AMERICAN JOURNAL OF PHARMTECH RESEARCH
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Development and validation of a method for densitometric analysis of umbelliferone, β-sitosterol and methoxsalen in polyherbal formulation

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ABSTRACT

Herbal medicines are generally available as a mixture of more than one plant constituent. It is important to quantify the maximum possible number of markers in such herbal formulations by which the quality of the formulations may be assessed. TLC densitometric methods were developed using High performance thin layer chromatography for the quantification of the three marker compounds, umbelliferone, β-sitosterol and methoxsalen, from the polyherbal tablet formulation. Solvent systems were optimized to achieve best resolution of the marker compounds from the other components of the sample. From the various solvent systems tried, the one containing toluene: ethyl acetate: methanol: formic acid (3: 3: 0.2: 0.8) gave best resolution of umbelliferone (R_f=0.35), β-sitosterol (R_f=0.47) and methoxsalen (R_f=0.6) in the presence of the other compounds in the polyherbal tablet sample and enabled the quantification of the marker compounds. The methods were validated in terms of precision, repeatability and accuracy. The method was found to be suitable for the quantification of these marker compounds in polyherbal formulation.

Keywords: densitometric, umbelliferone, β-sitosterol and methoxsalen.

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Received 13 December 2016, Accepted 09 January 2017

Please cite this article as: Deepa I *et al*., Development and validation of a method for densitometric analysis of umbelliferone, β-sitosterol and methoxsalen in polyherbal formulation. American Journal of PharmTech Research 2017.

INTRODUCTION

Therapeutic activity of herbal medicines depends on its phytochemical constituents. Accurate identification and quality reassurance is an essential prerequisite to make sure reproducible quality of herbal medicines.[1] Standardization is an imperative aspect for evaluating the quality and safety of polyherbal formulation as these formulations are combination of more than one herb to accomplish the desired therapeutic effect. Phytosterols like beta-sitosterol lower cholesterol levels by competing with cholesterol for absorption in the intestine. Having a similar structure to cholesterol, phytosterols compete with cholesterol of dietary and biliary origin for incorporation into micelles in the gastrointestinal tract.[2] Cholesterol displaced from the micelles is not absorbed and is destined for fecal excretion.

Elevated triglyceride levels are a risk factor for cardiovascular disease. Beyond LDL-C lowering, growing evidence suggest that phytosterols reduce triglyceride levels as well. The proposed mechanism behind the triglyceride lowering effect of phytosterols is due to a reduction in triglyceride rich VLDL particle produced by the liver. [3] Because it has very low systemic absorption it proves beneficial to lower the cholesterol level in the body. Increasing the intake of phytosterols may be a practical way to reduce coronary heart disease with minimum risk. The benzopyrans are a group of compounds whose members include coumarins and flavonoids.[4] Coumarins act by augmenting activity of Lecithin Acyl Transferase (LCAT), which regulates blood lipids. Lecithin acyl transferase (LCAT) plays a key role in the incorporation of free cholesterol into HDL and transferring it back to VLDL and LDL which are taken back later in liver cells. [5-6]

The aim of this study was, therefore, to develop a simple, economical, selective, precise, and reproducible high performance thin layer chromatographic (HPTLC) technique with densitometric UV detection for analysis of umbelliferone, β-sitosterol and methoxsalen in *Salvadora persica*, *Evolvulus alsinoides* and *Apium graveolens* extracts and polyherbal formulation as per ICH guidelines. The present investigation may be quite useful as these medicinal plants are highly valued as traditional system of medicine.

MATERIALS AND METHOD

HPTLC densitometric analysis was done using TLC scanner 3 with CATS software (CAMAG).

HPTLC finger printing profile:

HPTLC study of polyherbal tablet formulation was carried out along with the different marker compounds corresponding to the active ingredients to ensure the presence of active ingredients in

the formulation.

Reference Standards:

All the reference standards namely umbelliferone, β-sitosterol and methoxsalen used in the experiment were purchased from Sigma Aldrich, USA.

Apparatus

Spotting device:

Linomat V Automatic Sample Spotter (CAMAG, Muttenz, Switzerland)

Syringe: 100 µl (Hamilton, Bonaduz, Switzerland)

Thin layer chromatographic (TLC) chamber: Glass twin trough chamber (20 x 10 x 4 cm) (CAMAG)

Densitometer: TLC Scanner 3 linked to Win Cats software (CAMAG)

HPTLC plates: 20 x 10cm, 0.2 mm thickness precoated with silica gel 60 F_{254} (Merck, Mumbai)

Preparation of standard solution

The stock solutions of umbelliferone, β-sitosterol and methoxsalen were prepared in methanol. A stock solution of all the markers was prepared by dissolving 50 mg of accurately weighed markers in methanol and making up the volume to 50 ml of methanol. From these stock solutions, standard solutions of umbelliferone, β-sitosterol and methoxsalen at concentrations of (1-10 µg/ml) were prepared by transferring aliquots of stock solution to volumetric flasks and adjusting the final volume with methanol.

Preparation of sample solution

Accurately weighed 500 mg of polyherbal was dissolved in methanol. The solution was filtered through Whatman filter paper. The filtrate obtained was evaporated to dryness and the residue obtained was reconstituted in 10 ml of methanol.

Calibration curve for umbelliferone, β-sitosterol and methoxsalen

10 µl of each of the standard solutions of umbelliferone, β-sitosterol and methoxsalen at concentrations of (1-10 µg/ml) were applied in triplicate on to an HPTLC plate. The plates were developed in a solvent system of toluene: ethyl acetate: methanol: glacial acetic acid (3:3:0.2:0.8) up to a distance of 9 cm. After development, the plate was dried in air and was scanned densitometrically at 232 nm using absorbance reflectance mode by CAMAG Scanner 2 and Wincats software III. λ max of all three marker compounds fall in the range of 225-232 nm, hence 232 nm was chosen for scanning the three markers to record the peak. (Figure 1-3)

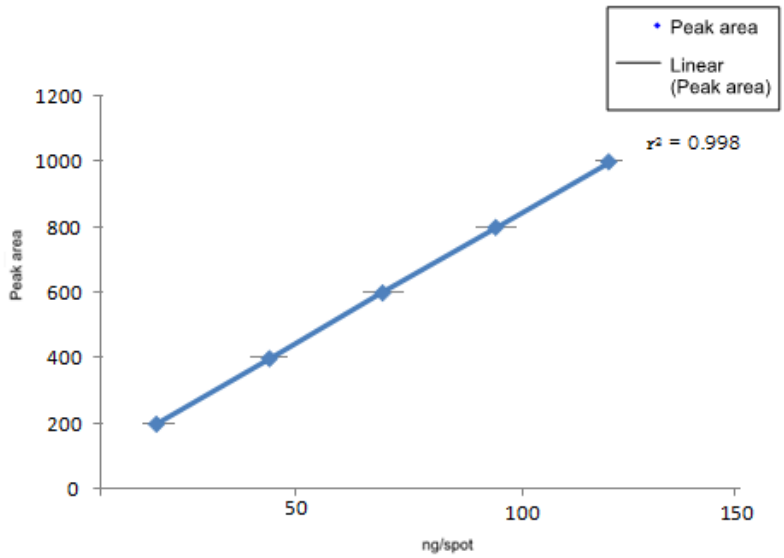

Figure 1: Calibration curve for Umbelliferone

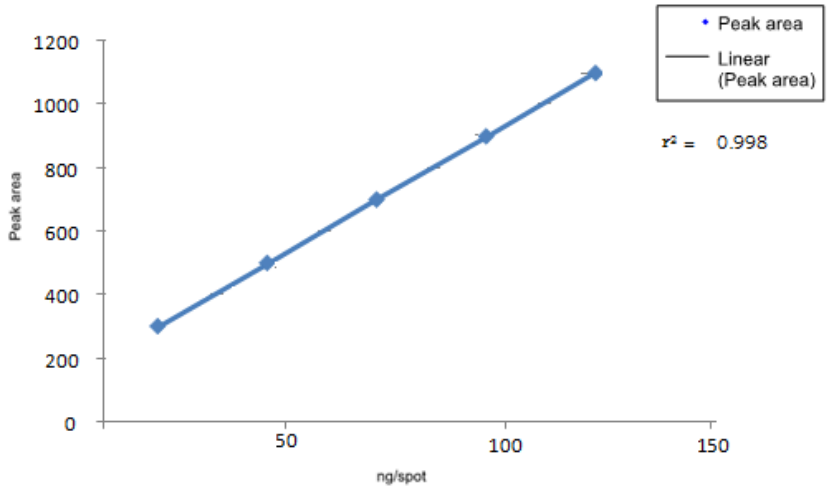

Figure 2: Calibration curve for β- sitosterol

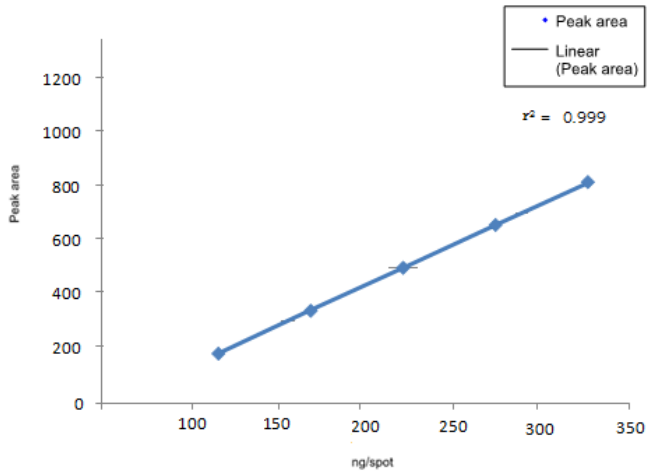

Figure 3: Calibration curve for Methoxsalen

Simultaneous quantification of umbelliferone, β-sitosterol and methoxsalen in polyherbal tablet

Sample solutions (10 µl) were applied in triplicate on a precoated HPTLC plate. The plate was developed in the same solvent system as above and scanned at 232 nm. The peak area and absorption spectrum was recorded. The amount of umbelliferone, β-sitosterol and methoxsalen was calculated using the respective calibration curve.

Validation of the method

ICH guidelines were (CPMP/ICH/381/95; CPMP/ICH/281/95) followed for the validation of the analytical procedure. The method was validated for precision and accuracy. Linear regression data from calibration curves have shown in Table-1.

Table 1: Linear regression data from calibration curves

Parameters	Umbelliferone	β- sitosterol	Methoxsalen
Linearity range	20-120 ng/spot	30-130 ng/spot	120-320 ng/spot
r^2	0.998	0.998	0.999
Slope ± S.D*	38.36 ± 0.6881	32.99 ± 0.7078	11.30 ± 0.1394
Intercept ± S.D	84.77 ± 53.59	-53.70 ± 61.57	-593.7 ±32.12
Confidence limit of slope [a]	36.45 to 40.27	31.03 to 34.95	10.91 to 11.69
Confidence limit of intercept*	-64.01 to 233.5	-224.6 to 117.2	-682.8 to -504.5
Sy.x	57.57	59.22	23.33

*p<0.001: Slope significantly different from zero

[a] 95%: Confidence limit

Sy.x: Standard deviation of residuals from line

Specificity

The peak purity of umbelliferone, β-sitosterol and methoxsalen was assessed by comparing their respective spectra at peak start, apex and peak end positions of the spot i.e., r (S, M) = 0.9986 and r (M, E) = 0.9983. Good correlation (r=0.9989) was also obtained between standard and sample spectra of umbelliferone, β-sitosterol and methoxsalen. (Figure 1-3)

Precision

Instrumental precision was checked by repeated scanning of the same spots 40, 80 and 120 ng/spot of umbelliferone (standard marker) three times within the same day to determine the intra-day variability. The RSD values were 0.217, 0.753 and 1.320 respectively. Similarly the inter-day precision was tested on the same concentration levels on two days and the RSD values were 0.804, 0.634 and 0.520 respectively.

Repeated scanning of 50, 90 and 130 ng/spot of β-sitosterol was done on the same day. The RSD values were found to be 0.528, 1.376 and 1.469 respectively. The RSD values during inter-day precision were found to be 1.277, 1.307 and 1.274 respectively.

Repeated scanning of 160, 240 and 320 ng/spot of methoxsalen was done on the same day. The RSD values were found to be 0.804, 1.958 and 1.650 respectively. The RSD values during inter-day precision were found to be were 1.221, 1.506 and 1.388 respectively. (Table- 2)

Table 2: Intra-day and inter- day precision data

Compound	Con. (µg/ml)	Intraday found Con. ± SD	%RSD	Interday found Con. ± SD	%RSD
Umbelliferone	40	39.95±0.087	0.753	40.18±0.323	0.804
	80	80.75±0.608	0.753	80.69±0.512	0.634
	120	119.75±1.581	1.320	120.47±0.63	0.520
β- sitosterol	50	49.94±0.264	0.528	49.60±0.633	1.277
	90	90.48±1.244	1.376	90.64±1.184	1.307
	130	131.08±1.926	1.469	130.92±1.668	1.274
Methoxsalen	160	157.20±1.263	0.804	156.08±1.905	1.221
	240	232.00±4.542	1.958	233.84±3.522	1.506
	320	324.83±5.360	1.650	325.49±4.519	1.388

Robustness

The robustness study was done by making small changes in the optimized method preparation like ± 5 % change in temperature, ± 0.01 ml mobile phase composition, ± 5 % amount of mobile phase ratio, time from spotting to chromatography, time from chromatography to scanning and plate pretreatment. There was no significant impact on analytical profile. (Table-3)

Table 3: Robustness data

Parameter	SD of peak area			% RSD		
	Umbelliferone	β-sitosterol	Methoxsalen	Umbelliferone	β-sitosterol	Methoxsalen
Mobile phase composition (± 0.01 ml)	1.760	1.125	1.538	0.758	0.653	0.225
Amount of mobile phase (± 5%)	1.556	1.378	1.265	0.164	0.835	0.325
Time from spotting to chromato-graphy	1.558	1.621	1.823	0.435	0.651	0.539
Time from chromato-graphy to scanning	1.643	1.059	1.238	0.139	0.589	0.325
Temperature (± 5%)	1.258	1.151	1.089	0.248	0.543	0.158
Plate pretreatment	1.116	1.119	1.253	0.135	0.765	1.251

N=6, Average of three concentrations, 40, 80, 120 ng spot $^{-1}$ for umbelliferone 50, 90, 130 ng spot $^{-1}$ for β- sitosterol 160, 240, 320 ng spot $^{-1}$ for methoxsalen

LOD (Limit of Detection) and LOQ (Limit of Quantification)

The signal to noise ratios of 3:1 and 10:1 were considered as LOD and LOQ respectively. (Table 4)

Table 4: LOD and LOQ data

Compound	LOD	LOQ
Umbelliferone	10 ng/ spot	20 ng/ spot
β- sitosterol	20 ng/ spot	30 ng/ spot
Methoxsalen	80 ng/ spot	120 ng/ spot

Recovery Studies

For the determination of recovery rates of umbelliferone, 59.76, 74.7 and 89.64 µg of pure marker were added to preanalyzed sample and quantitative analysis was performed. The recoveries were found between 99.29 and 99.38%. For the determination of recovery rates of β- sitosterol, 102.4, 128 and 153.6 µg of pure marker were added to preanalyzed sample and quantitative analysis was performed. The recoveries were found between 99.34 and 99.43%. For the determination of recovery rates of methoxsalen, 1780, 2225 and 2670 µg of pure marker were added to preanalyzed sample and quantitative analysis was performed. The recoveries were found between 99.48 and 99.68%. (Table-5)

Table 5: Recovery analysis

Compound	Amount in sample (µg)	Amount added (µg)	Amount found (µg)	Recovery (%)	Average (%)
Umbelliferone	74.7	59.76	124.5±4.50	99.29±0.05	99.29
	74.7	74.7	138.4±9.01	99.35±0.10	
	74.7	89.64	153.84±7.21	99.38±0.28	
β-sitosterol	128	102.4	229.5±5.50	99.34±0.03	99.45
	128	128	243.66±3.05	99.55±0.04	
	128	153.6	270.6±4.56	99.43±0.08	
Methoxsalen	2225	1780	3995±6.89	99.68±0.12	99.61
	2225	2225	4435±4.08	99.65±0.04	
	2225	2670	2486.66±6.11	99.48±0.06	

Quantitative Determination

The polyherbal formulation was analyzed by HPTLC. The content of each compound was determined by the corresponding regression equation. The results indicated that all three phytoconstituents are easily detected in sample analyzed. The polyherbal formulation found to contain of 0.291±0.012 %w/w of umbelliferone, 0.462±0.0693 %w/w of β- sitosterol, 7.73±1.6476%w/w of methoxsalen. (Table- 6)

Table 6: Phytoconstituents content in polyherbal formulation

Phytoconstituent	Content (%w/w)
Umbelliferone	0.291 ± 0.012
β- sitosterol	0.462 ± 0.0693
Methoxsalen	7.73 ± 1.6476

RESULTS AND DISCUSSION

HPTLC method was established for the quantification of the constituents umbelliferone, β-sitosterol and methoxsalen in polyherbal formulation. The proposed method was found to be suitable for estimation of these markers in polyherbal formulation as it is proved to be precise, reproducible, reliable, accurate and robust. HPTLC fingerprinting profile is very important parameter of herbal drug standardization for the proper identification of active constituents in medicinal plants.

HPTLC study of polyherbal tablet formulation was carried out along with the different marker compounds corresponding to the active ingredients to ensure their presence in the formulation. TLC densitometric methods were developed using High performance thin layer chromatography for the quantification of the three marker compounds, umbelliferone, β-sitosterol and methoxsalen, from the polyherbal tablet formulation. Solvent systems were optimized to achieve best resolution of the marker compounds from the other components of the sample. Of the various solvent systems tried, the one containing toluene: ethyl acetate: methanol: formic acid (3: 3: 0.2: 0.8) gave best resolution of umbelliferone (R_f=0.35), β-sitosterol (R_f=0.47) and methoxsalen (R_f=0.6) in the presence of the other compounds in the polyherbal tablet sample and enabled the quantification of the marker compounds. (Fig.4-5) The methods were validated in terms of precision and accuracy. The relationship between the concentration of standard solutions and the peak response was linear within a correlation coefficient of 0.998 for umbelliferone, 0.998 for β- sitosterol and 0.999 for methoxsalen. Precision studies were done as intra-day variability and inter-day variability. The RSD values during intra-day precision were found to be 0.217, 0.753 and 1.320 respectively. The RSD values during inter-day precision were found to be 0.804, 0.634 and 0.520 respectively for umbelliferone. The RSD values during intra-day precision were found to be 0.528, 1.376 and 1.469 respectively. The RSD values during inter-day precision were found to be 1.277, 1.307 and 1.274 respectively for β-sitosterol. The RSD values during intra-day precision were found to be 0.804, 1.958 and 1.650 respectively. The RSD values during inter-day precision were found to be were 1.221, 1.506 and 1.388 respectively for methoxsalen. The average percent recovery at three different levels was found. The recoveries were found between 99.29 and 99.38% for

umbelliferone. The recoveries were found between 99.34 and 99.43% for β- sitosterol. The recoveries were found between 99.48 and 99.68% for methoxsalen. The results indicated that all three phytoconstituents are easily detected in sample analyzed. The polyherbal formulation found to contain of 0.291 ± 0.012% w/w of umbelliferone, 0.462 ± 0.0693% w/w of β-sitosterol, 7.73 ± 1.6476% w/w of methoxsalen. The method was found to be suitable for the quantification of these marker compounds in polyherbal formulation.

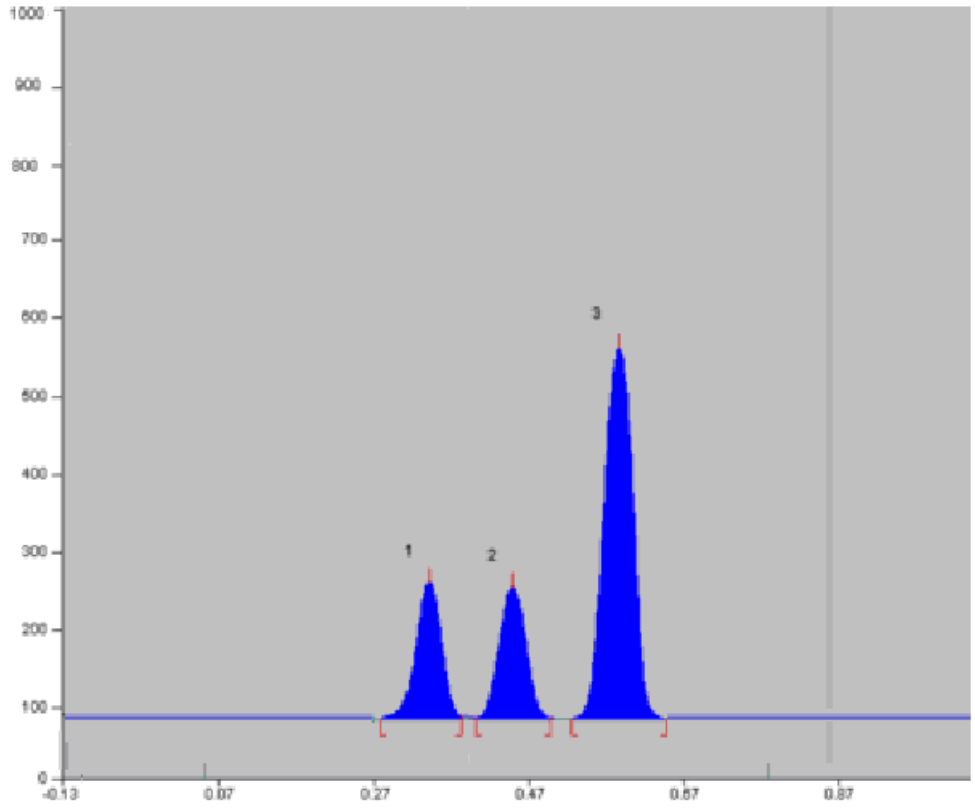

Figure 4: Densitogram of standard Umbelliferone, β- sitosterol and Methoxsalen

Peak 1: Umbelliferone (R_f =0.35)

Peak 2: β- sitosterol (R_f =0.47)

Peak 3: Methoxsalen (R_f =0.6)

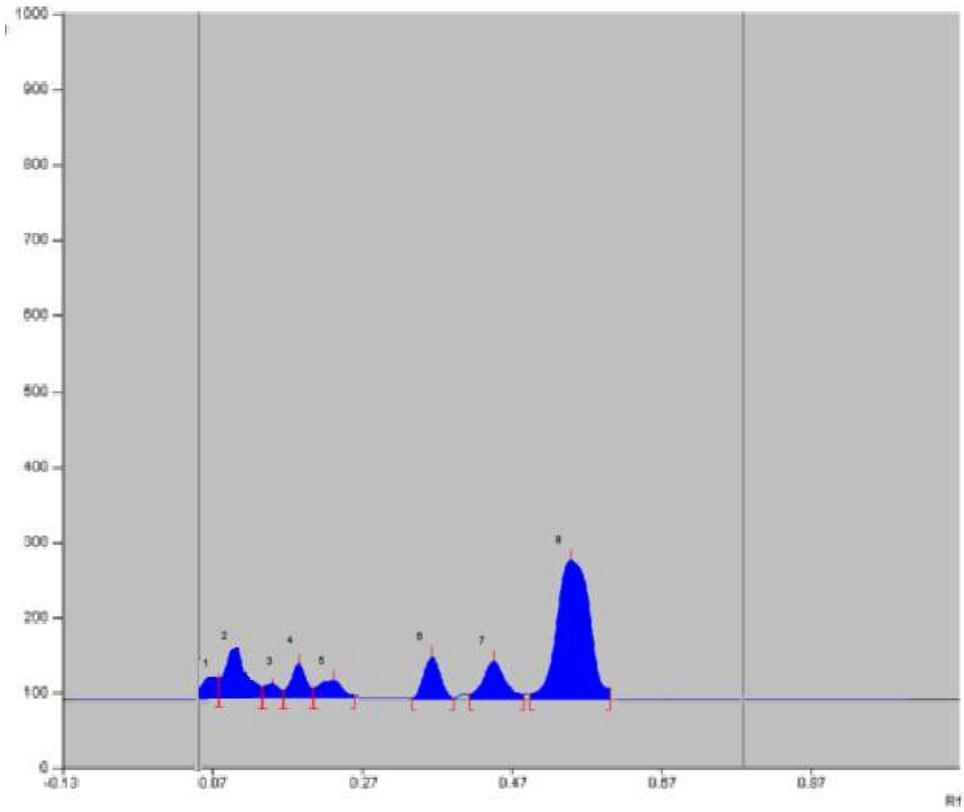

Figure 5: Densitogram of Polyherbal formulation

Peak 6: Umbelliferone (R_f =0.35)

Peak 7: β- sitosterol (R_f =0.47)

Peak 8: Methoxsalen (R_f =0.6)

Phytochemical assessment signifies the quality measurement, including preliminary phytochemical screening, chemo-profiling, and marker compound analysis employing innovative analytical techniques. HPTLC has been emerged as a significant tool for the qualitative, semiquantitative, and quantitative phytochemical analysis of the naturally occurring drugs.[7]

Standardization promises constant composition of all herbals including analytical operations for identification, markers and assay of active principles. [8] TLC and HPTLC are routinely used as valuable tools for qualitative determination of small amounts of impurities. [9] Different researchers have proposed that HPTLC method enables high-quality resolution and can be used for quantization of biomarkers.[10-12] HPTLC method was found to be simple, reliable, and convenient for routine analysis. [13-14]

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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