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Original article

Determination of ursolic acid in fractionated leaf extracts of *Ocimum gratissimum Linn* and in developed herbal hepatoprotective tablet by HPTLC

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ABSTRACT

Background: Ursolic acid was determined in dichloromethane and ethyl acetate fractions of methanolic extract of *Ocimum gratissimum* and in developed herbal hepatoprotective tablet by HPTLC method.
Methods: Hepatoprotective polyherbal formulation was prepared using five fractions of three plant extracts namely *O. gratissimum*, *Butea monosperma* and *Bauhinia variegata*. Among these three plants *O. gratissimum* contains ursolic acid. Chromatographic separation was performed on silica gel HPTLC plates with petroleum ether:ethyl acetate:acetone (8.2:1.8:0.1, v/v/v) as mobile phase. After drying, the plates were sprayed with 10% (v/v) ethanolic solution of sulfuric acid and heated to 120 °C for 3 min. Quantification was performed in absorbance/transmittance mode at a wavelength of 530 nm using a computer-controlled densitometer.
Results: The presented method was validated for linearity 400–1200 (ng/spot), intraday precision % C.V. (0.58–1.97), and interday precision % C.V. (1.46–2.22). Correlation coefficient (r^2 = 0.9960), detection limits as well as recovery values (97.5%–98.22%) were found to be satisfactory.
Conclusion: A good correlation was obtained among the standard, samples of polyherbal formulation and fractionated extract of *O. gratissimum* using HPTLC method.

1. Introduction

Ursolic acid is one of the biologically active compounds in *Ocimum gratissimum*. Ursolic acid has been isolated from the leaves.[1]

Ursolic acid is well known for its hepatoprotective effects in both acute chemically induced liver injury and chronic liver fibrosis and cirrhosis. They are still used alone or in combination with other hepatoprotective ingredients as oral medications. The beneficial effects of triterpenic acid on the liver could be due to its antioxidant and anti-inflammatory actions, and their effects on drug metabolizing enzymes. These triterpenoids are effective inducers of metallothionein, a small cysteine-rich protein acting like glutathione in the body's defense against toxic insults.[2] Oleanolic and ursolic acid are position isomers shown in Fig. 1.

Ursolic acid shows pharmacological properties like anti-inflammatory, hepatoprotective, antitumor, anti-HIV, antimicrobial, antifungal, antiulcer, gastroprotective, hypoglycemic and anti-hyperlipidemic activity.[3–6]

O. gratissimum (Lamiaceae) is a perennial, woody shrub that is a herbal medicine which has been practiced worldwide and distributed throughout India.[7] Traditionally, it is used in the treatment of diarrhea,[8,9] as a febrifuge and integral component of anti-malaria remedies,[10] mosquito/insect repellant, stomachic and general tonic, antiseptic in wound dressing, skin infections, conjunctivitis and bronchitis. '*Ocimum* tea', is dispensed as a remedy for fever and diaphoresis,[9] roots are used as sedative for children.[11]

Unfortunately, insufficient information is available concerning the distribution of ursolic acid in the *O. gratissimum* Linn (Lamiacea). The present study involves the determination of ursolic acid in both *O. gratissimum* leaves extracts and developed hepatoprotective tablet.

2. Material and methods

2.1. Apparatus

HPTLC system (Linomat 5, Camag, Switzerland) automatic sample applicator, TLC scanner IV (Camag), flat bottom and twin-

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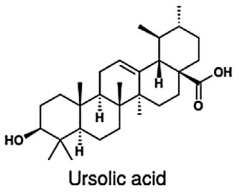

Fig. 1. Structure of ursolic acid.

trough developing chamber (15 × 10 cm), pre-coated silica gel aluminum plate (E. Merck, Darmstadt, Germany), electronic analytical balance, Shimadzu (AUX-220), micro syringe (100 μl) (Hamilton).

2.2. Reagents and standard

Ursolic acid was purchased from Yucca enterprises, Wadala, Mumbai and methanol AR grade from S.d. fine-Chem Ltd., Mumbai.

2.3. Plant materials

Polyherbal hepatoprotective tablet was prepared by using fractions obtained from alcoholic extracts of *Butea monosperma*, *Bauhinia variegata* stem bark and *O. gratissimum* leaves. All these ingredients were collected from Maliba Pharmacy College campus and were authenticated by Prof. Minoo H. Parabia, Department of Bioscience, Veer Narmad South Gujarat University, Surat. Voucher specimen (No: MPC/13032010/01, 02 and 03) has been deposited in the Department of Bioscience.

2.4. Extraction and fractionation

The powdered leaf of *O. gratissimum* was extracted with methanol at room temperature for seven days with shaking and stirring. Filtration was followed by evaporation of solvent using a rotary evaporator at low temperature and pressure. Crude methanolic extract was diluted with distilled water and the resultant mother solution was subjected to solvent–solvent partition using hexane, dichloromethane (DCM) and ethyl acetate (EtOAc).

2.5. Isolation of ursolic acid from polyherbal tablet

Weight accurately 500 mg equivalent of polyherbal tablet was transferred to the 10 ml volumetric flask and dissolved in 10 ml methanol. This solution was sonicated for 10 min and filtered through Whatman No. 1 paper to get solution containing 10 mg/mL. Concentrated solution obtained from polyherbal tablet was subjected to preparative TLC. Preparative TLC were performed on 20 cm × 10 cm TLC aluminum plate coated with 200 μm layer thickness of silica gel 60 F 254 using Petroleum ether:ethyl acetate:acetone (8.2:1.8:0.1, v/v/v) as mobile phase. After drying in a stream of warm air the plates were sprayed with 10% (v/v) sulfuric acid in ethanol, dried for 10 min and heated to 120 °C for 3 min. The quantification was carried out by densitometric scanning in absorbance mode at wavelength 530 nm. The silica in the respective marked area was scraped off and collected carefully in a test tube. The scraped silica, which contained the standard ursolic acid was extracted in methanol and filtered individually. The filtrates containing ursolic acid were evaporated under reduced pressure to obtain ursolic acid.

Melting point of ursolic acid was determined by open capillary method. Structural confirmation of the isolated ursolic acid was done by I.R. Spectroscopy and compared with that of standard.

2.6. Sample preparation

2.6.1. Preparation of standard solutions of ursolic acid

Stock solutions of ursolic acid were prepared by dissolving 20 mg ursolic acid in 100 ml of methanol (200 μg/mL). Standard solutions of concentration 400, 600, 800, 1000 and 1200 ng/ml were prepared by dilution of the stock solution with methanol.

2.6.2. Sample preparation from DCM and EtOAc fractions of O. gratissimum methanolic extract

Accurately weighed 100 mg of DCM and EtOAc fractions of *O. gratissimum* methanolic extract and polyherbal tablet were transferred to separate 10 ml volumetric flask and dissolved in 10 ml methanol. These solutions were sonicated for 10 min and filtered through Whatman No. 1 paper to get solution containing 10 mg/mL.

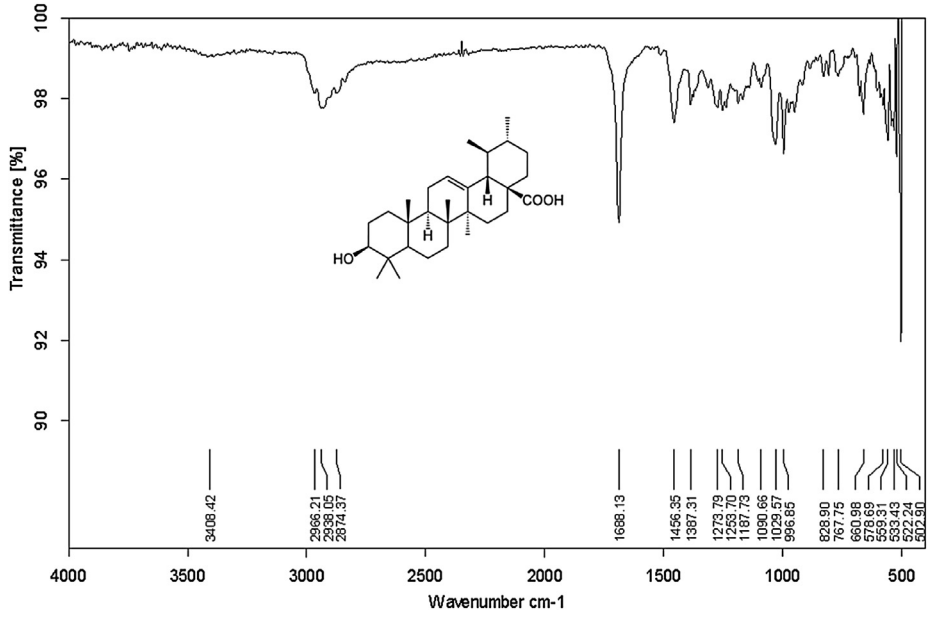

Fig. 2. IR spectrum of isolated compound (ursolic acid) from polyherbal tablet.

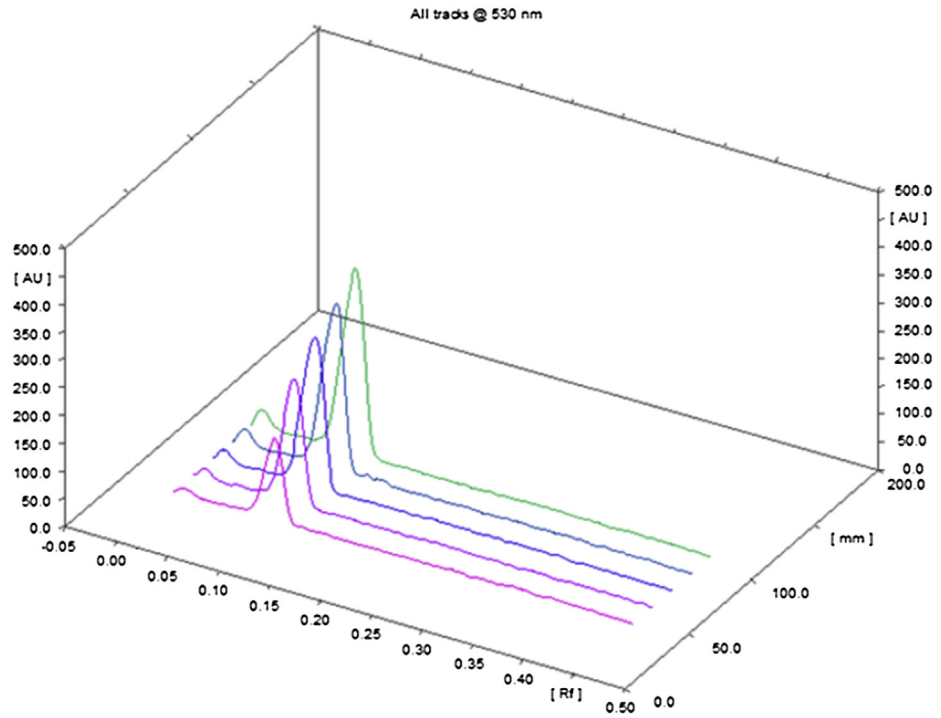

Fig. 3. 3D-Chromatogram of ursolic acid (200–1000 ng/spot).

2.6.3. Sample preparation from polyherbal tablet

Polyherbal tablet equivalent to about 100 mg of *O. gratissimum* extract was weighed and transferred to 50 ml volumetric flask containing 99.9% methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and supernatant was analyzed for ursolic acid content.[12]

2.7. Chromatographic conditions for ursolic acid

Pre-coated HPTLC plates 20 cm × 10 cm were washed with methanol and dried in a stream of hot air before use. Two micro litres of standard solutions, 10 μL of sample solutions were spotted using an automatic applicator as 6 mm long streaks [track distance: 15.4 mm, distance from the left edge: 15 mm] and allowed to dry.

2.8. Calibration curve of ursolic acid

Different volumes of stock solution (200 μg/mL) were spotted on the TLC plate to obtain concentration 400, 600, 800, 1000, 1200 ng/spot of ursolic acid.

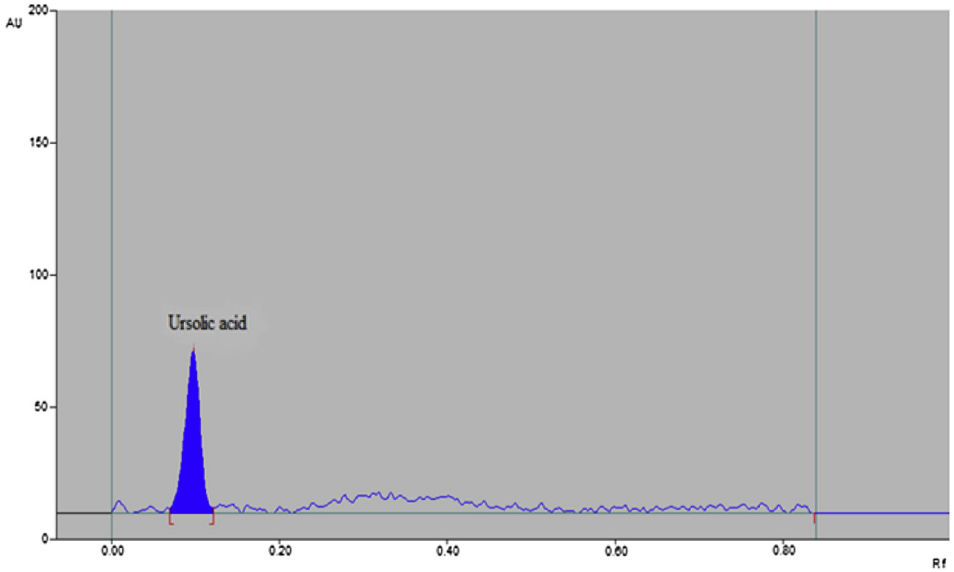

Fig. 4. Densitogram of ursolic acid (400 ng/spot) at 530 nm.

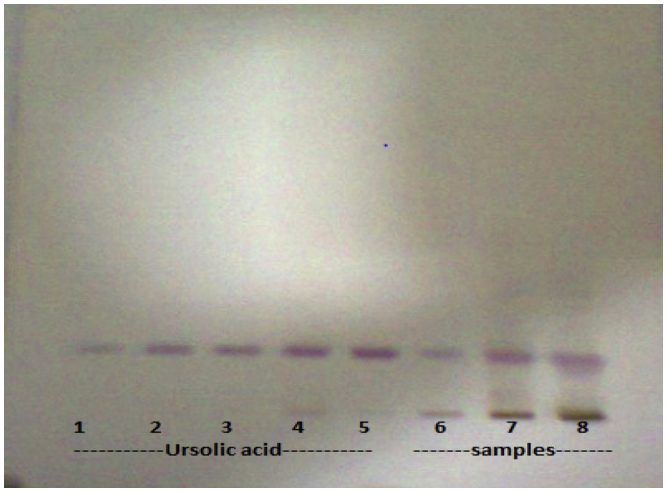

Fig. 5. Photograph of HPTLC plate: 1–5 (400–1200 ng/spot), calibration curve of ursolic acid, 6-polyherbal tablet, 7-dichloromethane, 8-ethyl acetate fractions of *Ocimum gratissimum* leaf.

2.9. Method validation

The proposed method was validated as per ICH guidelines.[13] Samples were prepared as per the earlier adopted procedure given in the experiment.

2.9.1. Linearity and range

Linearity is expressed in terms of correlation coefficient of linear regression analysis. The linearity response was determined by analyzing 5 independent levels of calibration curve in the range of 400–800 ng/spot of ursolic acid. The calibration curve of absorbance vs. concentration was plotted and correlation coefficient and regression line equations were determined.

2.9.2. Precision

Result of precision should be expressed as relative standard deviation (% R.S.D) or coefficient of variance (% C.V.).

2.9.2.1. Repeatability. Standard solutions were applied by Linomat 5 automatic sample applicator. Sample was spotted seven times for repeatability studies. The peak area obtained with each solution was measured and % C.V. was calculated.

2.9.2.2. Intraday precision. Mixed solution containing 600–1000 ng/spot of ursolic acid was analyzed three times on the same day and % C.V. was calculated.

2.9.2.3. Interday precision. Mixed solution containing 600–1000 ng/spot of ursolic acid was analyzed on three different days and % C.V. was calculated.

2.9.3. Accuracy

It was determined by calculating the recovery of ursolic acid by standard addition method.

Table 1
Linearity data for ursolic acid.

Ursolic acid (ng/spot)	Peak area
400	1205.47
600	2506.37
800	3904.28
1000	5126.22
1200	6122.73

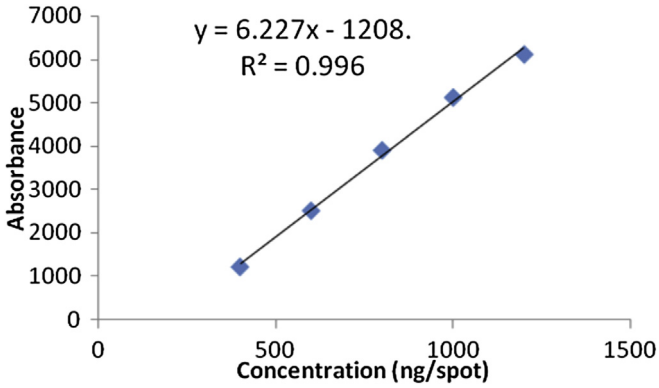

Fig. 6. Calibration curve for ursolic acid (400–1200 ng/spot).

Table 2
Repeatability data for estimation for ursolic acid.

Concentration (ng/spot)	Peak area
1000	5067.12
1000	5100.13
1000	5125.6
1000	5078.4
1000	5122.8
1000	5213.8
1000	5241.5
Average	5135.62 ± 66.84
% C.V.	1.30

2.9.4. Recovery studies

The accuracy of the method was established by performing recovery experiments at three different levels using the standard addition method. In 1 μL (1 μg/mL) of sample, known amounts of ursolic acid (400, 600, 800 ng per spot) standards were added by spiking. The values of percent recovery and average value of percent recovery for ursolic acid were calculated.

2.9.5. Limits of detection and limit of quantitation

The LOD and LOQ were estimated from the set of five calibration curves. The LOD and LOQ calculated as

$$LOD = 3.3 \times (SD/Slope)$$

$$LOQ = 10 \times (SD/Slope)$$

where,

SD = Standard deviation of the Y-intercepts of the five calibration curves.

Slope = Mean slope of the five calibration curves.

Table 3
Intraday precision data for estimation for ursolic acid.

Concentration of ursolic acid (ng/spot)	Peak area (±S.D.)	% C.V.
600	2455.59 ± 48.44	1.97
800	3884.37 ± 33.83	0.87
1000	5096.53 ± 29.82	0.58

Table 4
Interday precision data for estimation for ursolic acid.

Concentration of ursolic acid (ng/spot)	Peak area (±S.D.)	% C.V.
600	2551.84 ± 56.70	2.22
800	3904.55 ± 61.37	1.57
1000	5137.16 ± 75.38	1.46

Table 5
Recovery study for ursolic acid.

Concentration of ursolic acid in sample (ng/spot)	Amount of ursolic acid standard added (ng/spot)	Total concentration (ng/spot)	Mean concentration recovered (ng/spot)	% Recovery	% Recovery mean
200	200	400	198	99	97.50
200	200	400	195	97.5	
200	200	400	192	96	
200	400	600	387	96.75	98.16
200	400	600	396	99	
200	400	600	395	98.75	
200	600	800	582	97	98.22
200	600	800	596	99.33	
200	600	800	590	98.33	

Table 6
LOD and LOQ data for ursolic acid.

Mean slope (S)	6.227
S.D. of intercept (S)	−1208.
LOD (ng/spot)	5.99
LOQ (ng/spot)	18.17

Table 7
Estimation of ursolic acid in fractionated extracts of *Ocimum gratissimum* and tablet formulation.

Tracks	Rf values	Conc. (ng/10 mg)
Ursolic acid standards	0.10	–
Ursolic acid from EtOAc fraction of *Ocimum gratissimum*	0.10	399.55
Ursolic acid from DCM fraction of *Ocimum gratissimum*	0.10	372.66
Ursolic acid in polyherbal tablet (PTF)	0.11	255.14

3. Results and discussion

3.1. Identification of isolated compound from polyherbal tablet

Oleanolic and ursolic acids are position isomers.[14] The IR spectra of standard oleanolic acid and isolated compound from polyherbal tablet are displayed in Fig. 2 and were found to be comparable. Melting point of isolated compound was found to be 288 °C. IR spectra of isolated compound appears as a very intense absorption ribbon, around 3425 cm^{-1} due to OH group. A very intense absorption ribbon in between 2874 and 2966 cm^{-1}. At 1688 cm^{-1} appears a characteristic peak of carbonyl group (C═O). At 1456 cm^{-1} appears absorption ribbon from OH vibrations of planar distortion. At 1387 cm^{-1} appears a characteristic ribbon, which derives from C–H stretch and at 1108 cm^{-1} stretching vibrations of C–O group of carbonic acid. In this I.R. spectrum of isolated compound (ursolic acid) shown in Fig. 2, skeleton ring values are found to match with the reported values of oleanolic acid.[15]

3.2. Optimization of mobile phase

Various ratios of solvents were tried as a mobile phase and optimum mobile phase was selected was petroleum ether:ethyl acetate:acetone (8.2:1.8:0.1 v/v/v). This mobile phase allowed good resolution, dense, compact and well-separated spots at Rf value 0.10. Wavelength 530 nm for ursolic acid was used for quantification of the drug. Since there is only one peak seen, is shown in Fig. 3.

3.3. Quantification by HPTLC

3.3.1. Method development

In HPTLC chromatogram, all tracks for standard ursolic acid at wavelength 530 nm are shown in Figs 3 and 5. The Rf value of standard ursolic acid was found to be 0.10 and peak area was 2506.37 (Fig. 4).

3.3.2. Method validation

3.3.2.1. Linearity and range. Linearity was determined for ursolic acid at five concentration levels. The linearity was in the range 400–1200 ng/spot. Linearity data are depicted in Table 1 and Fig. 6. Correlation coefficient for calibration curve was 0.9960 and regression line equation for ursolic acid was $y = 6.227x - 1208$.

3.3.2.2. Precision. Precision considered at three level repeatability, interday and intraday precision.

3.3.2.2.1. Repeatability. The data for repeatability are shown in Table 2. The % C.V. for repeatability was found to be 1.30.

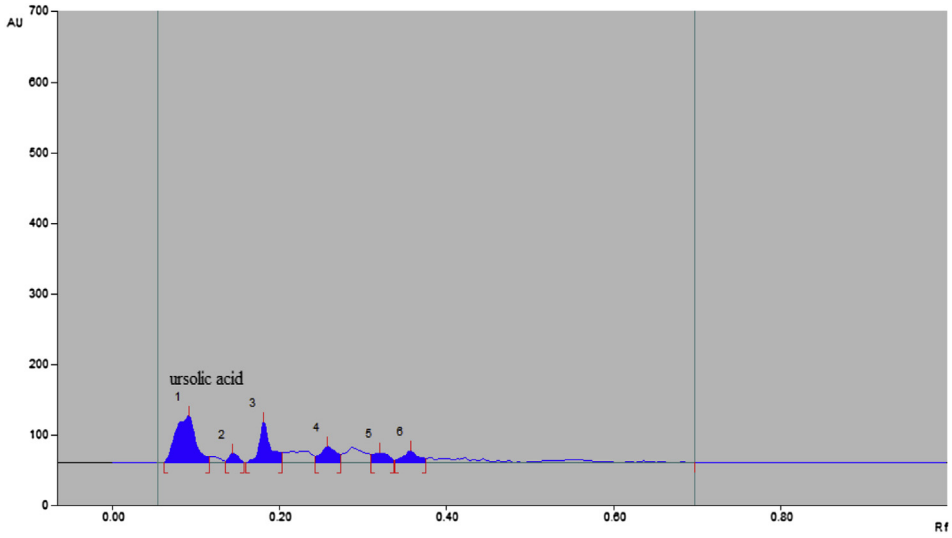

Fig. 7. Chromatogram of ethyl acetate fractions of *Ocimum gratissimum*.

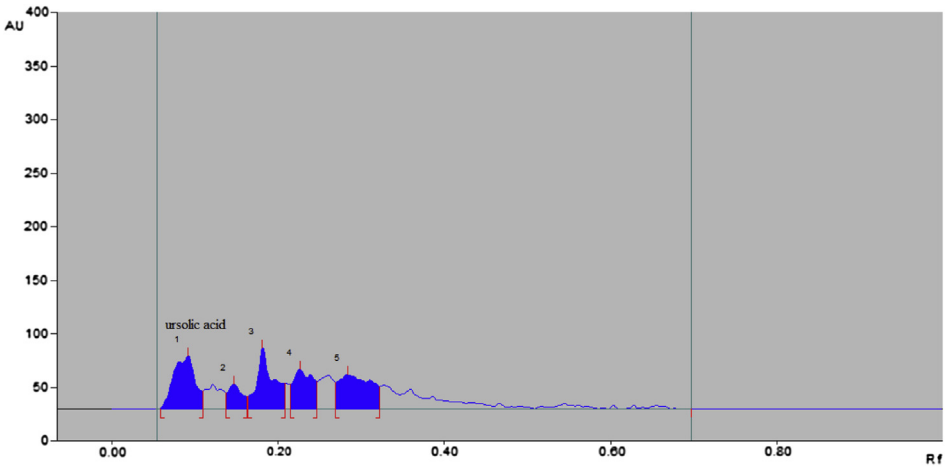

Fig. 8. Chromatogram of DCM fractions of *Ocimum gratissimum*.

3.3.2.2.2. Intraday precision. The data for intraday precision for ursolic acid are shown in Table 3. The % C.V. was found to be 0.58–1.97.

3.3.2.2.3. Interday precision. The data for interday precision for ursolic acid is shown in Table 4. The % C.V. for interday precision was found to be 1.46–2.22.

3.3.2.3. Accuracy. Accuracy of the method was confirmed by recovery at three level of standard addition. Percentage recovery for ursolic acid from DCM and ethyl acetate fractions of *O. gratissimum* and polyherbal hepatoprotective tablet was given in Table 5.

3.3.2.4. LOD and LOQ. Limit of detection and quantitation were determined by equation – LOD = 3.3 × (S/S) and LOQ = 10 × (S/S). LOD and LOQ results are shown in Table 6.

3.3.3. Estimation of ursolic acid in fractionated extracts of O. gratissimum and tablet formulation

The peak purity of ursolic acid from standard was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot. Good correlation (r^2 = 0.9960) was obtained between the standard and the samples of ursolic acid in the range 400–1200 ng/spot.

The identification of ursolic acid was done on the basis of Rf values. The concentrations of ursolic acid found using the presented method were found to be 399.55, 372.66 and 255.14 ng/10 mg in fractionated leaf extract of ethyl acetate fraction, dichloromethane fraction of *O. gratissimum* and polyherbal formulation respectively. The results are shown in Table 7.

Ethyl acetate fraction of *O. gratissimum* also showed six peaks, first peak of Ethyl acetate fraction Rf value 0.10 coincided with standard Rf value with peak area 1280 of standard (Fig. 7). The concentration of ursolic acid in Ethyl acetate fraction of *O. gratissimum* was found to be 399.55 (ng/10 mg).

Dichloromethane fraction also showed five peaks, the first peak with Rf value 0.10 coincided with standard Rf value with peak area was 1110 (Fig. 8). The concentration of ursolic acid in DCM fraction of *O. gratissimum* was found to be 372.66 (ng/10 mg).

Polyherbal tablet of PTF-2 formulation showed seven peaks. The Rf value (0.10) of third peak is coinciding with standard Rf value and its peak area was 935 (Fig. 9). The concentration of ursolic acid was found to be 344.53 (μg/10 mg).

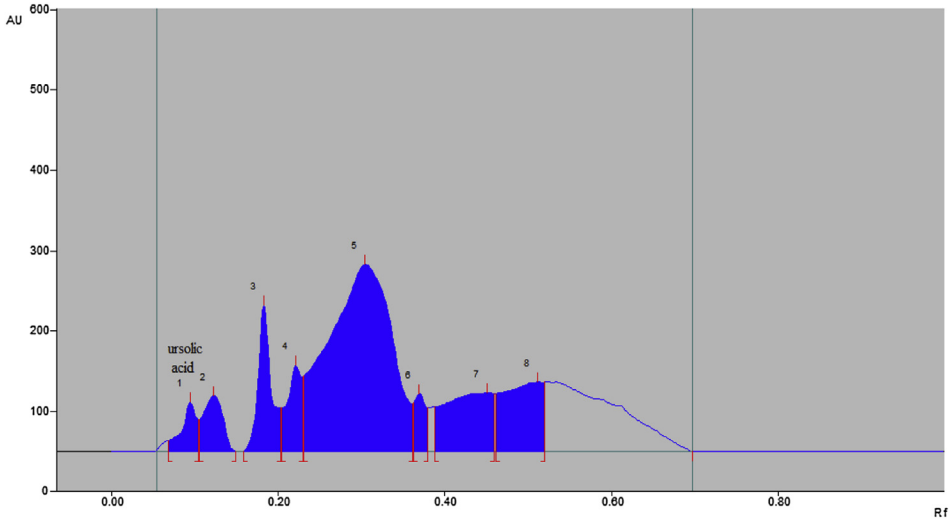

Fig. 9. HPTLC densitogram of polyherbal tablet showing ursolic acid.

Table 8
Summary of validation parameters.

Parameters	Result for ursolic acid
Linearity range	400–1200 (ng/spot)
Correlation coefficient	0.9960
Precision (% C.V.)	
Repeatability ($n = 7$)	1.30
Intraday precision ($n = 3$)	0.58–1.97
Interday precision ($n = 3$)	1.46–2.22
Accuracy (% recovery)	97.5–98.22
LOD (ng/spot)	5.99
LOQ (ng/spot)	18.17

3.3.4. Summary of validation parameters

The detailed summary of validation parameters is described in Table 8.

4. Conclusion

A good correlation was obtained among the standard, samples of polyherbal formulation and fractionated extract of *O. gratissimum*. An HPTLC method for quantitative estimation of ursolic acid present in fractionated leaf extract of *O. gratissimum* and polyherbal tablet has been developed and validated. The method can be used as an ursolic acid standard.

Conflicts of interest

All authors have none to declare.

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