CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.1211702

Available online at: http://www.iajps.com Research Article

SIMULTANEOUS QUANTIFICATION OF DIOSGENIN, OLEIC ACID, HARMINE AND KAEMPFEROL BY RP-HPLC IN THE FRUIT EXTRACT OF TRIBULUS TERRESTRIS L. AND ITS FORMULATION

Vikas V. Vaidya[1], Prakash L. Kondalkar[1*], Pushkar M. Pradhan[1], Sulekha R. Gotmare[2]

[1*]Department of Chemistry, Ramnarain Ruia College, Matunga, Mumbai -400019, Maharashtra, India

[2]Department of Analytical Chemistry, S.N.D.T. Women's University, Santacruz, Mumbai -400049, Maharashtra, India

Abstract:

A simple, accurate and reproducible RP-HPLC method has been developed for the simultaneous quantification of Diosgenin, Oleic acid, Harmine and Kaempferol from the methanolic extract of fruits of plant Tribulus terrestris L. and its marketed formulation. The Agilent 1290 Infinity II HPLC system was used for the analysis. The optimized conditions (Mobile phase A: 0.01 M Di-Potassium hydrogen phosphate buffer; pH 7.0 adjusted with ortho-phosphoric acid And Mobile phase B Acetonitrile: Methanol (80:20), gradient elution system on a C8 reversed-phase column with a flow rate of 1 mL/min. The separated compounds were detected at 201 nm using Agilent 1290 DAD FS (Diode Array Detector) The quantity of Harmine was found to be 0.013 % and 0.006%, Kaempferol was 0.011% and 0.005%, Oleic acid was 0.261 % and 0.115 % and Diosgenin was 0.084 % and 0.019% in plant and formulation respectively. The developed method was then validated and applied for quantification of four components in a formulation containing Tribulus terrestris L. extract. This method can also be used as a quality control tool for other formulations or dietary supplements containing the extract of Tribulus terrestris.

Keywords: *Tribulus terrestris L., Harmine, Kaempferol, Oleic acid, Diosgenin, Simultaneous quantification*

Corresponding author:

Mr. Prakash L. Kondalkar,

Student, Department of Chemistry,
Ramnarain Ruia College, Matunga, Mumbai -400019,
Maharashtra, India
Email[1]: prakash.kondalkar@gmail.com*
Contact no[1]: 09819077163*

QR code

Please cite this article in press Prakash L. Kondalkar et al., ***Simultaneous Quantification of Diosgenin, Oleic Acid, Harmine and Kaempferol by RP-HPLC in the Fruit Extract of Tribulus Terrestris L. and It's Formulation,*** *Indo Am. J. P. Sci, 2018; 05(03).*

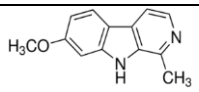
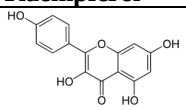
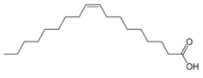
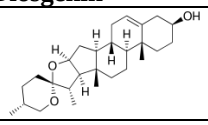
INTRODUCTION:

Tribulus terrestris L. is a valuable herb known for its application in the folk medicine in various parts of the world. Indian ayurveda system of medicine has recognized the importance of this herb long ago and used in tonics, under the Sanskrit name, "gokshura" to energize, vitalize and improve sexual function and physical performance in males. This plant is extremely rich in substances having potential biological significance, including: saponins, flavonoids, alkaloids, and other nutrients. Gokshura is extremely efficacious in most of the urinary tract disorders because it promotes the flow of urine, cools and soothes the membranes of the urinary tract, and aids in the expulsion of urinary stones and gout. Goksura effectively controls the bleeding, in large doses, it imparts the laxative action, hence is used as an adjunct in the treatment of piles. It is commonly used in treating diabetes, urinary calculi, dysuria, gout and sexual debility [1-5].

Medicinal plants have played a key role in world health especially in rural areas. It is a complicated job to standardize thousands of plant extracts with respect to their medicinal value and constituents. To maintain the quality of traditional medicines, WHO has issued guidelines for quality control methods of medicinal plant materials. Chromatographic and spectroscopic techniques are the most commonly used methods in standardization of herbal medicines but the herbal system is not easy to analyse because of their complexity and variations of chemical composition. Extremely valuable are techniques like high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), mass spectrometry (MS), high-performance liquid chromatography (HPLC), LC-MS, and GC-MS [6-9].

Many forms of raw plant material and herbal drugs derived from *Tribulus terrestris L.* are distributed in herbal market; however, the content of bioactive components in these products have not necessarily been quality-controlled. Therefore, a simple, low-cost, and rapid method for screening and quantitating bioactive components is strongly desired. Literature survey revealed that no method has been reported for simultaneous quantitation of Diosgenin, Oleic acid, Harmine and Kaempferol from methanolic extract of fruits of *Tribulus terrestris L.* Therefore, the aim of the study was to develop a rapid, precise and reproducible RP-HPLC method for quantification of Diosgenin, Oleic acid, Harmine and Kaempferol from *Tribulus terrestris L.* plant materials that can be used to determine their content in commercial herbal drugs. The proposed method was validated in accordance with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [10-18].

Table 1: Structures and properties of bioactive components

Component	Harmine	Kaempferol	Oleic acid	Diosgenin
Structure				
Molecular formula	$C_{15}H_{12}N_2O$	$C_{15}H_{10}O_6$	$C_{18}H_{34}O_2$	$C_{27}H_{42}O_3$
Molecular weight	212.25	286.24	282.46	414.6
pKa	6.44	6.44	4.99	Neutral
Group	Alkaloid	Flavonoid	Essential fatty acid	Saponin

MATERIALS AND METHOD:

Collection of plant

Tribulus terrestris L. were collected from Padadhari, around 30 km away from Rajkot, Gujarat, India in the month of December and it was authenticated with specimen No. 10291(2) of H. Santapau at 'Blatter Herbarium' in St. Xavier's college, Mumbai-400001

Preparation of plant material

The fruits were washed thoroughly with tap water. The fruits were dried initially using tissue paper to remove excess of water and later were air dried thoroughly under shade at room temperature to avoid direct loss of phytoconstituents from sunlight. The shade dried material was powdered using grinder and sieved through an ASTM 80 mesh. It was then homogenized to fine powder and stored in an air-tight container for further analysis.

Preparation of the fruit extracts

About 5 gm of dried fruit powder of *Tribulus terrestris L.* was weighed into a round bottom flask. 150 ml of mixture of 2 N hydrochloric acid and methanol in the ratio of 20:80 was added to the flask and the mixture

was refluxed at controlled 80 ° C on a boiling water bath for about 6 hrs. The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India) and extracted with chloroform (50 ml×3). The three chloroform extracts were combined and rinsed thrice times with 2 N NaOH and then rinsed thrice with distilled water. The extract was then passed through a filter bed of Na_2SO_4 to eliminate any remaining water. The samples were concentrated to dryness by evaporating the solvent at reduced pressure on Rotavapor buchi at 60° C and reconstituted the residue to final 50 ml volume in volumetric flask. This solution was further used for assay.

Preparation of the formulation extracts

About 10 gm of formulation Gokhshuradi Guggul containing *Tribulus terrestris L.* was weighed into a round bottom flask. 150 ml of mixture of 2 N hydrochloric acid and methanol in the ratio of 20:80 was added to the flask and the mixture was refluxed at controlled 80 ° C on a boiling water bath for about 6 hrs. The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India) and extracted with chloroform (50 ml×3). The three chloroform extracts were combined and rinsed thrice times with 2 N NaOH and then rinsed thrice with distilled water. The extract was then passed through a filter bed of Na_2SO_4 to eliminate any remaining water. The samples were concentrated to dryness by evaporating the solvent at reduced pressure on Rotavapor buchi at 60° C and reconstituted the residue to final 50 ml volume in volumetric flask. This solution was further used for assay.

Reagents and standards

All chemicals and solvents used were of analytical grade and purchased from Merck (Darmstadt, Germany). Analytical standards Diosgenin, Oleic acid, Harmine and Kaempferol were procured from Sigma-Aldrich (Bengaluru, India).

Chromatographic conditions

HPLC was performed on Agilent 1290 Infinity II HPLC system. The system was equipped with quaternary pumps, auto-sampler. The column oven temperature was maintained at 40°C throughout the analysis. The detection was carried out using Agilent 1290 DAD FS (Diode Array Detector). The method involves use of a Phenomenox reversed-phase Prodigy C8 column with length 150 mm and 5.0 µ particle size of stationary phase. Different compositions of solvents were tried as mobile phase in both isocratic and gradient mode. Finally a gradient of Buffer Mobile phase A 0.01 M Di-Potassium hydrogen phosphate buffer; pH 7.0 adjusted with ortho-phosphoric acid And Mobile phase B Acetonitrile: Methanol (80:20) was selected which gave a good resolution between the sample components. The flow rate was maintained at 1 mL/min and the separated components were detected at 201 nm. Gradient program used was

Table 2: Mobile phase gradient programme

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (mL/min)
0	60	40	1.00
3	60	40	1.00
18	10	90	1.00
23	10	90	1.00
25	60	40	1.00
30	60	40	1.00

Validation of the Method

ICH harmonized tripartite guidelines were followed for the validation of the developed analytical method [9].

Specificity

During the experiments an UV scan ranging from 200 to 400 nm in the time window of the analytes using PDA detector was performed with the aim of revealing eventual interfering compounds and evaluating the selectivity of the method. Specificity of the intended method was established by comparing the HPLC retention time and absorption spectra of target peaks from the analysed samples with those of the reference compounds. Specificity test was carried out by applying 5 µL of *Tribulus terrestris L* .fruits methanolic extract and 5 µL formulation; and 5 µl of each standard solution (1000 µl/ml of Diosgenin, Oleic acid) and 2 µL of each standard solution (1000 µl/ml of Harmine, Kaempferol), 5 µL of diluent and mobile phase

Precision

The variability of the method was studied by carrying out repeatability and intermediate precision. Repeatability was carried out in same laboratory, on same day, by analysing quality control samples containing the mixture of Diosgenin, Oleic acid, Harmine and Kaempferol using optimized chromatographic conditions. The experiment for inter-day precision was carried out using quality control samples of Diosgenin, Oleic acid, Harmine and Kaempferol on different days.

System suitability

System suitability experiment was performed by injecting six consecutive injections Standard solution

containing mixture of (80 µg/mL) of each bioactive marker, namely Diosgenin and Oleic acid and (40 µg/mL) Harmine and (20 µg/mL) Kaempferol during the start of the method validation. Values with % CV of ≤2% were accepted.

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

ICH defines the limit of detection (LOD) is the lowest concentration of an analyte that can be detected under the operational conditions of the method but not necessarily quantitated as an exact value. The limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy, under the operational conditions of the method.

Linearity

The Linearity of a method is the measure of how well a calibration plot of detector response against concentration approximates to a straight line. Seven concentration levels of each marker were selected for linear dynamic range for experiment. For Harmine, concentration levels of 4.01 µg/mL to 80.26 µg/mL were selected. Concentrations of Kaempferol were 1.71 µg/mL to 34.16 µg/mL, Concentrations of Oleic acid were 8.15 to 162.94 µg/mL, For Diosgenin, concentrations of 8.03 µg/mL to 160.58 µg/mL, were selected for linear dynamic range for experiment.

Assay

5 microliters of sample solution i.e. *Tribulus terrestris L.* fruits extract and 5 microliters of formulation were injected six times separately and analysed using the optimized chromatographic conditions. Peak areas were recorded for each analyte of interest and the amount of all the four analytes (Harmine, Kaempferol, Oleic acid and Diosgenin) was calculated by use of the calibration plot.

Recovery

The recovery experiment was carried out to check if there is any interference of other constituents with the peaks of Diosgenin, Oleic acid, Harmine and Kaempferol present in fruits of *Tribulus terrestris L.* and formulation Gokhshuradi Guggul containing extract of *Tribulus terrestris L.* Accuracy of the method was established by carrying out recovery experiment at three different levels, using standard addition method. To 5 µl fruits extract and 5 µl of formulation, known amounts of pure standards of Diosgenin, Oleic acid, Harmine and Kaempferol were added at different levels. The sample was then analysed by HPLC method using the developed optimized chromatographic conditions. Each sample was analysed in three replicates and the amounts of Diosgenin, Oleic acid, Harmine and Kaempferol recovered for each level, were determined. The value of percentage recovery for the four components was then calculated.

Recovery (%) = [(amount found – original amount) / amount added] x 100. Values within the range of 85 – 115% were accepted.

Robustness

Robustness of the method was studied by determining the effects of small variations of mobile phase pH (±0.2%), flow rate (1.00 ± 0.1 mL/min), column oven temperature (40 ^{0}C $\pm$ 2 ^{0}C). Effect of these deliberate changes on the response (area) and retention time of QC samples of Harmine, Kaempferol, Oleic acid and Diosgenin was observed during the analysis. The results were expressed in terms of % mean difference. Values within a difference range of ±5% were accepted.

Solution Stability

The stability of the stock solutions of all the three standards was evaluated by storing the solutions in refrigerator at 2-8 ºC for 72 hours and then comparing the results against freshly prepared stocks for each standard. Samples in triplicate were also subjected to bench top stability at 0 hrs, 24hrs, 48 hrs and 72 hrs respectively. Values within a difference range of ±5% were accepted.

RESULTS AND DISCUSSION:

Optimization of the Chromatography

Initial trial experiments were conducted to select a suitable mobile phase for accurate analysis of the standards. Of the various mobile phases tried in both isocratic and gradient mode, Buffer: acetonitrile in gradient mode gave the best resolution between the three. HPLC chromatograms corresponding to the four standards, *T. terrestris* L. extract and marketed formulation are represented in Figure 2 to 4. All the analytes exhibited the UV absorption at 201 nm.

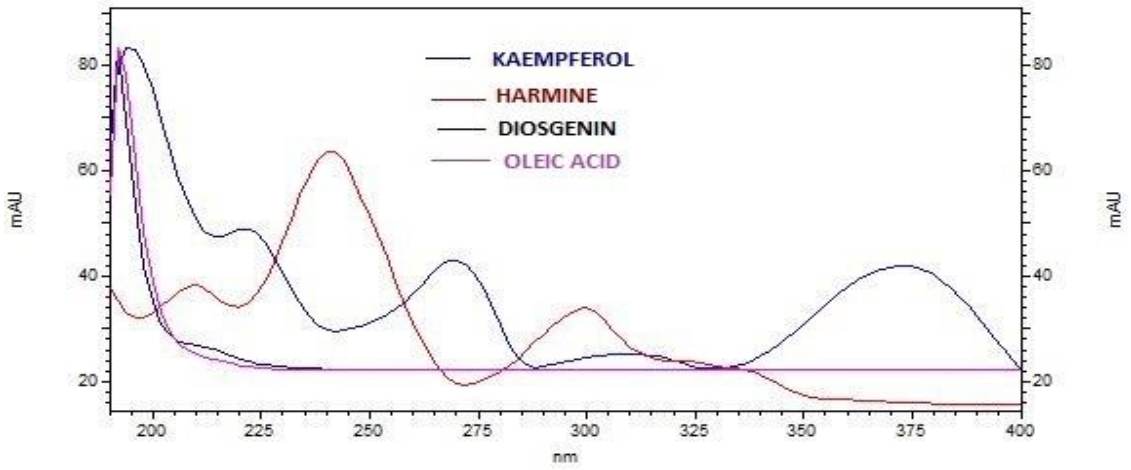


Fig. 1: Overlay of UV spectra

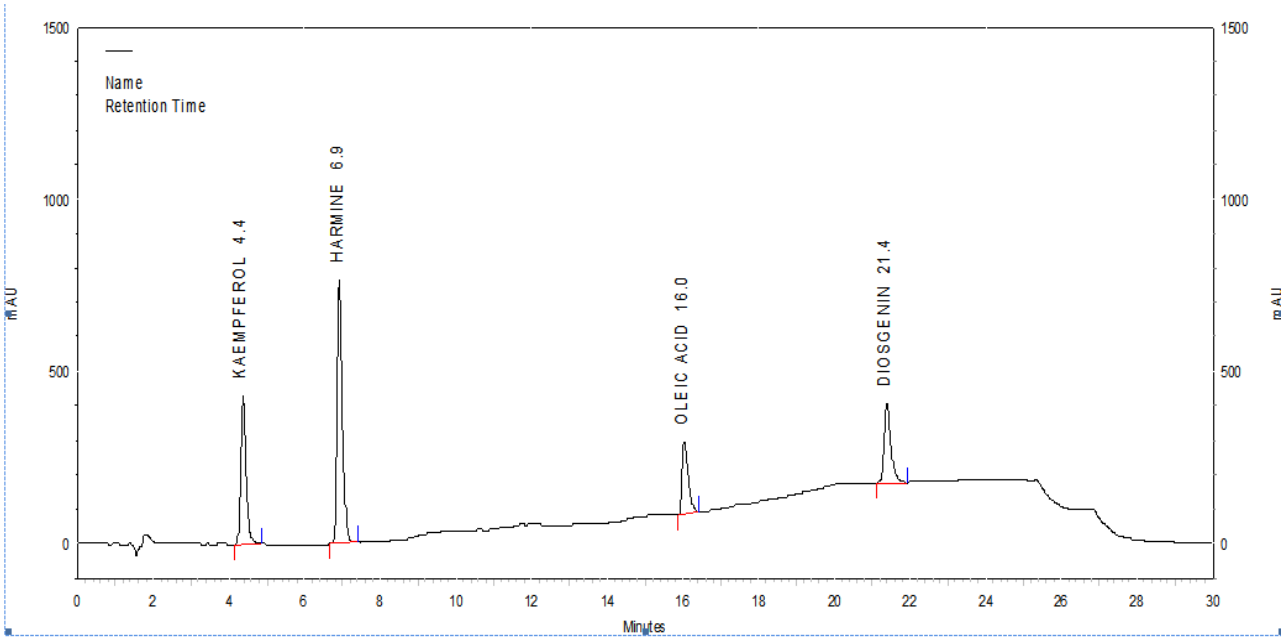


Fig. 2: Typical chromatogram of method development

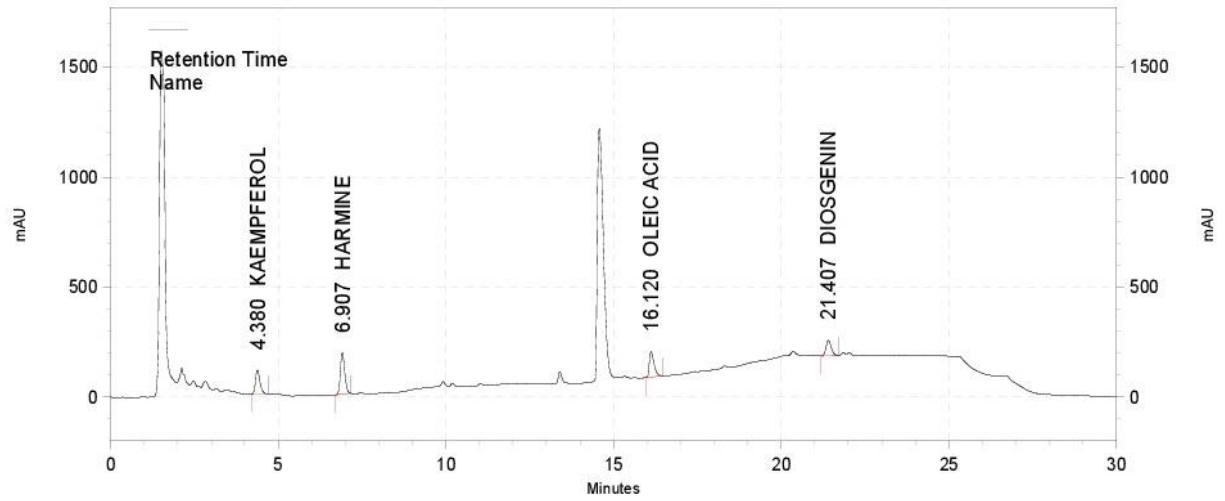


Fig. 3: Typical chromatogram of plant sample

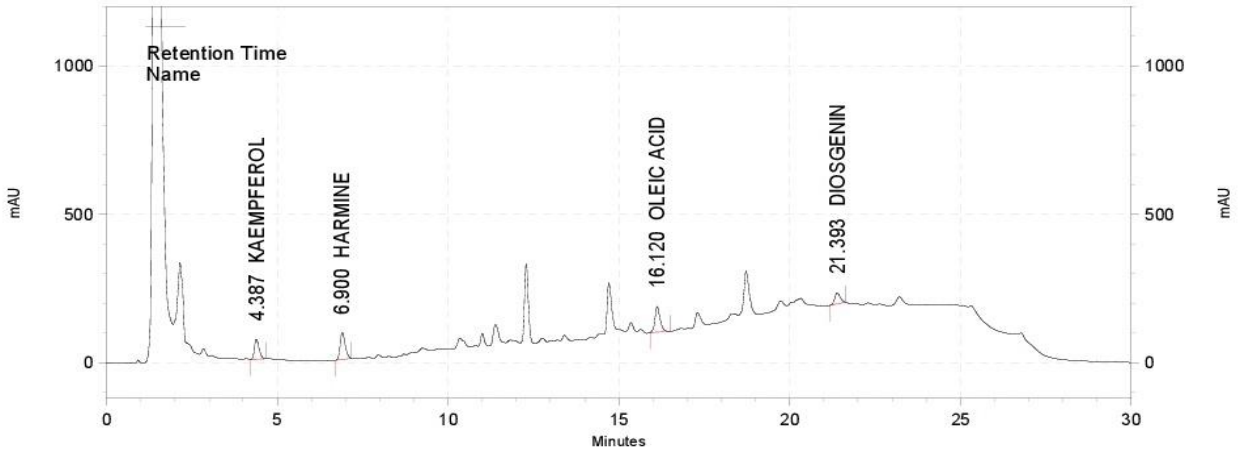


Fig. 4: Typical chromatogram of formulation sample

Method Validation

Selectivity and Specificity

During the UV scan no appreciable difference was found in the spectra of reference standards and the analysed samples. Hence, the method demonstrated a high degree of selectivity.

Linearity

The method was found to be linear from 4.01-80.26 µg/mL for Harmine, 1.71-34.16 µg/mL for Kaempferol, 8.15-162.94 µg/mL for Oleic acid and 8.03-160.58 µg/mL for Diosgenin respectively. The correlation coefficient was found to be ≥0.995 for all the four components. Results of regression analysis are summarized in Table 3.

Table 3: LOD, LOQ and Linearity

Compound	RT*	Regression equation	r^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Kaempferol	4.4	Y= 3279875 X -1397029	0.9999	1.71-34.16	0.30	0.85
Harmine	6.9	Y=2482458 X +378073	0.9994	4.01-80.26	0.78	2.01
Oleic acid	16.1	Y=398461 X -39035	0.9998	8.15-162.94	2.03	4.07
Diosgenin	21.4	Y=475667 X +771565	0.9996	8.03-160.58	2.00	4.01

RT* Retention time

Sensitivity

Sensitivity of the method was affirmed in terms of LOD and LOQ for Harmine, Kaempferol, Oleic acid and Diosgenin respectively. The results are represented in Table 2. The values for both LOD and LOQ were low which indicated that the method is capable of detecting and quantifying trace amount of the four components in plant samples.

Precision

In the repeatability study intra-day and inter-day precision of the HPLC method were investigated using replicate injection (*n*=3) of quality control samples of all the three standards. The developed method was found precise with % CV<2%.

Stability

Stock solution stability study of all the three standards stored for the period of 72 hours at 2-8°C showed % CV <2% with the % mean difference within ±5%. During bench top stability study, similar results were obtained. Stability studies showed that For Harmine, Kaempferol, Oleic acid and Diosgenin were found stable for at least 6.0 h at room temperature and 72 hours at 2-8°C of storage condition.

Robustness

Proposed method was not influenced by the factors considered for robustness study. Change in flow rate, mobile phase pH and column oven temperature affected the retention time of the four analytes but the area results were satisfactory since % CV was <2% with % mean difference <5%.

Recovery

The recovery values are summarized in Table 4. for all the four components were within acceptable limits (85.0 to 115.0%). This indicated that the method was reliable and accurate.

Table 4: % Recovery in fruit extract of Tribulus terrestris L. fruit and Formulation extract

Level		50%			100%			200%		
		i	ii	iii	i	ii	iii	i	ii	Iii
Harmine	Spiked conc.(µg/mL)	20.02	20.02	20.02	40.05	40.05	40.05	80.10	80.10	80.10
	% Recovery*	101.31	100.34	101.16	96.44	96.65	102.28	96.43	95.83	97.42
	% Recovery**	93.01	96.82	98.76	93.52	93.73	95.22	93.32	95.97	98.19
Kaempferol	Spiked conc.(µg/mL)	8.53	8.53	8.53	17.06	17.06	17.06	34.12	34.12	34.12
	% Recovery*	102.22	102.40	100.61	92.56	95.91	95.35	89.60	88.96	91.09
	% Recovery**	92.84	90.92	97.80	87.63	90.63	90.91	90.84	94.04	90.55
Oleic acid	Spiked conc.(µg/mL)	40.02	40.02	40.02	80.04	80.04	80.04	160.08	160.08	160.08
	% Recovery*	94.08	95.69	90.54	97.49	92.75	99.14	98.98	96.29	95.93
	% Recovery**	93.25	95.99	99.34	96.44	92.93	94.15	92.90	94.96	94.78
Diosgenin	Spiked conc.(µg/mL)	40.04	40.04	40.04	80.09	80.09	80.09	160.18	160.18	160.18
	% Recovery*	91.39	94.89	96.43	96.28	96.74	99.01	93.02	99.41	98.81
	% Recovery**	98.07	99.72	98.12	92.07	87.36	95.26	91.93	91.76	91.76

* % Recovery in fruit extract

** % Recovery in formulation extract

Assay

The assay value for samples of *Tribulus terrestris L.* fruit powder was found to be 0.013%, 0.011 %, 0.261 % and 0.084% for Harmine, Kaempferol, Oleic acid and Oleic acid respectively, while for the formulation, it was found to be 0.006 %, 0.005%, 0.115% and 0.019% for Harmine, Kaempferol, Oleic acid and Diosgenin respectively. The method is specific for all the four components because it resolved all standards well in the presence of other phytochemicals in *Tribulus terrestris L.* The proposed HPLC method was found to be suitable for qualitative and simultaneous quantitative analysis of Harmine, Kaempferol, Oleic acid and Diosgenin in the methanolic extract of *Tribulus terrestris L.*

Table 5: Summary of method validation parameters

Parameter		**Harmine**	**Kaempferol**	**Oleic acid**	**Diosgenin**
Specificity		Specific	Specific	Specific	Specific
Precision*		0.26%	0.32%	0.88%	0.78%
Robustness*	Flow rate (1 ml/min ± 0.1)	0.42%	0.55%	1.08%	0.92%
	mobile phase pH (7±0.2)	0.88%	1.12%	1.22%	1.32%
	Column temperature (40 ^{0}C ± 2^{0}C)	0.64%	0.88%	0.89%	0.58%
Recovery (Plant)**		98.65%	95.41%	95.66%	96.22%
Recovery (Formulation)**		95.39%	91.80%	94.97%	94.00%
Stability at RT***		6hrs	6hrs	6hrs	6hrs
Stability at 2-8° C		72 hrs	72 hrs	72 hrs	72 hrs

*Values are average % CV

** Values are average % Recoveries of all levels of concentrations

*** Room temperature

CONCLUSION:

A precise, accurate and reproducible HPLC method is validated for simultaneous quantification of four bioactive markers Harmine, Kaempferol, Oleic acid and Diosgenin. Proposed HPLC method can be used as an analytical tool for quality evaluation of plants and formulations containing Harmine, Kaempferol, Oleic acid and Diosgenin as chemical markers. It is an efficient method to screen *Tribulus terrestris L.* fruit samples in order to assess its quality and authenticity. Hence, it can be demonstrated that HPLC is a powerful practical tool for comprehensive quality control of plant raw materials and its formulations.

REFERENCES:

1. Amin A, Lotfy M, Shafiullah M, Adeghate E. The protective effect of Tribulus terrestris L.in diabetes. Ann N Y Acad Sci. 2006; 1084: 391-401.
2. Dinchev D., Janda B., Evstatieva L., Oleszek W., Aslani M. R., Kostova I. Distribution of steroidal saponins in Tribulus terrestris from different geographical regions. Phytochemistry. 2008; 69(1):176–186.doi: 10.1016/j.phytochem.2007.07.003.
3. Devi DJ, Ramesh CU. Different chemo types of gokhru (Tribulus terrestris): a herb used for improving physique and physical performance. Int J Green Pharmacy 2008; 3: 158–161.
4. F. Roghani-Dehkordi, M. Roghani, and T. Baluchnejadmojarad, "Diosgenin mitigates streptozotocin diabetes-induced vascular dysfunction of the rat aorta: the involved mechanisms," Journal of Cardiovascular Pharmacology, vol. 66, no. 6, pp. 584–592, 2015.
5. Ganzera M., Bedir E, Khan IA. Determination of steroidal saponins in Tribulus terrestris L.by reversed-phase high performance liquid chromatography and evaporative light scattering detection. J Pharm Sci 2001; 90: 1752–1758.
6. Gauthaman K., Adaikan P. G., Prasad R. N. V. Aphrodisiac properties of Tribulus Terrestris extract (Protodioscin) in normal and castrated rats. Life Sciences. 2002; 71(12):1385–1396. doi: 10.1016/s0024-3205(02)01858-1.
7. General Chapter <1225>, Validation of compendial methods, United States Pharmacopeia 32, National Formulary 27, Rockville, Md., USA, The United States Pharmacopeial Convention, Inc (2009).
8. General Chapter, Chromatography, United States Pharmacopeia 32, National Formulary 27,

Rockville, Md., USA, The United States Pharmacopeial Convention, Inc (2009).
9. Gupta PK, Nagore DH, Kuber VV, Purohit S. 2012. A validated RP-HPLC method for the estimation of Diosgenin from polyherbal formulation containing Tribulus terrestris Linn. Asian J Pharm Clin Res. 5:91–94.
10. H. J. Fang, K. S. Bi, Z. Z. Qian et al., "HPLC-DAD-ELSD determination of five active components in Tribulus terrestris L. Chinese," Journal of Pharmaceutical Analysis, vol. 32, article 6, 2012.
11. G. Saravanan, P. Ponmurugan, M. A. Deepa, and B. Senthilkumar, "Modulatory effects of diosgenin on attenuating the key enzymes activities of carbohydrate metabolism and glycogen content in streptozotocin-induced diabetic rats," Canadian Journal of Diabetes, vol. 38, no. 6, pp. 409–414, 2014.
12. ICH Q2R1: Validation of Analytical Procedures: Text and Methodology. Proceeding of the International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use, Geneva, Switzerland, 1996.
13. J. Niño, D. A. Jiménez, O. M. Mosquera, and Y. M. Correa, "Diosgenin quantification by HPLC in a Dioscorea polygonoides tuber collection from Colombian flora," Journal of the Brazilian Chemical Society, vol. 18, no. 5, pp. 1073–1076, 2007
14. Kole PL, Venkatesh G, Kotecha J, Sheshala R. 2 011. Recent advances in sample preparation techniques for effective bioanalytical methods. Biomed Chromatogr. 25:199–217.
15. Kozlova OI, Perederiaev OI and Ramenskaia GV. Determination by high performance chromatography, steroid saponins in a biologically active food supplements containing the extract of Tribulus terrestris. Voprosy Pitaniia 2011; 80(6): 67-71.
16. Louveaux A, Jay M, El Hadi OTM and Roux G. Variability in flavonoid content of four Tribulus terrestris. Journal of Chemical Ecology 1998; 24(9): 1465-1481.
17. Lehmann RP, Penman KG and Halloran KG. Comparison of photometric and HPLC-ELSD analytical methods for Tribulus terrestris. Revista de Fitoterapia 2002; 2(S1): 217.
18. L. Xu, Y. Liu, T. Wang et al., "Development and validation of a sensitive and rapid non-aqueous LC-ESI-MS/MS method for measurement of diosgenin in the plasma of normal and hyperlipidemic rats: a comparative study," Journal of Chromatography B, vol. 877, no. 14-15, pp. 1530–1536, 2009.
19. N. Gong, B. Zhang, F. Hu et al., "Development of a new certified reference material of diosgenin using mass balance approach and Coulometric titration method," Steroids, vol. 92, pp. 25–31, 2014.
20. Obreshkova D, Pangarova T, Milkov S and Dinchev D. Comparative analytical investigation of Tribulus terrestris preparations. Pharmacia 1998; 45(2): 11.
21. Oh JH, Lee YJ. 2014. Sample preparation for liquid chromatographic analysis of phytochemicals in biological fluids. Phytochem Anal. 25:314–330.
22. Q. Shi, B. Y. Yu, L. S. Xu, and G. J. Xu, "Determination of three hydrolytic flavonoid aglycones in Tribulus terrestris and atriplexcentralasiatica by RP –HPLC," Chinese Journal of Pharmaceutical Analysis, vol. 19, pp. 75–77, 1999.
23. Sun B.-S., Gu L.-J., Fang Z.-M., et al. Simultaneous quantification of 19 ginsenosides in black ginseng developed from Panax ginseng by HPLC-ELSD. Journal of Pharmaceutical and Biomedical Analysis. 2009; 50 (1):15–22. doi: 10.1016/j.jpba.2009.03.025.
24. Soni H, Patgiri B, Bhatt S. 2014. Quantitative determination of three constituents of Rasayana Churna (a classical Ayurvedic formulation) by a reversed phase HPLC. Int J Res Ayurveda Pharm. 5:17–22.
25. Shiquan X, Ruihai L. 2015. Content comparison of flavonoids in Tribulus terrestris from different habitats. China Pharmacist. 18:1671–1673.
26. Vol. 126. The book has no author, it's a publication of Govt. of India; 1989. Ayurvedic Pharmacopoeia of India, 1st Ed, Vol. 1. Govt of India, Ministry of Health and Family Welfare Gokshura (Rt.) pp. 49–52.
27. V. K. Manda, B. Avula, Z. Ali et al., "Characterization of in vitro ADME properties of Diosgenin and Dioscin from Dioscorea villosa," Planta Medica, vol. 79, no. 15, pp. 1421–1428, 2013.
28. Wang Y, Ohtani K, Kasai R and Yamasaki K. Steroidal saponins from fruits of Tribulus terrestris. Phytochemistry 1997; 45(4): 811-817.
29. Wu TS, Shi LS, Kuo SC. Alkaloids and other constituents from Tribulus terrestris. Phytochemistry. 1999; 50:1411–5.