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Estimation of ursolic acid and oleanolic acid from leaves of *Plumeria obtusa* by HPTLC method after iodine derivatization

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ABSTRACT

Ursolic acid and Oleanolic acid are an anti-cancer and anti-inflammatory pentacyclic triterpenoids, an active constituent of plant Plumeria obtusa. Ursolic acid and Oleanolic acid content in Plumeria obtusa has not been reported till date. Thus HPTLC method was developed for quantification of Ursolic acid and Oleanolic acid in leaves of Plumeria obtusa. The separation of Ursolic acid and Oleanolic acid were performed on aluminum backed silica gel G 60 F_{254} as stationary phase. Mobile phase system optimized was Hexane: Ethyl acetate: Methanol (8.2: 1.8: 0.5 v/v/v) with pre-saturation time of 45 min. Spectrophotometric evaluation was performed at 540 nm. Ursolic acid and Oleanolic acid are chemical isomers and they not separated as such. Ursolic acid and Oleanolic acid were resolved at R_f 0.28 ± 0.03 and 0.41 ± 0.03 respectively by pre chr omatographic derivatization with iodine. The polynomial regression of method was found to be within range 400-1400 ng/spot with mean percentage recovery of 97.59 ± 0.99% for ursolic acid and 1200-4200 ng/spot with mean percentage recovery of 96.61 ± 0.83% for Oleanolic acid. The amount of Ursolic acid and Oleanolic in leaves were found to be 0.197 % and 0.0253 % respectively.

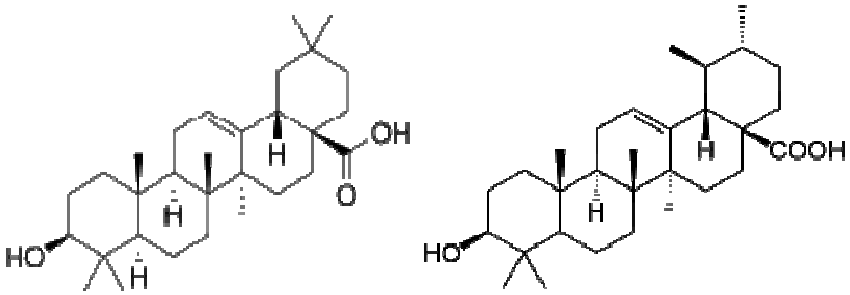
Keywords: Ursolic acid, Oleanolic acid, *Plumeria obtusa*, HPTLC method.

INTRODUCTION

Plumeria obtusa belongs to family Apocynaceae. Locally it called as Frangipani or Champa. Indigenous to Central America, Southern Mexico, Southest Asia. *Plumeria obtusa.* The leaves are dark green in color and glossy. Leaves are elliptical and obovate. Apex is rounded. Flowers are white in color with yellow central portion and rounded obovate petals. The seeds produce the white, red, pink, yellow and multicolored blooms. You can recognize Plumeria seed by locating a pod that splits open on the tree [1].

The major chemical constituents reported from *P. obtusa* are Ursolic acid, Oleanolic acid, betulinic acid, neriocoumaric acid, obtusalin, obtusin, obtusic acid, obtusidin,α-amyrin, plumieride coumarate, isoplumieride, plumericin, kaneroside, oleandrin, fulvoplumerin [2,3]. Methanolic extract of leaves shows significant anti-inflammatory, antioxidant, Free Radical Scavenging Activities and antimicrobial properties. Methanolic extract of bark shows significant anti tumor activity [4].

Ursolic acid and Oleanolic acid showed anti-inflammatory, hepatoprotective, antihyperlipidemic and anti cancer [5-7]. Content of Ursolic acid and Oleanolic acid not reported till date in *Plumeria obtusa*. So, quantification of Ursolic acid and Oleanolic acid in Plumeria obtusa performed by HPTLC method.



Oleanolic acid **Ursolic acid**

MATERIALS AND METHODS

Materials

(a) *Plant material* – The leaves of Plumeria obtusa collected from L. M. College of pharmacy botanical garden, Ahmedabad, Gujarat, India. The samples were authenticated by botanist. The sample was dried at room temperature in shade and stored at 25 ^{0}C in air tight container and powdered to 40 meshes whenever required.
(b) *Standard compound* – Gifted by Dr. Sindhu Ezhava, L. M. College of Pharmacy.
(c) *Chemicals* – All chemicals used were analytical grade.

Apparatus

(a) *Spotting device* – Linomat IV automatic sample spotter (camag, Switzerland)
(b) *Syringe* – 100 µL (Hamilton)
(c) *TLC Chamber* – Glass twin trough chamber for 10 × 10 cm plates (Camag)
(d) *Scanner* – TLC Scanner 3 linked to cats4 software (Camag)
(e) *HPTLC plates* - TLC Aluminum sheets pre-coated with silica gel 60 F_{254} 20×20 cm^2 layer thickness- 0.2 mm (Merck, Germany)

Detection Method

(a) *Prechromatographic derivatization* [8]
The plates were developed in glass chamber with 1% iodine solution in chloroform to a distance of 1.2 cm, plate was removed and the start zone was covered by aluminium foil and the plates were placed in dark for 10 min. When the reaction was complete, the plates were dried in a stream of warm air to remove the excess of iodine.
(b) *Derivatization* [9]
After development, Plate was dried in stream of hot air and was derivatized with 1% Methanolinc sulphuric acid solution. Then it was heated for 5 minutes at 110 ^{0}C in Hot air oven and plates are analyzed.

Sample solution

➢ An accurately weighed fine *Plumeria obtusa* leaves powder (1 gm) was transferred to 250 mL RBF. Petroleum ether (20 mL × 3) was added and the mixture was refluxed for 2 hr at 70 °C on constant temperature water bath. Filtered through Whatman filter paper no.42. The marc was dried. It was refluxed with Methanol (20 mL × 3) 3 hr at 80 °C on constant temperature water bath. Filtered through Whatman filter paper no.42. The filtrate was concentrated upto 10 mL.

Standard solutions

Preparation of stock solution of Ursolic acid
➢ Accurately weighed Ursolic acid (2 mg) was transferred to 10 mL volumetric flask, dissolved in and diluted with Methanol up to the mark (200µg/mL).
Preparation of stock solution of Oleanolic acid
➢ Accurately weighed Oleanolic acid (2 mg) was transferred to 10 mL volumetric flask, dissolved in and diluted with Methanol up to the mark (200µg/mL).
Preparation of working standard
➢ Stock solution of Ursolic acid (2 mL) and stock solution of Oleanolic acid (6 mL) was transferred to a 10 mL volumetric flask and diluted with Methanol up to the mark. (Ursolic acid 40 µg/mL and Oleanolic acid 120 µg/mL)

Preparation of calibration curve of Ursolic acid and Oleanolic acid

Standard solutions were applied (6mm, distance between the tracks, 12mm) on HPTLC plate using Linomat IV as following (*Table-1*). Pre chromatographic derivatization performed as above. The plate was developed in twin trough chamber with mobile phase Hexane: Ethyl acetate: Methanol (8.2:1.8:0.5 v/v/v) for a distance 8.0 cm at 25 ± 2 ^{0}C and 40% relative humidity. The plate wad dried at room temperature in air and derivatized with 1%

Methanolinc sulphuric acid solution. Then it was heated for 5 minutes at 110 ^{0}C in Hot air oven and scanned at 540 nm in Absorption/Reflection mode using tungsten lamp. The calibration curves of Ursolic acid and Oleanolic acid were obtained by plotting peak areas vs applied concentration.

Table – 1 calibration curve for Ursolic acid and Oleanolic acid

Standard Id	Volume (µL) used for spotting	Concentration of Ursolic acid (ng/spot)	Concentration of Oleanolic acid (ng/spot)
S1	10	400	1200
S2	15	600	1800
S3	20	800	2400
S4	25	1000	3000
S5	30	1200	3600
S6	35	1400	4200

Quantification

Quantification of Ursolic acid

3 µL of sample was applied in duplicate on an HPTLC plate along with calibration curve (*figure – 1).* The plate was developed and scanned as described above.

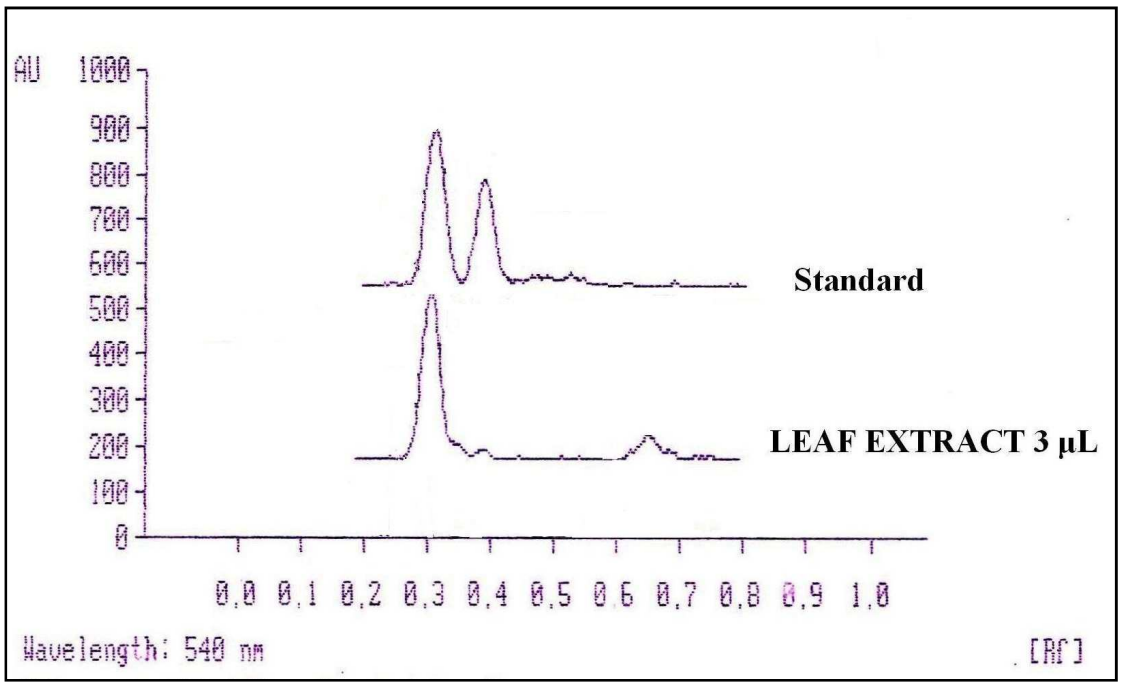


Figure - 1 Chromatogram for Quantification of Ursolic acid from Leaf extract of Plumeria obtuse

Quantification of Oleanolic acid

50 µL of sample was applied in duplicate on an HPTLC plate along with calibration curve (*figure – 2*). The plate was developed and scanned as described above.

Validation of the Method [10]

ICH Guidelines were followed for the validation of the analytical method. The method was validated for precision, repeatability, linearity and accuracy. Instrumental precision for scanner was checked by repeated scanning of same spot of Ursolic acid (1400 ng/spot) and Oleanolic acid (4200 ng/spot) and expressed as relative standard deviation (RSD). Instrumental precision for spotter was checked by spotting Standard solution of Ursolic acid (400 ng/spot) and Oleanolic acid (1200 ng/spot) on precoated TLC plate six times. The areas of six spots were measured and results were expressed as RSD. Variability of method was studied by analyzing standard solution containing 400, 1000 and 1400 ng/spot Ursolic acid and 1200, 3000, 4200 ng/spot of Oleanolic acid on same day (intraday precision) and on three different days (interday precision) and results were expressed as RSD. Limit of Detection (LOD) and Limit of Quantification (LOQ) were evaluated by applying different dilutions of standard solutions. Accuracy of the method was assessed by performing recovery studies at 3 different levels (approximately 50, 100 and 150% addition of Ursolic acid and Oleanolic acid). The recoveries and average recoveries were calculated.

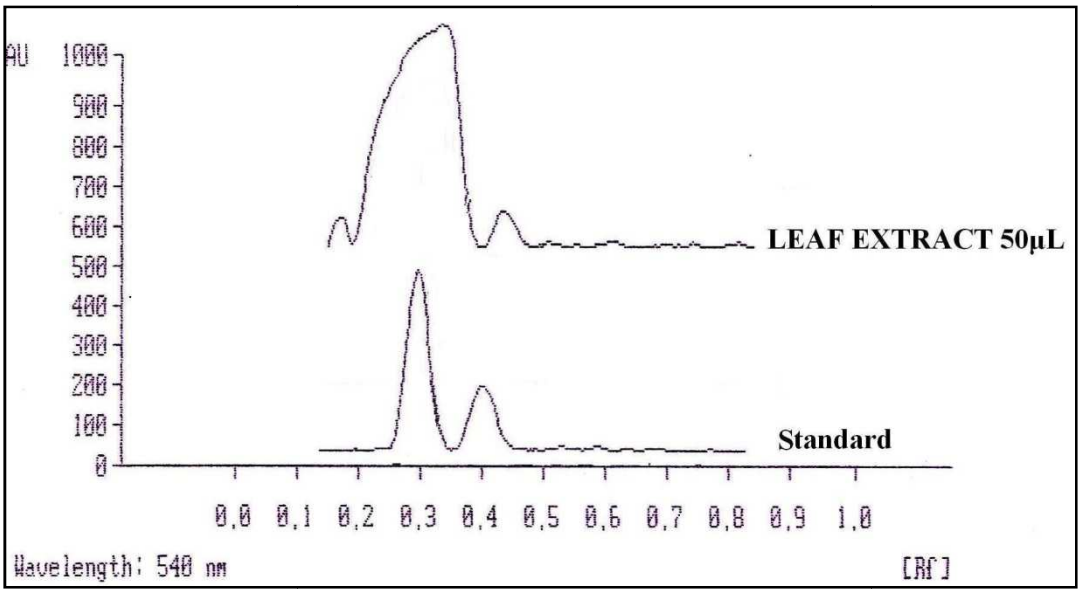


Figure – 2 Chromatogram for Quantification of Oleanolic acid from Leaf extract of Plumeria obtusa

RESULTS AND DISCUSSION

We developed a new method for quantification of Ursolic acid and Oleanolic acid with pre chromatographic derivatization with iodine. The optimized mobile phase resolved with following R_f values: Ursolic acid (0.28) and Oleanolic acid (0.41) *figure-3*.

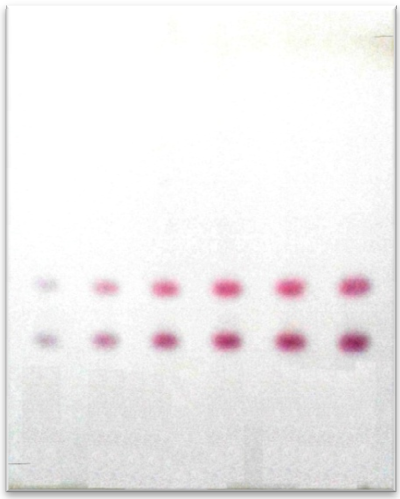


Figure – 3 TLC plate showing resolution between standard Ursolic acid (R_f = 0.28) and Oleanolic acid (R_f = 0.41)

The identity of the bands of Ursolic acid (figures 4A and 4B) and Oleanolic acid (figures 5A and 5B) in sample extract were confirmed by R_f value and comparing spectra with reference standard using camag TLC Scanner 3 with cats4 software.

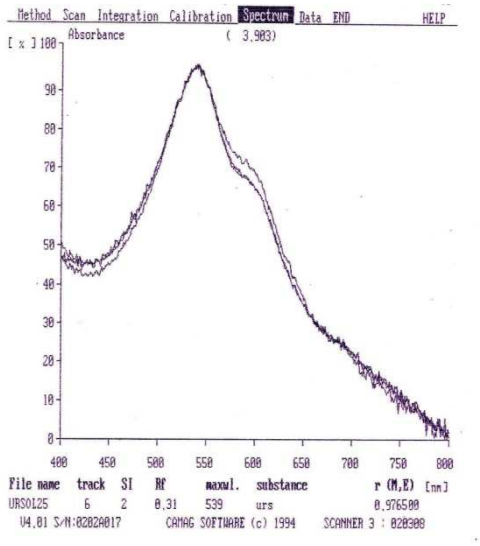


Figure-4 (A) Overlay UV absorption spectra of Ursolic acid in Standard at the start, middle and end position

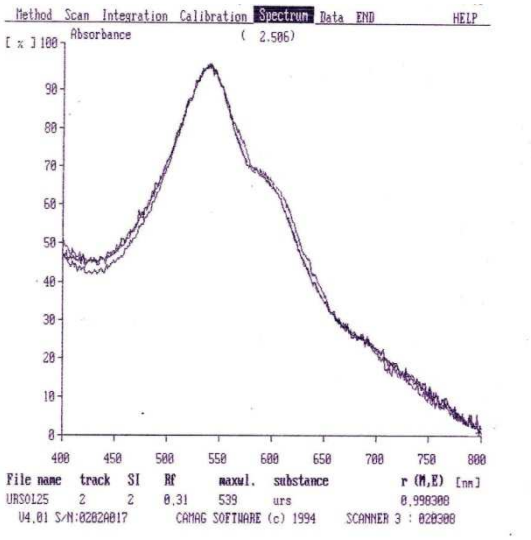


Figure-4 (B) Overlay UV absorption spectra of Ursolic acid in Sample at the start, middle and end position

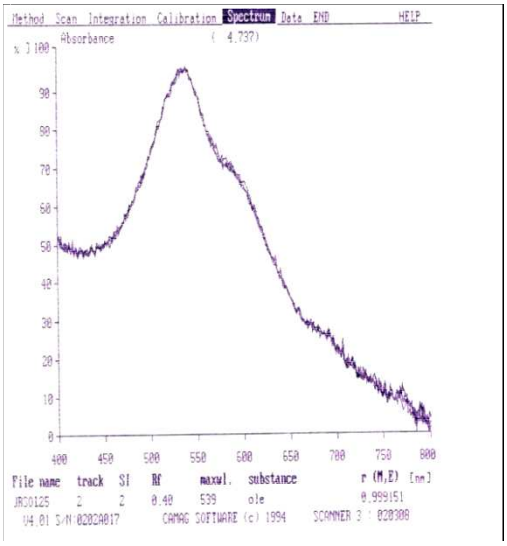


Figure-5 (A) Overlay UV absorption spectra of Oleanolic acid in Standard at the start, middle and end position

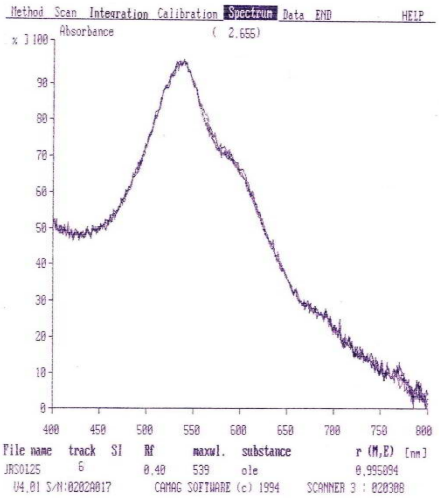


Figure-5 (B) Overlay UV absorption spectra of Oleanolic acid in Sample at the start, middle and end position

The methods were validated in terms of precision, repetability, linearity and accuracy (*Table-2*).

Table – 2 Method validation parameters

Sr. No.	Parameters	Ursolic acid	Oleanolic acid
1	**Limit of Detection**	160ng/spot	320 ng/spot
2	**Limit of Quantification**	389.65 ng/spot	960.31ng/spot
3	**Linearity range**	400-1400 ng/spot	1200-4200 ng/spot
4	**Correlation Coefficient**	0.9968	0.9939
5	**Precision**		
	Repeatability (CV)		
	a. Repeatability of scanner	0.24%	0.60%
	b. Repeatability spotter	0.82%	0.89%
	Reproducibility (CV)		
	a. Intraday Precision	0.40 - 1.46%	0.60 - 1.19%
	b. Interday Precision	1.00 - 2.58%	1.18 - 2.32%
6	**Accuracy (% Recovery)**	97.59 ± 0.99%	96.61 ± 0.83%
7	**Specificity**	Specific	Specific

Table – 3 Recovery for Ursolic acid

Conc. Of Ursolic acid in extract (ng/Spot)	Amount Spiked of Standard Ursolic acid(ng)	Total conc. Of Ursolic acid(ng)	Actual Conc. Of standard Ursolic acid (ng) Recovered (n=3) Mean ± SD	% Recovery Mean ± % RSD (n=3)
405	200	605	194.67 ± 5.13	97.24 ± 0.73
405	400	805	390.33 ± 10.02	97.67 ± 1.25
405	600	1005	587.67 ± 9.02	97.85 ± 1.00
	Avg. % recovery			97.59 ± 0.99

Table – 4 Recovery for Oleanolic acid

Conc. Of Oleanolic acid in extract (ng/Spot)	Amount Spiked of Standard Oleanolic acid(ng)	Total conc. Of Oleanolic acid(ng)	Actual Conc. of standard Oleanolic acid (ng) Recovered (n=3) Mean ± SD	% Recovery Mean ± % RSD (n=3)
1270	600	1870	574.67 ± 19.01	95.76 ± 1.02
1270	1200	2470	1168.00 ± 19.47	97.09 ± 0.79
1270	1800	3070	1745.67 ± 20.82	96.99 ± 0.68
	Avg. % recovery			96.61 ± 0.83

Linearity ranges for Ursolic acid and Oleanolic acid were found to be 400 – 1400 ng/spot and 1200 – 4200 ng/spot respectively with correlation coefficients (r values) of 0.9968 and 0.9939 respectively. The intraday and interday precision expressed as RSD indicate that proposed method is precise and reproducible. The LOD values were found 160 and 320 ng respectively and LOQ values were 389.65 and 960.31 ng respectively. The average recoveries at 3

different levels of Ursolic acid and Oleanolic acid were found to be 97.59 (*Table – 3*) and 96.61% (*Table – 4*) respectively.

The content of Ursolic acid and Oleanolic acid in *Plumeria obtusa* was quantified by proposed HPTLC method were found to be 0.197 % and 0.0253 % respectively.

CONCLUSION

HPTLC Method successfully applied for quantification of Ursolic acid and Oleanolic acid in *Plumeria obtusa* leaves.The method proves to be helpful in separation of chemical isomer Ursolic acid and Oleanolic acid. The developed method is simple, specific, precise and accurate. This Method can be use be use for quantification of Ursolic acid and Oleanolic acid In other plants.

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