

# Estimation of ursolic acid from *Urtica dioica* L. using validated HPTLC method

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## ABSTRACT

*Urtica dioica* L. (Urticaceae, leaves) is commonly used in traditional systems of medicine for the treatment of a wide range of disorders. The present work emphasizes on a validated HPTLC method for estimation of ursolic acid from *U. dioica* leaves and its available formulation. Chromatographic separation was achieved on silica gel 60 F<sub>254</sub> TLC plate with toluene: ethyl acetate: formic acid (7:3:0.1, v/v/v) as a mobile phase. Detection of ursolic acid was carried out by derivatizing the plate with Liebermann Burchard reagent at 110°C for 10 min. Camag TLC scanner 4 equipped with winCATS software was used for densitometric scanning at 366 nm. The accuracy of the method was checked by conducting various validation parameters according to ICH guidelines. The method was found applicable to evaluate the impact of regional variation on ursolic acid content in *U. dioica* leaves. The research also highlights estimation of ursolic acid from a marketed herbal formulation of *U. dioica* leaves. The described HPTLC method was found useful for quantitation of bioactive marker ursolic acid and can be used as a routine quality control tool for the assessment of botanicals.

## INTRODUCTION

*Urtica dioica* L. (Urticaceae) is a traditional Ayurvedic herb known as *Vrishchhiyaa-shaaka* (Khare, 2007). *U. dioica* L. is a common Himalayan species which produces allergenic substances causing oedema and inflammation in humans. The plant has become a source of folk medicine for the management of a wide spectrum of ailments (Ozen and Korkmaz, 2003). Leaves of the plant have many medicinal properties and have been used for hundreds of years in world traditional medicine for treating diseases such as eczema, sexual disorders, rheumatism, diabetes, hyperlipidemia, anaemia, alopecia, nephritis, jaundice, menorrhagia, immune disorders, etc (Kataki *et al.*, 2012; Bnouham *et al.*, 2003; Khare, 2007; Tahri *et al.*, 2000; Khare *et al.*, 2012). Leaves are reported to possess linolenic acid, lutein, neoxanthin, violaxanthin, lycopene,  $\beta$ -sitosterol, rutin, quercetin, ursolic acid, etc (Wetherilt, 1992). Literature survey reveals that, chromatographic characterization of *U. dioica* leaves (in terms of

ursolic acid content) using validated HPTLC method has not been reported so far. Based on the therapeutic activities of ursolic acid (Figure 1, a pentacyclic triterpenoid carboxylic acid) like anti-inflammatory, antitumor, antibacterial, antifungal, hepato-protective, hypoglycemic etc (Liu, 1995), it was used as a marker to evaluate the quality of *U. dioica* leaves using a validated HPTLC method. Although ursolic acid is not a plant specific marker, it was chosen for its proven therapeutic efficacy against various ailments for the quality evaluation of *U. dioica* leaves.

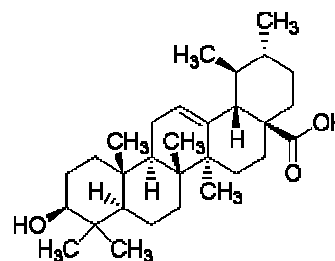


Fig. 1: Structure of Ursolic acid

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## MATERIALS AND METHODS

### Collection of plant materials and processing

Plant material collected from Pauri Garhwal, Uttarakhand was authenticated by NISCAIR, New Delhi (Auth 6546) and a voucher specimen was deposited for future reference. In order to study the impact of regional variation on ursolic acid content, the samples were also collected from two other geographical regions of India like Srinagar and Nainital (Uttarakhand). Samples were shade dried, powdered, sieved through BSS sieve (85 mesh) and stored in air-tight containers. A marketed herbal formulation of *U. dioica* leaves was purchased from local market.

### Chemicals and reagents

Analytical grade solvents were procured from Merck Specialities Pvt. Ltd., Mumbai. Ursolic acid (purity  $\geq 98.5\%$ ) was procured from Sigma-Aldrich Private Limited, India.

### Chromatographic characterization

#### Extraction of phytochemical constituents from *U. dioica* samples

Accurately weighed (0.25 g) powdered sample was extracted with methanol (10.0 mL). The sample was vortexed for 1-2 min, kept standing overnight at room temperature and filtered through Whatmann filter paper No. 1 (E. Merck, India). The filtrate was subjected to HPTLC analysis. Similar extraction procedure was followed for plant samples collected from different regions of India and herbal formulation of *U. dioica*.

#### Preparation of standard stock solution, calibrant and quality control samples

Stock solution of ursolic acid (1000.0  $\mu\text{g/mL}$ ) was prepared in methanol. Seven calibrant samples ranging from 5.0-100.0  $\mu\text{g/mL}$  and three quality control samples of ursolic acid namely low, mid and high (6.5, 25.0, 80.0  $\mu\text{g/mL}$  respectively) were prepared in methanol using the stock solution.

#### Optimized chromatographic conditions for estimation of ursolic acid from *U. dioica* samples

The HPTLC system used consisted of CAMAG TLC Scanner 4 supported by winCATS software version 1.4.7 equipped with CAMAG Linomat 5 sample spotter and CAMAG Reprostar 3 system for photo-documentation. A Denver analytical balance (Goettingen, Germany) was used to weigh standard and samples. Chromatographic separation of the phytochemical constituents was achieved on TLC plate (E. Merck) pre-coated with silica gel 60 F<sub>254</sub> (0.2 mm thickness) on aluminium sheet support.

For separation of ursolic acid from *U. dioica* samples, the samples (10.0  $\mu\text{L}$  each) along with the standard ursolic acid (10.0  $\mu\text{g/mL}$ ) were spotted on TLC plate as bands (8.0 mm wide) and at a distance of 15.0 mm from the edges under similar instrumental conditions. Plate was developed up to a distance of 85.0 mm in CAMAG twin trough glass chamber pre-saturated with mobile phase toluene: ethyl acetate: formic acid (7: 3: 0.1, v/v/v) for 15

min. After development, the plate was dried in a current of air at room temperature. The plate was derivatized using Liebermann Burchard reagent and dried in an oven preset at 110 °C for 10 min. For densitometric scanning, the source of radiation used was mercury lamp (366 nm). All measurements were performed at 22  $\pm$  1°C. Plate was photodocumented at 366 nm.

#### Estimation of ursolic acid from *U. dioica* samples

Relative response for the characteristic band of ursolic acid in *U. dioica* samples and its formulation was obtained and the content of ursolic acid in each sample was determined using the regression equation obtained through regression analysis of calibration curve.

### Method validation

The HPTLC method for estimation of ursolic acid was validated as per ICH guidelines (Shailajan *et al.*, 2012) for the parameters like sensitivity, linearity, precision, recovery, specificity and ruggedness.

### Statistical analysis

Microsoft Excel-2007 was used for the statistical evaluation of results.

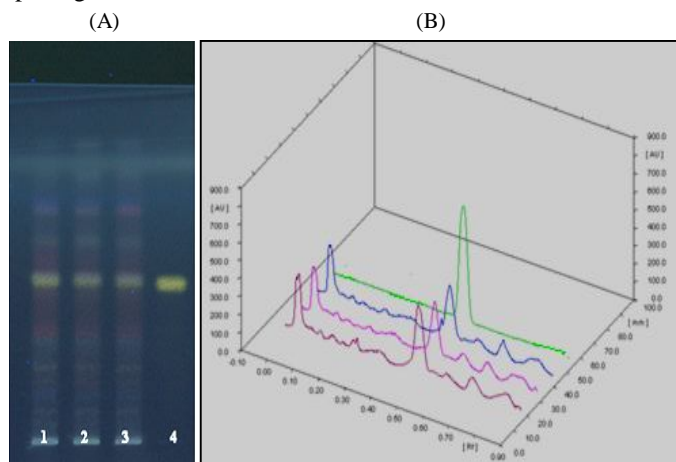
## RESULTS AND DISCUSSION

HPTLC methods are commonly applied for the identification, assay or content uniformity of herbal raw materials and their formulations (Shailajan *et al.*, 2012; 2013a; 2013b). The therapeutic benefits of *U. dioica* leaves are largely based on folkloric rather than scientific evidences (Khare, 2007; 2012). Considering the biological importance of this plant, a rapid, simple and accurate HPTLC densitometric method was developed for quantitation of ursolic acid from *U. dioica* leaves.

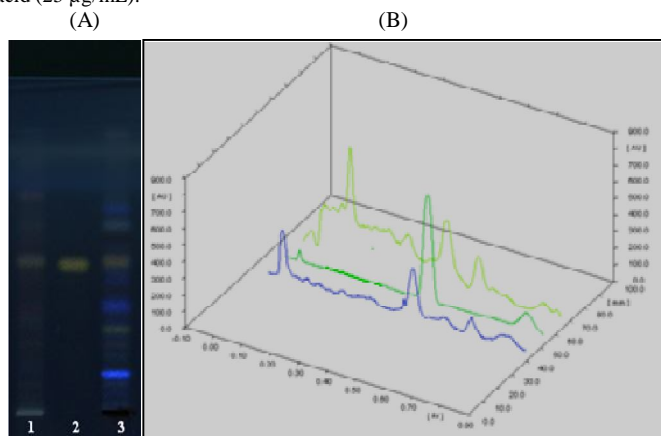
Of the various solvent systems tried, mixture containing toluene: ethyl acetate: formic acid (7:3:0.1, v/v/v) gave the best resolution of ursolic acid ( $R_f = 0.45$ ) from the other components present in the methanolic extract. The identity of band of ursolic acid in plant matrix was confirmed by comparing the  $R_f$  values and colour of the characteristic band with that of the standard ursolic acid. The plate photo and overlay of the chromatograms of *U. dioica* collected from different geographical regions of India with ursolic acid is shown in Figure 2A and B. Figure 3A and B shows the plate photo and overlay of the chromatograms of *U. dioica* leaves (collected from Srinagar) and its formulation along with the standard ursolic acid.

The method was validated as per ICH guidelines and was found to be linear over the range of 5.0-100.0  $\mu\text{g/mL}$  of the standard. The method was found to be precise during intra-day and inter-day precision studies. The method was also found to be sensitive with limit of detection and limit of quantification values 2.5 and 5.0  $\mu\text{g/mL}$  respectively. The average recovery for quality control samples of ursolic acid was found to be 99.07% (Table 1). The method was also found rugged for the parameters like change

in analyst, change in mobile phase composition and change in spotting volume.



**Fig. 2:** HPTLC plate photo (A) and overlay of the chromatograms (B) of *U. dioica* collected from different geographical regions of India with ursolic acid at 366 nm. Track details: 1) Pauri garhwal, 2) Srinagar, 3) Nainital, 4) Ursolic acid (25 µg/mL).



**Fig. 3:** HPTLC plate photo (A) and overlay of the chromatograms (B) of *U. dioica* leaves and its formulation with ursolic acid at 366 nm. Track details: 1) sample from Srinagar, 2) Ursolic acid (25 µg/mL), 3) Formulation containing *U. dioica* leaves.

**Table 1:** Results of method validation parameters for estimation of ursolic acid using HPTLC technique.

Parameters	Results
LOD and LOQ (µg/mL)	2.5 and 5.0
Linearity (µg/mL)	5.0 – 100.0
Regression equation	$y = 30.80x + 132.50$
Mean coefficient of determination ( $r^2$ )	0.999
<b>System suitability (% CV, n = 6)</b>	
$R_f$	0.90
Area	0.37
<b>Precision (% CV, n = 3)</b>	
Intra day	0.31-0.93
Inter day	0.09-1.35
<b>Recovery using QC samples (% Mean ± SD, n = 3)</b>	
Low	98.55
Mid	99.04
High	99.62
<b>Stability</b>	
Standard Stock Solution short term stability (For 12 h)	Stable at ( $4 \pm 1^0\text{C}$ )
Standard Stock Solution long term stability (For 15 days)	Stable at ( $4 \pm 1^0\text{C}$ )
Bench top stability (For 6 h)	Stable at ( $22 \pm 2^0\text{C}$ )
Ruggedness	Rugged

Using regression equation ( $y = 30.80x + 132.5$ ), the exact content of ursolic acid was determined from different samples of *U. dioica* (Table 2). Method was applied to determine the ursolic acid content in *U. dioica* leaves collected from different geographical regions. Sample collected from Srinagar had the maximum content of ursolic acid whereas sample collected from Pauri garhwal had the least. This clearly indicates that different geographical locations have significant effect on the content of phytochemical constituents. The impact of regional variation on the ursolic acid content of *U. dioica* leaves was clearly evident from HPTLC analysis and the results were in compliance with the recently published reports (Shailajan *et al.*, 2012; 2013a; 2013b). Method was also found applicable to determine the content of ursolic acid from a marketed herbal formulation of *U. dioica* leaves. The ursolic acid content in the formulation was found to be  $0.150 \pm 0.02$  mg/g.

**Table 2:** Ursolic acid content in the fruits of *Urtica dioica* collected from different geographical regions of India.

Regions	Content of ursolic acid in mg/g
Srinagar	$0.140 \pm 0.022$
Pauri garhwal	$0.125 \pm 0.031$
Nainital	$0.133 \pm 0.045$

Values are Mean  $\pm$  S.D., n=3.

Thus, the developed HPTLC method was found to be suitable for the quantification of ursolic acid in *U. dioica* samples. With the growing demand of herbal drugs in the market and with the increased belief in the usage of herbal drugs, the developed method can be used as a powerful quality control tool for botanical identification of this plant and to detect adulterants in plant raw materials (if any). On the basis of the concentration of ursolic acid, leaves of *U. dioica* can be selected from a region which gives maximum content and this may be supported by efficacy studies. Multiple samples must be analyzed from a region showing maximum content of bioactive marker in order to study the intra-regional variations.

## CONCLUSION

Results of the current study could be used by industries for the characterization of *U. dioica* samples and its formulation in order to check their uniformity. Using such validated methods, *U. dioica* leaves with precise quality can be encouraged in herbal industries. With the growing demand of herbal drugs, this standardization tool will help in maintaining the quality and batch to batch consistency of this medicinally important plant. The proposed HPTLC method for quantitative monitoring of ursolic acid in *U. dioica* leaves is rapid, simple, accurate, selective, and economic which can be used for routine quality testing. The method can also be useful for quality evaluation of some other plants containing ursolic acid as a marker.

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