, urinary obstruction, increased frequency and turbidity of urine, calculus, excessive vaginal discharge, gout, disease due to vata dosa/neurological disease and vitiation of semen [2]. In this study, we report the development of a simple, optimized and validated HPTLC method for the simultaneous extraction and estimation of piperine and diosgenin in Goksuradi guggulu. Pippali, the fruits of long pepper, obtained from *Piper longum* (Family Piperaceae) and marica the fruits of black pepper, obtained from *Piper nigrum* is a typical ayurvedic complementary component whose benefit is its antioxidant properties. The berries of pippali and marica are used as diuretic, vermifuge, emmenagogue and antiseptic. The major active constituent responsible for its pharmacological action is the alkaloid piperine [3]. Gokshura commonly called as Gokhru are the fruits of *Tribulus terrestris*. Fam. Zygophyllaceae. Its main constituents include the steroidal saponins and its hydrolysed products which contain diosgenin, hecogenin and neotigogenin. The fruits possess a great diuretic activity and contractile effect. The aqueous extract has shown activity against the host prevalent gram negative bacteria in urinary infection [4]. Methods so far reported for analysis includes the extraction and estimation of piperine alone in *Piper nigrum* and *P. longum* in the solvent system hexane, ethyl acetate and glacial acetic acid [5]. Also there have been reports of analysis of piperine in various Ayurvedic formulations by HPTLC [6-8]. Simultaneous estimation of diosgenin and levodopa for a polyherbal formulation in the solvent system toluene, ethylacetate, formic acid, glacial acetic acid has been reported [9]. TLC method was developed for simultaneous detection and quantification of diosgenin and sarsapogenin [10]. Literature reports the determination of diosgenin on a RP C-18 column in the mobile phase acetonitrile and water [11]. There are reports on quantification of diosgenin on crude drugs but literature gives only few information on simultaneous estimation of diosgenin with other synthetic or natural molecules [12]. In this paper we report a methanolic extraction of piperine and diosgenin from Ayurvedic formulations; further we have also analysed them by HPTLC in the same solvent system but at different λ max values.

2. MATERIALS AND METHODS

2.1 Plant Material, Samples, Chemicals and Solution

The plant drugs used in the formulation of Goksuradi Guggulu were purchased from crude drug market in Mumbai, India and were identified morphologically using references from standard text [13]. A laboratory formulation of Goksuradi Guggulu was prepared as per the

procedure given in Ayurvedic formulary of India and was labeled as L1 [1]. The marketed formulations M1 and M2 were purchased from local pharmacy store. Standard Diosgenin (CAS 512-04-9, 97%) was purchased from Sigma-Aldrich, of Spain. Standard piperine was isolated from the fruits of *Piper longum* as per the extraction procedure mentioned in Indian Herbal Pharmacopoeia. The purity and structure of piperine was confirmed by melting point, HPLC, HPTLC and spectral analysis [14,15]. The purity of piperine was >99.0% by HPLC and, hence, was regarded as suitable for use as reference standards for analysis. All the solvents and chemicals used were of analytical grade. Precoated silica gel aluminium plate GF-254 procured from E Merck, Mumbai was used for TLC and HPTLC studies.

2.2 Sample Preparation

2.2.1 Extraction of biomarkers from crude drug

Approximately 1gm each of Pipali fruit powder, Marica fruit powder and Goksura fruit was gently refluxed in 60 ml of methanol for 30 minutes and filtered through Whatman filter paper (No.41, pore size :20-25 µm). The residue was refluxed again with 40 ml methanol for 1 hr and filtered. The combined filtrates for each individual crude drug was evaporated to make final volume of 10ml. 1ml of this solution was diluted to 10ml with methanol. This was used as a test solution for determination of piperine content in pippali fruit (PL1) and marica fruit (PM1) and diosgenin content in goksura(G) fruit.

2.2.2 Extraction of biomarkers from formulation

6 tablets each of L1, M1 and M2 was gently powdered and about one gram of the powdered sample was subjected to extraction of the marker compounds similar to that of individual crude powders.

2.3 Chemical Analysis of Formulation by HPTLC

2.3.1 Chromotographic conditions

HPTLC analysis was performed on aluminium-backed silica gel 60 GF254 plates (10 cm×10 cm, with 20 µm thickness E. Merck, Germany) with a Camag Linomat V (Switzerland). Samples were applied as 6 mm bands by use of a Camag Linomat V sample applicator fitted with a microlitre syringe. Linear ascending development of the plates to a distance of 80 mm was performed in the solvent system toluene: hexane: ethyl acetate (6.8:0.2:3) in a twin-trough glass chamber previously saturated with mobile phase vapor for 10 min at 25ºC. A constant application rate of 150 nL/sec was employed. After development, the plate was scanned using Camag TLC scanner 3 and wincats software 1.4.4.6337 with a slit dimension of 5 X 0.45 nm and scan speed 200 mm/sec. A deuterium lamp was used as source of radiation and the wave length used was 340nm for piperine and194nm for diosgenin.

2.3.2 Calibration curve of markers

Stock solution of standard piperine (100 µg ml^{-1}) and Diosgenin (1000 µg ml^{-1}) were prepared in methanol. For calibration, different concentrations of piperine (20 - 100 µg ml^{-1}) and diosgenin (100-300 µg ml^{-1}) were prepared individually from respective stock solution. 10 µl of each of the solution was applied in triplicate to the plate. After development of the plates; peak area and concentration data was treated by linear regression analysis.

2.3.3 Estimation of piperine and diosgenin in the test samples

The test samples (PL1, PM1, G1, L1, M1 and M2) prepared as mentioned above were applied as bands and developed using optimized chromatographic conditions which was similar to the standards. The plate was scanned twice once at 194nm for analysis of diosgenin and next at 340nm for piperine analysis. The area of the peak that corresponds with the Rf of standards was recorded and the amount present in the test solution was calculated from the regression equation obtained from the calibration plot.

2.4 Validation

The method validation was carried out according to the statistical method of validation ICH Q2R1 [16]. The linearity of the method was checked between 0.2-1.0 µg ml^{-1} for piperine and 1.0–3.0 µg ml^{-1} for diosgenin and concentration was plotted against peak area. Instrumental precision was checked by scanning the same piperine spot (0.6 µg $spot^{-1}$) and diosgenin (1.5 µg $spot^{-1}$) spot seven times. The repeatability of the method was confirmed by separate analysis of seven spots of standard piperine and diosgenin (0.6 µg $spot^{-1}$ each for piperine and 1.5 µg $spot^{-1}$ for diosgenin). Intra and interday variations were determined by analysing three different concentrations of standard and test solution and also by analysing them over a period of three days. The analysis was carried out seven times and all the results were expressed as mean ± % RSD (Relative Standard Deviation).

The accuracy of the method was established by measurement of the recovery at three different levels using the standard addition method. Three sets each of 4 volumetric flasks were taken for the recovery study. To 1 ml of L1, M1 and M2 (Prepared as given above) are taken respectively in each of the three sets containing 4 volumetric flasks. To this Diosgenin stock solutions(0.1 mg mL^{-1}) and piperine stock solution (0.1 mg mL^{-1}) equivalent to 50%, 100% and 200% of the initial amount present in the sample were added individually to 2^{nd}, 3^{rd} and 4^{th} volumetric flasks of each set. The first volumetric flask in each of the set contains only the test sample and not the standard drug. The volume in each flask was made upto 10ml. 10µl of each of the test solution was applied as bands and developed using optimized chromatographic conditions which was similar to the standards.

Limits of detection (LOD) and quantification (LOQ) were determined by the standard deviation (SD) method from the slope (S) of the calibration plot and the SD of a blank sample, by use of the equations LOD = 3.3 × SD/S and LOQ =10 × SD/S [16].

3. RESULTS AND DISCUSSION

3.1 Method Development

The mobile phase composition was optimized to establish a suitable and accurate densitometric HPTLC method for analysis of diosgenin and piperine in the same solvent system. The solvent system combination of toluene: hexane: ethyl acetate (6.8:0.2:3) resulted in a sharp, symmetrical and well resolved peak at Rf 0.37 for piperine under 340nm and at Rf 0.53 for diosgenin at 194nm.

3.2 Calibration

The calibration plots were linear in the amount ranging from 0.2-1µg spot^{-1} for piperine and 1.0 -3.0 µg spot^{-1} for diosgenin. The correlation coefficient (R2) was 0.9979 and 0.9915 for piperine and diosgenin respectively. The linear regression equation was Y = 11870x + 493 for piperine and Y=1188.4x + 726 for diosgenin, where Y is response and X is the concentration of standard drug. The standard deviation of the slope and intercept value for piperine was 0.9300 and 0.9782 respectively and for diosgenin it was 0.6913 and 0.5999 respectively.

3.3 Validation

The method when validated in terms of instrument precision gave a % RSD of 0.923 for piperine and 0.648 for diosgenin. The repeatability of the method was confirmed by repeated analysis of the same spot and the % RSD for piperine was 0.223% and 0.356% for diosgenin. Results from determination of intraday and interday precision, for both the standards expressed as mean±% RSD is shown in Tables 1 and 2. A % RSD lower than 2 indicated that the method was precise. The LOD and LOQ were found to be 0.0052286 µg and 0.0158443 µg for piperine and 0.017724 and 0.05371 µg for diosgenin respectively. These values indicated adequate sensitivity of the method. The accuracy of the method, measured as recovery, showed an average % recovery for piperine as 97.61 for L1, 98.83 for M1 and 98.90 for M2. For diosgenin, the average % recovery was 94.33 for L1, 93.76 for M1 and 94.25 for M2 (Table 3 and 4).

Table 1. Precision data of the method for piperine

Sample	Amount (µg)	Intraday (n=3)		Interday (n=3)	
		Mean±%RSD	SD	Mean ±%RSD	SD
Standard	0.4	0.3940±0.35	0.0014	0.4121±0.63	0.0026
	0.6	0.5912±0.52	0.0031	0.5854±0.25	0.0015
	0.8	0.7910±0.45	0.0036	0.8015±0.31	0.0025
Pippali Fruit	1.8003	1.7589±0.23	0.0041	1.8005±0.11	0.0021
Marica Fruit	5.4005	5.4006±0.15	0.0032	5.4005±0.06	0.0015
L1	0.3602	0.3575±0.58	0.0021	0.3607±0.60	0.0022
M1	0.3600	0.3586±0.33	0.0012	0.3602±0.52	0.0019
M2	0.3400	0.3419±0.49	0.0017	0.3392±0.64	0.0022

Table 2. Precision data of the method for diosgenin

Sample	Amount (µg)	Intraday (N=3)		Interday (N=3)	
		Mean ± %RSD	SD	Mean±%RSD	SD
Standard	1.5	1.5213±0.03	0.0006	1.4896±0.05	0.0008
	2.0	2.0032±0.10	0.0021	2.0018±0.07	0.0016
	2.5	2.5225±0.02	0.0006	2.4988±0.03	0.0009
Goksura	0.5101	0.5125±0.41	0.0021	0.5109±0.23	0.0012
L1	0.5606	0.5589±0.14	0.0008	0.5631±0.26	0.0015
M1	0.5404	0.5395±0.40	0.0022	0.5463±0.34	0.0019
M2	0.5202	0.5215±0.13	0.0007	0.5216±0.42	0.0022

Table 3. Recovery studies of piperine in Goksuradi guggulu

Sample	Initial amount (µg/ml)	Externally added piperine (µg/ml)	Recovered Piperine (µg/ml)	% recovery	SD	%RSD
L1	36.02	0	0	0	0.0012	0.003
	36.02	18	16.99	94.44	0.0029	0.0170
	36.02	36	35.66	99.08	0.0015	0.0042
	36.02	72	71.23	98.93	0.0026	0.0024
M1	36.00	0	0.00	00	0.0011	0.0030
	36.00	18	17.85	99.17	0.0034	0.0190
	36.00	36	35.34	98.18	0.0026	0.0073
	36.00	72	71.15	98.81	0.0021	0.0019
M2	34.00	0	0	00	0.0005	0.0014
	34.00	17	16.74	98.48	0.0036	0.0215
	34.00	34	33.56	98.72	0.0027	0.0080
	34.00	68	66.56	97.88	0.0022	0.0021

Table 4. Recovery studies of diosgenin in Goksuradi guggulu

Sample	Initial amount (µg/ml)	Externally added diosgenin (µg/ml)	Recovered Diosgenin (µg/ml)	% recovery	SD	%RSD
L1	56.06	0	0	0	0.0026	0.0046
	56.06	28.00	26.46	94.50	0.0035	0.0132
	56.06	56.00	52.30	93.40	0.0018	0.0034
	56.06	112.00	110.58	98.73	0.0021	0.0013
M1	54.04	0	0	0	0.0015	0.0027
	54.04	27.00	25.16	93.20	0.0026	0.0103
	54.04	54.00	51.08	94.60	0.0021	0.0041
	54.04	108.00	106.65	98.75	0.0015	0.0009
M2	52.02	0	0	0	0.0021	0.0040
	52.02	26.00	24.53	94.35	0.0032	0.0132
	52.02	52.00	49.50	95.20	0.0026	0.0052
	52.02	104.00	103.45	99.47	0.0015	0.0010

3.4 Quantification of Piperine and Diosgenin in the formulations

The peaks of piperine and diosgenin in test samples (L1, M1 and M2) were identified by comparing their Rf values with those obtained by chromatography of the standard under the same conditions. An overlay of UV spectra of the test samples over the standard indicated that there was a good correlation between both the spectra obtained at each position (Figs. 1 and 2).

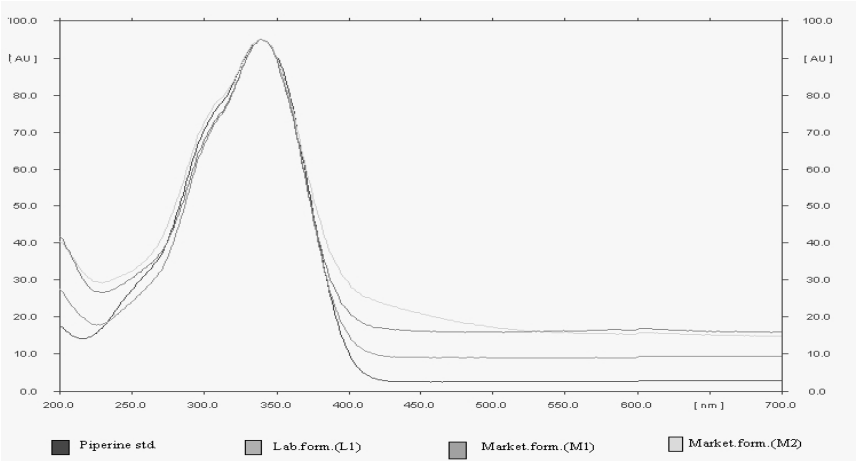

Fig. 1. An overlay spectrum of standard piperine over test sample at 254 nm

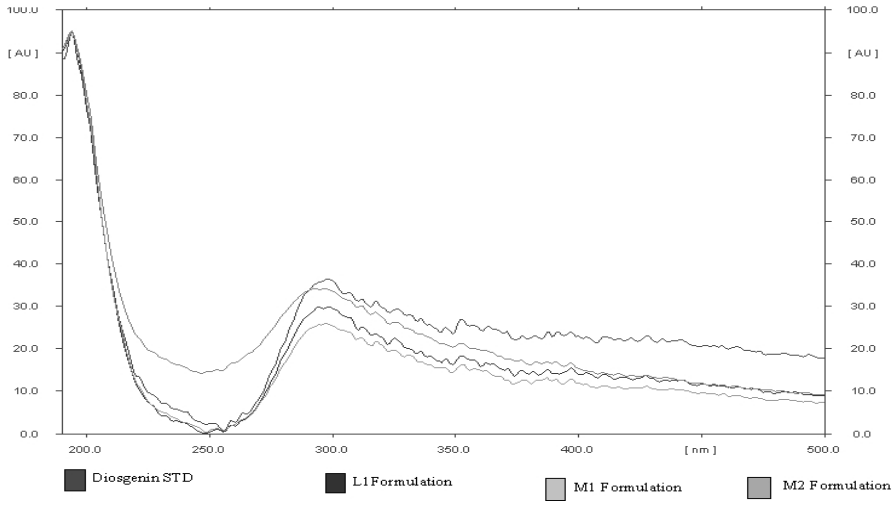

Fig. 2. An overlay spectrum of standard diosgenin over test sample at 254 nm

The content of piperine in pippali fruit was obtained from the calibration curve and was found to be 1.80% w/w which was found to be within the limits given in literature i.e.1-2%w/w [5]. Whereas piperine content in marica fruit was found to be 5.40% w/w which was within the limits 5-9%w/w [5] and diosgenin content in goksura fruit was found to be 0.5101% w/w. As per the formula of Goksuradi guggulu, the total piperine content (obtained from pippali powder and marica powder) should be 0.3740%w/w. The L1 formulation on analysis gave piperine content of 0.3602 %w/w (Fig.3), where as marketed formulations M1 and M2 gave a content of 0.3602 (Fig.4) and 0.3600 %w/w respectively. The formula of Goksuradi guggulu contains the aqueous extract of guggulu which theoretically should contain 0.7419%w/w of diosgenin. But the total quantity of diosgenin depends upon the aqueous extraction of the powder. The L1 formulation on analysis gave diosgenin content of 0.5606 % w/w (Fig.5), where as marketed formulations M1 and M2 gave a content of 0.5404 (Fig.6) and 0.5202 % w/w respectively.

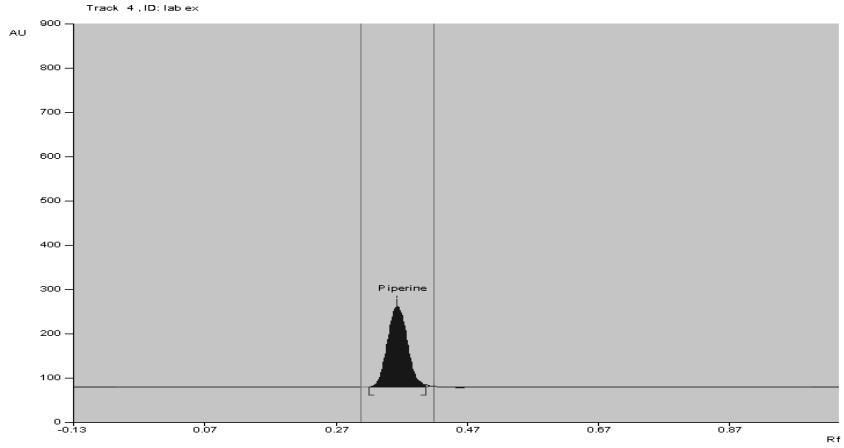

Fig. 3. Densitometric HPTLC of laboratory formulation (L1) for piperine at 340 nm

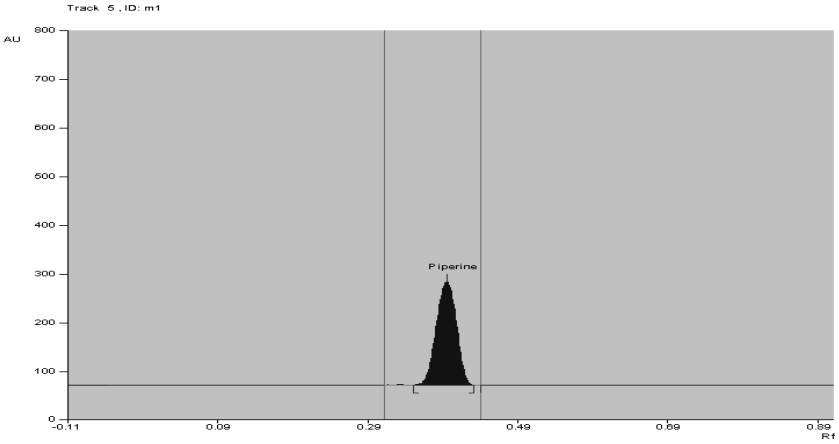

Fig. 4. Densitometric HPTLC of marketed formulation (M1) for piperine at 340 nm

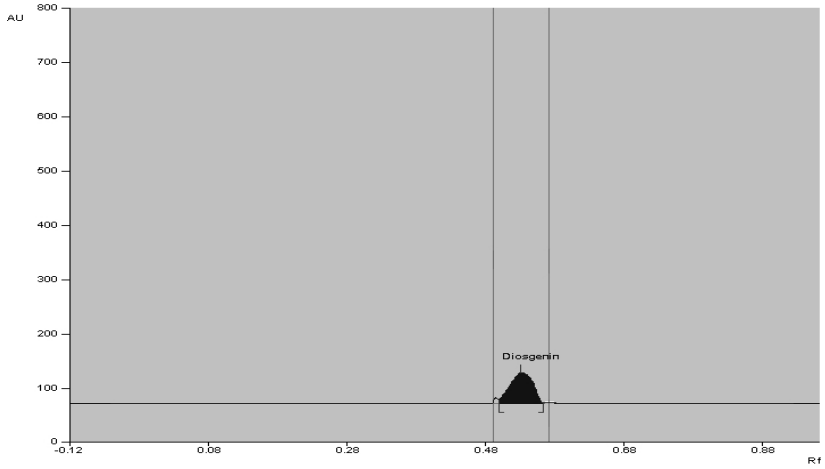

Fig. 5. Densitometric HPTLC of laboratory formulation (L1) for diosgenin at 254 nm

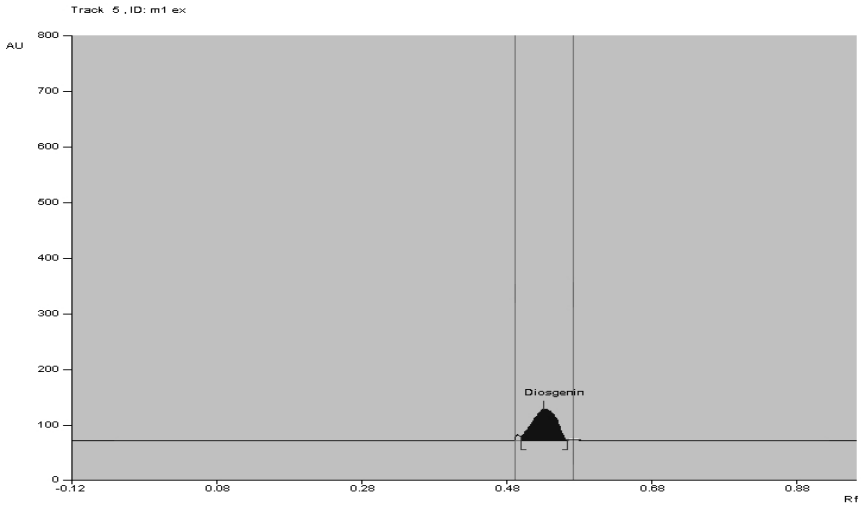

Fig. 6. Densitometric HPTLC of marketed formulation (M1) for diosgenin at 254 nm

4. CONCLUSION

The developed HPTLC technique is quite simple, accurate, precise, reproducible, and sensitive. The method was developed taking into consideration the standardization of an Ayurvedic formulation Goksuradi guggulu using the markers piperine and diosgenin. This method explored the use of simultaneous extraction and estimation of both markers in the formulation. The developed HPTLC method is cost effective and can be used for routine analysis of these two markers in Goksuradi guggulu as well as other formulations containing the same.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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