RESEARCH PAPER

A VALIDATED, PRECISE TLC-DENSITOMETRY METHOD FOR SIMULTANEOUS QUANTIFICATION OF LAWSONE, GALLIC ACID AND DIOSGENIN IN MARKETED HERBAL FORMULATION

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Abstract

Background *Lawsonia inermis*(henna) and *Trigonella foenum-graecum* are an important medicinal plants in natural products research for its diverse pharmacological activities. Major phytoconstituents obtained from henna are lawsone and gallic acid with dyeing and antioxidant activity respectively. Whereas, diosgenin found in seeds of *Trigonella foenum-graecum* is a very important steroid generally found in herbal formulations. Standardization plays a vital role in the herbal drug industry for maintaining the quality, hyphenated analytical techniques such as HPTLC, HPLC, GC–MS, and LC–MS were used to assess the efficacy, safety, and purity of herbal preparations. In the current analysis, a specific, simple, and rapid semi-automated TLC method was developed to quantify lawsone, gallic acid and diosgenin in a marketed herbal formulation, and the same was validated based on (ICH)-Q2-(R1) guidelines.

Results This study revealed that the powder formulation contains 463 ng/spot of lawsone, 312.5 ng/spot of gallic acid and 13.33 ng/spot of diosgenin.

Conclusion A simple, specific, and reproducible semi-automated TLC method was developed and validated suc-successfully as per (ICH)-Q2-(R1) guidelines and can be utilized for analysing marketed herbal formulations containing *Lawsonia inermis*(henna) and *Trigonella foenum-graecum***.**

Keywords-Lawsone, gallic acid, diosgenin, Validation, HPTLC

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Introduction:

Lawsone is chemically called 2-hydroxy-1,4-naphthoquinone, mainly obtained from *Lawsonia inermis*. It is a plant ubiquitous to North Africa, the Middle East, and South Asia. It is locally known as henna, madurang, manghati, goranti, and madayantika, and is one of the most frequently employed dyes, therefore, it has been proposed for usage as a non-oxidizing hair coloring agent[1][2]. Lawsone has several pharmacological actions, notably anti-inflammatory, antioxidant, antibacterial, antifungal, and anticancer activities. Traditionally, it was used to treat conditions of the skin such as eczema, psoriasis, and fungal infections [3]. Contrary to other antioxidants including vitamin C and E, β-carotene, and flavonoids which protect against oxidative stress, lawsone has an improved efficacy and potential safety profile. Henna is well recognized for its beneficial properties against dandruff (Malassezia) [2]

Gallic acid is a phenolic compound present in almost all naturally occurring compounds like black and green tea, gallnuts, pomegranates, oranges, berries, etc. It is extremely important due to its antibacterial, antifungal, and antioxidant effects [4]

Diosgenin is a well-known steroidal sapogenin produced by the hydrolysis of the saponin dioscin, which can be derived from a variety of plants, including Dioscorea, Trigonella, Costus, and Smilax species [5]. It is a steroidal sapogenin with intriguing bioactivity that has been studied for many years. In fact, this compound has anti-inflammatory and antioxidant characteristics and can be utilized for the management of allergic conditions. It is also being studied for anti-

infectious properties against fungi, bacteria, protozoa, and viruses [6].

Standardization of herbal formulations aids in the establishment of consistent criteria and intrinsic qualities that ensure safety, efficacy, quality, and reproducibility. According to the definition provided by the American Herbal Product Association, "Standardization refers to the body of information and controls necessary to produce material of reasonable consistency [7]. Nowadays, in the herbal medicinal sector, standardization is an essential aspect of quality control for obtaining adequate raw materials, preserving the quality and purity of final goods, and so on [8]. HPTLC, HPLC, MS, LC-MS, and GC-MS were some of the analytical techniques used in this study [9]. Based on the TLC principle, HPTLC is an essential separation method with several benefits such as reduced exposure to toxic solvents, improved sample application, reduced usage of mobile phase, less analysis time, less likelihood of environmental pollution, and higher separation efficiencies [10]. Planar chromatographic methods (TLC and HPTLC) were extensively utilized for quality monitoring of herbal formulations. It facilitates in the qualitative, quantitative, and semi-quantitative analysis of phytoconstituents found in various herbal formulations developed and distributed by different companies. Thin-layer chromatographic techniques have been shown in numerous studies to effectively guarantee the quality and purity of commercially available herbal products.. Patel et al. developed a HPTLC method for analysing lawsone in Trichup herbal hair powder by employing toluene: ethyl acetate: glacial acetic acid (8:1:1 v/v/v) as the mobile phase. In this study, lawsone was validated, quantified successfully, and stability studies were also performed [11]. Garg et al. successfully developed a method for the estimation of lawsone in *Lawsonia inermis* (Henna) obtained from two natural habitats and dye products collected from local market using mobile phase as toluene: ethyl acetate: formic acid (22:16:2 v/v/v) [12]. A simple and affordable HPTLC method was established by Athar Ali et al. for the simultaneous analysis of colchicine and gallic acid , which are present together in a complex commercial polyherbal drug (Habb-e-Irqun-Nisa) in tablet dosage forms with ethyl acetate: acetonitrile: water: formic acid: N-dimethylformamide (7.5:1:0.5:0.5:0.5 v/v) as mobile phase [13]. Naveen Choudhary et al. developed HPTLC method for standardization of biomarker compound lupeol and diosgenin in Mutrakrichantak churna using a mobile phase as isopropyl alcohol: n-butanol (5:5 v/v) [14]. These studies highlighted the importance of planar chromatographic techniques in the standardization of marketed herbal formulations, and marker compounds play an important role in this.

A thorough literature review revealed that multiple analytical procedures were used to estimate lawsone, diosgenin, and gallic acid individually. However, to the best of our knowledge, no analytical approach exists for standardizing marketed herbal formulations that contain lawsone, diosgenin, and gallic acid simultaneously. As a result, in the current work, a rapid and easy semi-automated TLC approach was developed to identify and estimate three important phytoconstituents lawsone, gallic acid and diosgenin (Fig. 1) in a marketed formulation, as well as for use in the determination of formulation quality and purity. The established approach has been validated according to the (ICH)-Q2-(R1) guidelines.

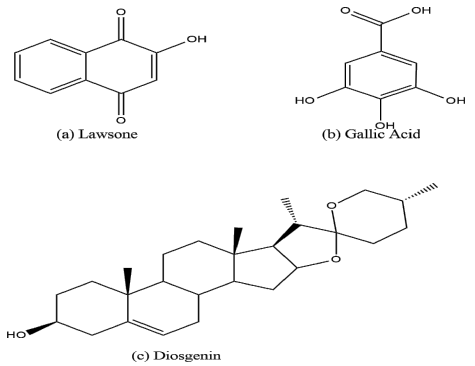

Fig. 1 Structures of lawsone, gallic acid and diosgenin

Methods

Instruments

The TLC visualizer 2, Linomat V applicator, and TLC scanner 4 made by CAMAG (Switzerland) are all included with the VisionCATS software (version 4.0). We purchased TLC silica gel 60F254 plates from Merck in Germany. Sartorius (Germany) and Biotechnics India (India) supplied the analytical balance and hot air oven.

Reagents and standard substances

Selected marketed formulation

A hair paste that was purchased from the distributor served as the chosen marketing sample. [15]. The sample was kept at normal room temperature and out of direct sunlight.

Standard and sample preparation

The standard solution was prepared by weighing accurately 10 mg of each of lawsone, diosgenin and gallic into separate 100 ml volumetric flasks. Volume was made up using HPLC grade methanol to 100 ml and sonicated to obtain stock solutions of 0.1 mg/ml of each. For diosgenin, 1 ml of the above stock solution was transferred to a 10 ml volumetric flask and volume was made upto 10 ml to obtain 0.01 mg/ ml of standard solution. Hence, the standards obtained were as follows: lawsone

(0.1 mg/ml), diosgenin (0.01 mg/ml) and gallic acid (0.1 mg/ml).

For sample solution, 100 mg was accurately weighed and 2 ml of HPLC methanol was added. This solution was sonicated for 15 minutes. Further, this solution was filtered through Whatman filter paper and stored it in an eppendorf tube at normal room temperature.

Optimization of the analytical conditions

TLC silica gel $60F_{254}$ plates were utilized as the stationary phase in the current study. To identify an appropriate mobile phase for analysis, different solvent combinations were examined. Following a thorough literature review, it was decided that different combinations of toluene, ethyl acetate, and formic acid would be employed to determine the ideal mobile phase composition. Based on the separation pattern obtained from preliminary TLC analysis/observation, a mixture of ethyl acetate, toluene and formic acid (5: 4: 1) (v/v/v) was selected as the final mobile phase and was employed throughout the analysis.

Semi-automated TLC conditions

On each TLC silica gel $60F_{254}$ plate, lawsone, gallic acid and diosgenin standards and sample were applied at the rate of 150 nL S^{-1} by utilizing a Linomat V applicator. The applied band length was 8 mm, and the application was about 1 cm from the bottom and left edges of the plate. After application, the development of the plates was done in CAMAG twin trough chambers to a distance of 70 mm with the selected mobile phase composition which was previously saturated for 20 min. After that, the developed plates were air-dried, and the fingerprint profile was generated by placing the plates in TLC visualizer 2. Then, the plates were scanned using a TLC scanner 4 at λ = 280 nm, 272 nm and 430 nm for lawsone, gallic acid and diosgenin, respectively. The wavelengths were optimized by performing spectrum scanning in the 190– 500 nm wavelength range, and the obtained data were compared with the maximum wavelengths mentioned in the literature (lawsone- 277, 278 nm)[12], [16], (Gallic acid- 254, 280 nm)[17], [18] and (diosgenin- 275, 366 nm)[19], [20]. The conditions for densitometric scanning (VisionCATS version 4.0) were: scanning speed 20 mm/s, data resolution 100 µm/step, and slit dimension 6.00 × 0.45 mm. From densitometric analysis, the retardation factor (Rf) for lawsone, gallic acid and diosgenin was found satisfactory. For quantitative analysis, the obtained values for the peak areas were utilized.

Method validation

Method validation is an integral part of any analytical experiment/procedure for getting accurate and reproducible results. This process is performed by checking the following parameters: system suitability test, linearity, LOQ, LOD, accuracy (recovery), precision (intra- and inter-day precision) and robustness.

System suitability test

A system suitability test (SST) is a necessary step to make sure that a chromatographic system works well and produces reliable results. Any concentration from the working range is selected, and six replicates are applied.

Linearity

In any analytical method, linearity is a crucial parameter that indicates its ability to produce test findings that are directly proportional to the content (concentration) of the analyte present in the sample [21]. The standard stock solutions containing lawsone, gallic acid, and diosgenin have been diluted to achieve linearity of standard solutions (taking into account the quantitation limit) in the concentration range of 100–700, 100–700, and 20–50 ng/spot, respectively, in order to quantify the analytes present in the formulations. The standards were applied in triplicate to generate the calibration curve. The correlation coefficient (r^2), intercept, and slope of the calibration curves were estimated to obtain the method's linearity.

Detection limit (LOD)

The detection limit (LOD) is defined as the lowest amount of the analyte detected in the prepared sample. LOD can be calculated by using the following equation:

LOD = 3.3 × standard deviation of the response /slope of the calibration curve

Quantitation limit (LOQ)

The quantitation limit (LOQ) is defined as the lowest amount of the analyte quantified with sufficient accuracy and precision. It can be calculated by using the following equation:

LOQ = (10 × standard deviation of the response) /slope of the calibration curve

For the following study the LOD and LOQ was generated by the system itself.

Specificity

The specificity of the method was evaluated by applying the diluent, standard, sample and, mobile phase on each track. The track is checked for any interference at the Rf of the drug.

Accuracy (recovery)

The accuracy of any analytical method is defined as the degree of agreement between the values regarded to be true and the amount of the substance in the test samples obtained through the analysis

[21], [22]. For estimating the percentage of recovery of lawsone, gallic acid and diosgenin in the sample, 240 ng/spot (80%), 300 ng/spot (100%), 360 ng/spot (120%) of lawsone and gallic acid standards and 24 ng/spot (80%), 30 ng/spot (100%) and 36 ng/spot (120%) of diosgenin were applied on the TLC plate. After that, the percentage of recoveries was calculated and documented.

Precision

The precision of any analytical method was defined as the degree of agreement between a set of measurements obtained from multiple sampling of the same homogenous sample under the specified conditions [21], [22]. The standard solutions were applied in triplicate at three different concentration levels three times at 72-hour intervals in the current study to conduct inter-day precision studies of the developed method. For intra-day precision studies, the standard solutions were applied in triplicate at three different concentration levels three times within two-hour intervals on the same day.

Robustness

The reliability of an analysis of intentional modifications in method parameters can be ascertained by conducting robustness research, which is necessary during method development. To test the robustness of the suggested approach, the following modifications were made in the current study: varying quantities of mobile phase compositions were used (Ethyl acetate: toluene: formic acid = 5.5:3.5:1, 5: 4.5: 0.5); the saturation time to 10 and 30 minutes; mobile phase volume to 8 and 12 ml. It was observed that the developed method gave reproducible results in all varying conditions.

Results

The marketed formulation (paste) was collected from the distributor. The stock solutions of the standards were prepared as such to obtain 0.1 mg/ml of lawsone and gallic acid and 0.01 mg/ml for diosgenin dissolved in HPLC grade methanol. In the present study, TLC silica gel $60F_{254}$ plates were used as a stationary phase, and a combination of ethyl acetate: toluene: formic acid (5:4:1 v/v/v) was utilized as a mobile phase. The developed fingerprinting profile showed that lawsone, gallic acid and diosgenin were present in the selected formulation. Both lawsone and gallic acid were densitometrically detected at λ = 280 and 272 nm (Fig. 2 and 3) respectively. Derivatization was required for detection of diosgenin in which the plate was dipped in anisaldehyde sulphuric acid reagent and then heated at 100˚C for 5 minutes. Diosgenin was densitometrically detected at 430 nm (Fig 4). The absorption spectrum and the peak areas were recorded and documented for analysis. From spectrum data, it was observed that in the wavelength range of 190–500 nm, absorption maxima for lawsone, gallic acid and diosgenin were observed at 280, 272 and 430 nm, respectively. So, these three wavelengths were selected for further quantification. The specificity of the method was determined by comparing the spectra of lawsone, gallic acid and diosgenin in the selected formulations with the standards. The peaks obtained by densitometric scanning were easily identifiable, symmetrical, well resolved, and no interference was observed at the Rf of the standards. From densitometric scanning, the obtained Rf values for lawsone, gallic acid and diosgenin were 0.63, 0.41, and 0.72, respectively (Fig. 5 and 6)

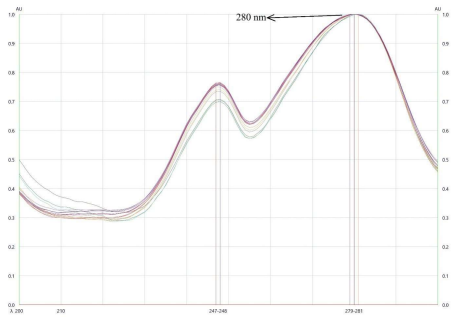

Fig.2 Spectrum data of lawsone in the selected formulation at 280 nm

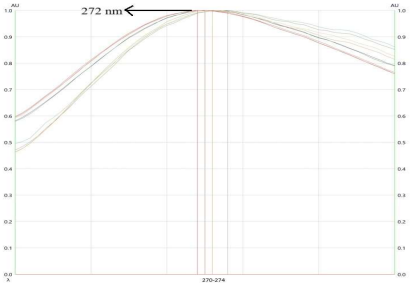

Fig.3 Spectrum data of gallic acid in the selected formulation at 272 nm

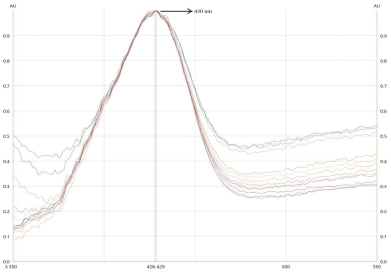

Fig.4 Spectrum data of diosgenin in the selected formulation at 430 nm

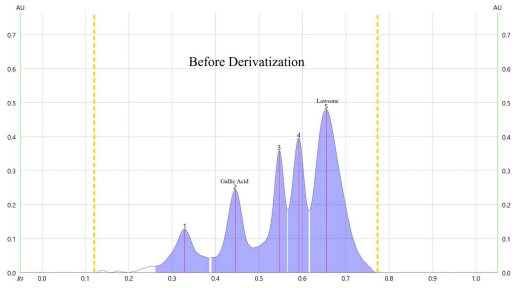

Fig.5 HPTLC chromatogram of lawsone and gallic acid in formulation before derivatization

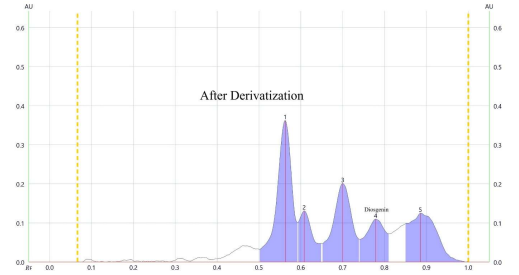

Fig.6 HPTLC chromatogram of diosgenin in formulation after derivatization

Further, the developed method was validated as per the guidelines mentioned by (ICH)-Q2-(R1). The system suitability test was done, and the %RSD was found under the limit(Table 1). To determine the linearity of the developed method, standard solutions of increasing concentration (100-700 ng/spot for lawsone and gallic acid and 20-50 ng/spot for diosgenin) were applied on TLC silica gel $60F_{254}$ plate. The calibration curve was generated (Fig. 7,8 and 9, Tables 2,3 and 4), and a linear relationship was established(Table 5). From the calibration curve, the obtained equations were Y = 1E-05x + 0.0069 , Y = 8E-06x + 0.0002, and Y = 9E-05x + 0.0002, correlation coefficients (R^2) = 0.9911, 09922 and 0.9983 , coefficient of variances (CV) = 0.23, 0.51 and 0.28% for lawsone, gallic acid and diosgenin, respectively (Fig. 7,8 and 9, Tables 2,3 and 4). The obtained R^2 value indicates a strong correlation between the concentrations of phytocompounds and peak areas.

Table 1 Data for system suitability test (SST)

Standard	Conc(ng/spot)	Mean	RSD(%)
Lawsone	500	0.01842	1.09
Gallic acid	500	0.00550	1.48
Diosgenin	30	0.00092	1.64

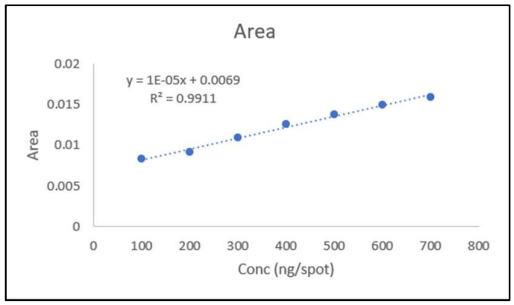

Fig. 7 Calibration curve of lawsone standard

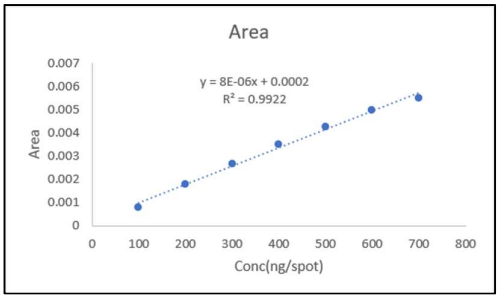

Fig. 8 Calibration curve of gallic acid standard

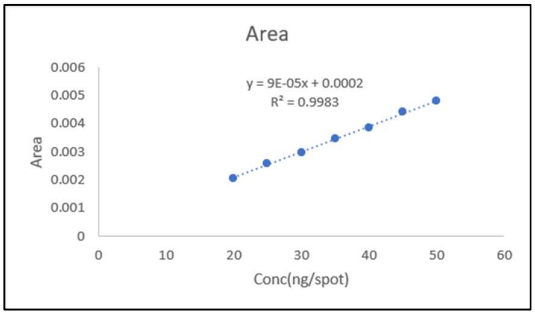

Fig. 9 Calibration curve of diosgenin standard

Table 2 Linear regression data of lawsone

Sr No	Conc.(ng/spot)	Area
1	100	0.00836
2	200	0.00912
3	300	0.01097
4	400	0.01255
5	500	0.01376
6	600	0.01494
7	700	0.01594

Table 3 Linear regression data of gallic acid

Sr No	Conc.(ng/spot)	Area
1	100	0.00077
2	200	0.00177
3	300	0.00267
4	400	0.00349

5	500	0.00425
6	600	0.00498
7	700	0.0055

Table 4 Linear regression data of diosgenin

Sr No	Conc.(ng/spot)	Area
1	20	0.00204
2	25	0.00259
3	30	0.00298
4	35	0.00346
5	40	0.00385
6	45	0.00441
7	50	0.00481

Table 5 Method validation parameters

Sr No	Parameters	Lawsone	Gallic acid	Diosgenin
1	Wavelength(nm)	280	272	430
2	Linearity range concentration (ng/spot)	100–700	100-700	20-50
3	Coefficient of variances (CV)	0.23	0.51	0.28
4	Correlation coefficients (R^2)	0.9911	0.9922	0.9983
5	Limit of detection (ng/spot)	174.1	127.8	2.904
6	Limit of quantitation (ng/ spot)	448.1	328.8	7.472
7	Reproducibility	Reproducible	Reproducible	

Table 6 Intra and interday precision of lawsone

Conc(ng/spot)	Mean area	RSD(%)
Intra-day		
300	0.01229	1.59
400	0.0133	1.16
	67	
500	0.014554	1.80
Inter-day		
300	0.012440	1.39
400	0.013333	0.66
500	0.014266	0.89

Table 7 Intra and interday precision of gallic acid

Conc(ng/spot)	Mean area	RSD(%)
Intra-day		
200	0.00186	0.37
300	0.00281	1.51
400	0.00366	1.84
Inter-day		
200	0.00018	0.66

	6	
300	0.00276	1.92
500	0.00453	1.85

Table 8 Intra and interday precision of diosgenin

Conc(ng/spot)	**Mean area**	**RSD(%)**
Intra-day		
25	0.00247	1.53
30	0.00254	1.59
40	0.00291	0.33
Inter-day		
25	0.00159	1.56
30	0.00246	1.65
40	0.00290	0.69

The LOD and LOQ values were calculated by the software, and the obtained values were 174.1 ng/spot and 448.1 ng/spot for lawsone, 127.8 ng/spot and 328.8 ng/spot for gallic acid, 2.904 ng/spot and 7.472 ng/spot for diosgenin, respectively (Table 5). The developed approach underwent inter-day and intra-day precision investigations; intra-day studies were performed in triplicate at three-hour intervals, while inter-day studies were performed in triplicate at three consecutive days (Tables 6,7 and 8). The developed method was evaluated for recovery (accuracy) studies. For this purpose, the selected formulation. The calculated % of recovery for lawsone was: 97.55%, for gallic acid: 95.24% and for diosgenin: 95.59%; hence, the developed method was accurate) (Tables 9,10 and 11). By adjusting the approach parameters, robustness studies were also conducted, and the findings obtained showed no significant modifications. (Tables 12, 13 and 14).

Table 9 Accuracy (recovery) data of lawsone obtained from the formulation

Formula	**Area obtained from software**	**Recovery(%)**
3 bands of 100% sample	0.01153	97.55
	0.01202	
	0.01199	
3 bands of 100% sample+ 80% standard	0.01975	
	0.01970	
	0.01977	
3 bands of 100% sample+ 100% standard	0.01990	
	0.01982	
	0.01990	
3 bands of 100% sample+ 120% standard	0.02035	
	0.02033	
	0.02031	
2 bands of 80% standard	0.00822	
	0.00839	
2 bands of 100% standard	0.00837	
	0.00840	
2 bands of 120% standard	0.00960	
	0.00882	

Table 10 Accuracy (recovery) data of gallic acid obtained from the formulation

Formula	**Area obtained from software**	**Recovery(%)**
3 bands of 100%	0.0027	95.24

sample	0.0026	
	0.0027	
3 bands of 100% sample+ 80% standard	0.0055	
	0.0055	
	0.0055	
3 bands of 100% sample+ 100% standard	0.0056	
	0.0056	
	0.0052	
3 bands of 100% sample+ 120% standard	0.0061	
	0.0065	
	0.0066	
2 bands of 80% standard	0.0029	
	0.0031	
2 bands of 100% standard	0.0031	
	0.0032	
2 bands of 120% standard	0.0047	
	0.0043	

Table 11 Accuracy (recovery) data of diosgenin obtained from the formulation

Formula	**Area obtained from software**	**Recovery(%)**
3 bands of 100% sample	0.0012	95.59
	0.0014	
	0.0015	
3 bands of 100% sample+ 80% standard	0.0032	
	0.0034	
	0.0038	
3 bands of 100% sample+ 100% standard	0.0044	
	0.0043	
	0.0043	
3 bands of 100% sample+ 120% standard	0.0046	
	0.0044	
	0.0045	
2 bands of 80% standard	0.0028	
	0.0028	
2 bands of 100% standard	0.0025	
	0.0025	
2 bands of 120% standard	0.0043	
	0.0032	

Table 12 Data of robustness study of lawsone

Mobile phase composition (Toluene: ethyl acetate: formic acid)(v/v/v)		
Volume	Mean Standard area	Mean Sample area
5.5: 3.5: 1	0.00971	0.01475
4: 5: 1 (Normal)	0.00958	0.01508
4:4.5:0.5	0.00938	0.01546
RSD(%)	1.72	2.35
Saturation time(in minutes)		
Time	Mean Standard area	Mean Sample area
10	0.01266	0.00163
20 (Normal)	0.01216	0.00164
30	0.01254	0.00166
RSD(%)	2.09	3.18
Mobile phase volume(ml)		
Volume	Mean Standard area	Mean Sample area
8	0.01232	0.00167
10 (Normal)	0.01222	0.00163
12	0.01273	0.00163
RSD(%)	2.18	0.73

Table 13 Data of robustness study of gallic acid

Mobile phase composition (Toluene: ethyl acetate: formic acid)(v/v/v)		
Volume	Mean Standard area	Mean Sample area
5.5: 3.5: 1	0.00240	0.00288
4: 5 1 (Normal)	0.00230	0.00286
4:4.5:0.5	0.00240	0.00280
RSD(%)	2.37	1.42
Saturation time(in minutes)		
Time	Mean Standard area	Mean Sample area
10	0.00325	0.00385
20 (Normal)	0.00334	0.00384
30	0.00337	0.00392
RSD(%)	1.77	1.13
Mobile phase volume(ml)		
Volume	Mean Standard area	Mean Sample area
8	0.00243	0.00330
10 (Normal)	0.00243	0.00328
12	0.00246	0.00330
RSD(%)	0.83	0.35

Table 14 Data of robustness study of diosgenin

Mobile phase composition (Toluene: ethyl acetate: formic acid)(v/v/v)		
Volume	Mean Standard area	Mean Sample area
5.5: 3.5: 1	0.00329	0.00163
4: 5: 1 (Normal)	0.00330	0.00166
4:4.5:0.5	0.00334	0.00163
RSD(%)	0.88	1.00
Saturation time(in minutes)		

Time	Mean Standard area	Mean Sample area
10	0.00329	0.00163
20 (Normal)	0.00330	0.00164
30	0.00338	0.00166
RSD(%)	1.48	1.03
Mobile phase volume(ml)		
Volume	Mean Standard area	Mean Sample area
8	0.00311	0.00167
10 (Normal)	0.00323	0.00163
12	0.00311	0.00163
RSD(%)	2.23	1.23

After that, the developed method was successfully employed to estimate lawsone, gallic acid and diosgenin in the selected marketed formulation. From this study, the phytocompounds were quantified as following lawsone- 463 ng/spot, gallic acid- 312.5 ng/spot and diosgenin- 13.33 ng/spot.

Table 15 Quantity of lawsone, gallic acid and diosgenin in 3µl spot of sample

Name	**Conc (ng/spot)**
Lawsone	463
Gallic acid	312.5
Diosgenin	13.33

Discussion

Lawsonia inermis and *Trigonella foenum-graecum* are important medicinal plants, Furthermore, there were a number of herbal cosmetic formulations on the market that included *Lawsonia inermis* leaf powders, extracts, or oils, as well as *Trigonella foenum-graecum* seed powder. Nevertheless, no particular analytical technique has been described for standardizing the same, and planar chromatographic methods for marker-based standardization would be a better option for this. Hence, there is a need to develop a suitable analytical method that helps to perform qualitative and quantitative analysis of two major marker compounds of *Lawsonia inermis,* lawsone and gallic acid and one major marker of *Trigonella foenum-graecum* named diosgenin in the marketed herbal formulations for routine quality control analysis. Hence, in the current study, the marketed formulation manufactured by a company was collected, and quantitative estimation of lawsone, gallic acid and diosgenin was done in this formulation by using a semi-automated TLC method. Lawsone (Rf value 0.63), gallic acid (Rf value 0.41) and diosgenin (Rf value 0.72) were identified in all of the formulation at the wavelengths of 280, 272 and 430 nm, respectively and quantified in the formulation. For performing this analysis, different mobile phases were employed, but based on the separation pattern, ethyl acetate: toluene: formic acid (5:4:1 v/v/v) was selected for final quantification. This method passed all the parameters of analytical validation as per the guidelines prescribed in (ICH)-Q2-(R1) and gave reproducible results. The peak shapes were found to be good. Hence, the developed semi-automated TLC method can be utilized for the routine quality control analysis of marketed herbal formulations of *Lawsonia inermis* and *Trigonella foenum-graecum* effectively, and its advantages are specificity, accuracy, and simplicity.

Conclusion

The results of this investigation demonstrated that the established HPTLC method was accurate, simple, precise, and specific in determining the amounts of lawsone, gallic acid, and diosgenin in a particular marketed herbal product. According to this study, all three are recognized and measured in the formulation. The quality of marketed herbal formulations comprising *Lawsonia inermis* leaf powders, extracts, or oils, as well as *Trigonella foenum-graecum* seed powder, can be preserved with the aid of the quantitative and qualitative analysis of the concentration of lawsone, gallic acid, and diosgenin. The accuracy, precision, and dependability of the devised approach were proven by validation by the requirements outlined in (ICH)-Q2-(R1).

Abbreviations

TLC Thin-layer chromatography
HPTLC High-Performance Thin-Layer Chromatography
HPLC High Performance Liquid Chromatography
LOD Limit of Detection
LOQ Limit of Quantitation
R^2 Correlation coefficient
CV Coefficient of variance
LC–MS Liquid Chromatography–Mass Spectrometry
GC–MS Gas Chromatography–Mass Spectrometry
Rf Retardation factor
mg Milligram
ng/spot nanogram/ spot
ICH International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
mL Millilitre
v/v/v Vol/vol/vol
µL Microlitre
MS Mass spectrometry
nm Nanometre
mm Millimetre
nm/s Nanometre/second
nm/step Nanometre/step
µm/step Micrometre/step min. Minute

hrs Hours

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Author contributions

AP prepared the methodology, performed the experiments, and conducted the statistical analysis. The manuscript draft was prepared by AP and PC supervised and edited the manuscript. The final version of the manuscript was approved by all of the authors.

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Availability of data and materials

The data sets utilized and analysed during the present study can be available upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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