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Original Article

# Simultaneous Quantification of Bioactive Triterpene acids (Ursolic acid and Oleanolic acid) in Different Extracts of *Eucalyptus globulus (L)* by HPTLC Method

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**ABSTRACT**

**Objective:** To develop a novel analytical method for simultaneous determination of two triterpenic acids by high-performance thin layer chromatography in methanol and dichloromethane extracts of *Eucalyptus globulus* leaf. Ursolic acid was also isolated from *Eucalyptus globulus* leaf. **Materials and Methods:** Two triterpenic acids (ursolic and oleanolic acid) were extracted using methanol and dichloromethane as the extraction solvents. Study for total triterpenoids present in *Eucalyptus globulus* leaves was carried out which shows considerable amount of terpenoids present. Because of the similarity of chemical structure, the prechromatographic derivatization was necessary to separate these triterpenic acids. The samples were treated by 1% iodine solution in chloroform directly on the chromatographic plate and developed with the mobile phase consisting of petroleum ether, ethyl acetate and acetone (7.8:2.2:0.1, v/v/v). After drying, the plates were sprayed with 10% (v/v) ethanol solution of sulfuric acid and heated to 120 °C for 3 min. Quantification was performed in absorbance/transmittance mode at a wavelength of 345 nm. The developed HPTLC method was validated for linearity, precision and accuracy. **Results:** Correlation coefficient ($r^2 > 0.99$), R.S.D. values, detection limits as well as recovery values were found to be satisfactory. Ursolic acid was isolated from *E. globulus* leaves. The identification of isolated ursolic acid was done on the basis of Rf value (0.26) for HPTLC and peak interpretation for FT-IR. **Conclusion:** The method has been successfully applied in the analysis of both triterpenic acids in medicinal herbs.

**Key words:** Ursolic acid, Oleanolic acid, HPTLC, Iodine derivatization, Triterpenes.

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## INTRODUCTION

Triterpenoids are an interesting group of compounds in nature. Oleanolic acid and ursolic acid are triterpenoid compounds that exist widely in food, medicinal herbs and other plants.[1] Ursolic acid (3-hydroxy-urs-12-en-28-oic acid) and its isomer, oleanolic acid (3-hydroxy-olea-12-en-28-oic acid) are bioactive compounds with confirmed pharmacological properties. In recent years they became the subject of many publications because of their various activities combined with low toxicity.[2]

*Eucalyptus* spp. (family Myrtaceae) originated in Australia, but these plants now grow in almost all tropical and sub-tropical areas and are cultivated in many other climates. Much research has been conducted on the medicinal properties of *Eucalyptus* spp. The leaf extract or essential oil from the leaves of *Eucalyptus* spp. has been reported to possess antifungal, antibacterial, mosquito repellent and antioxidant properties.[3]

In the case of *Eucalyptus* spp., it has been reported that the lipophilic extracts of *E. globulus* outer bark contain high amounts of several triterpenic acids with ursane and oleanane skeletons, namely ursolic and oleanolic acids.[4] These triterpenic acids are recognized as promising compounds for the development of new multi-targeting bioactive agents [5-8] For example, oleanolic and ursolic acids show significant anti-inflammatory, [9-16] anticancer, [17] Anti-Platelet Aggregation, [18-19] Anti-HIV/AIDS,[20-21] Anti-Mycobaterium Tuberculosis, [22-27] anti-proliferative [28] and hepatoprotective [29-31] properties in laboratory animals.

Literature describes HPLC [31,32-42] HPTLC [43-47] micellar electrokinetic chromatography (MEC) [48-49] and Thin-layer chromatography has been also described methods for analysis of ursolic acid and oleanolic acid alone as well as simultaneously from other plants and extracts but oleanolic and ursolic acids are position isomers are shown in Figure.1 and their separation by TLC is rather difficult. There are some chromatographic systems to determine these triterpenic acids reported in literature but none of them enable their separation in eucalyptus leaves. On the other hand, modern TLC is powerful analytical technique, especially useful to analysis of plant material because large number of samples can be chromatographed simultaneously and the samples without any pretreatment can be applied. In case of compounds

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with similar chemical structure, sometimes the pre-chromatographic derivatization can be helpful in their determination. There are a lot of examples of use the specific chemical derivatization, for example, esterification was employed in analysis of primary, secondary and tertiary alcohols and hydrolysis (acidic or alkaline) were used in determination of flavonoids, triterpenes and cardenolide glycosides.[50]

However, there is no published report describing separation of ursolic acid and oleanolic acid from methanolic and DCM extracts of *Eucalyptus globulus* leaves. The yield of the lipophilic extractives of bark extracted with dichloromethane was good with previous results of *E. globulus*[4] that's why DCM was selected for further study.

Pre-chromatographic derivatization was required because of chemical structural similarity of ursolic acid and oleanolic acid. HPTLC is a well-known and versatile separation method which shows a lot of advantages in comparison to other separation techniques. HPTLC is the simplest separation technique today available to the analyst. HPTLC layer is more homogeneous and thinner resulting in improved resolution, shorter analysis time and suitable for in situ quantification.

## MATERIALS AND METHODS

### *Apparatus*

HPTLC system (Linomat 5, Camag, Switzerland) automatic sample applicator, TLC scanner IV (Camag), flat bottom and twin- trough developing chamber (15 X 10 cm), Darmstadt, Germany), pre-coated silica gel, aluminum plate (E. Merck, electronic, analytical balance, Shimadzu (AUX-220), micro syringe (100 ml) (Hamilton).

### *Reagents and standard*

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

### Standard and sample preparation

Stock solutions of ursolic acid and oleanolic acid were prepared by dissolving 10 mg of each compound in 100 mL of methanol (final concentration 1000 µg/ml). Standard concentration of 20 µg/ml of both compounds were prepared by dilution of stock solutions with methanol.

To quantification 15 gm of dry powdered leaves of eucalyptus globulus were extracted with methanol and dichloromethane for 7 hrs in soxhlet apparatus. The obtained extracts were evaporated to dryness and 10 mg residue was dissolved in10 ml methanol separately, which were further diluted to get 100 µg/ml concentration.

### Estimation of total triterpenoids by colorimetric method

Accurately measured quantity of plant extracts were dissolved in 25 ml of ethanol. A volume of 0.2 ml of ethanol solution was transferred in a graduated test tube and it was evaporated to dryness in a boiling water bath. A volume of 0.3 ml of 5% vanillin/glacial acetic acid and 1ml of perchloric acid solution were added. The sample solutions were heated at 60˚C for 45 min and cooled in an ice water bath to the ambient temperature. A volume 5ml of glacial acetic acid was added. The absorbance of the samples was measured at 548 nm. The same procedure was repeated for preparation of standard ursolic acid. The percentage of total triterpenoids was calculated from the calibration curve.[51]

### Estimation of UA in DCM extracts

Accurately weighed 5.0 gm samples in 25 ml 50% v/v methanol, was heated to ensure complete dissolution. A volume of 75 ml water was added and mixed thoroughly. It was transferred to a 250 ml RBF and 10 gm $H_2SO_4$ was zadded and refluxed 6-8 hr. The contents were cooled & transferred into separating funnel. About 25 ml chloroform was added, shaken for a while & allowed to stand for layer separation. The chloroform layer was transferred to another separator and aq. Acidic layer once again washed with 25 ml chloroform. The separated chloroform layer was mixed with earlier washing. Both chloroform washings (50 ml) were washed with water till acid free (2-3 washing). Acid free chloroform layer was dried over anhydrous sodium sulphate and after filtration; chloroform is evaporated to dryness in a pre-weighed beaker. The residue in beaker was finally dried at 80˚C under vacuum to constant weight. This gives the quality of total triterpinic acids for calculating the percentage of ursolic acid.[52]

### Chromatographic conditions

In simultaneous estimation pre-coated silica gel 60 $F_{254}$ aluminium plates of 10 x 10 cm and 20 × 10 cm (Merck, Germany) were used as stationary phase.

Twenty five micro litres of mixture of standard solutions, 20 µL of both methanolic and DCM extracts solutions were spotted using an Linomat V semiautomatic sample applicator (Camag, Switzerland) under nitrogen at 6 mm band length and 15 mm distance from left edge and from bottom and 10 mm distance from centres of tracks.

### Prechromatographic derivatization

The plates were developed in glass chamber with 1% iodine solution in chloroform to a 1.5 cm, plate was removed and the start zone was covered by aluminium foil and the plates were placed in dark for 10 minutes. When the reaction was complete, the plates were dried in a stream of warm air to remove the excess of iodine.

### Chromatography and determination

The pre-derivatized plates were developed with a mixture of Petroleum ether : ethyl acetate : acetone (7.8 :2.2:0.2) (v/v/v) as mobile phase on a distance of 7.5 cm. after drying in a stream of warm air the plates were sprayed with 10% (v/v) $H_2SO_4$ in ethanol, dried for 10 min and then heated to 110˚ C for 5 min.

The quantification was carried out by densitometric scanning (Camag TLC scanner IV) at absorbance transmittance at $\lambda$ = 345 nm (slit distance: 4.00 x 0.30 mm)

Derivatization and determination were performed under controlled conditions at room temperature (27 $\pm$ 2˚C)

## RESULTS AND DISCUSSION

The % yields obtained with methanolic and DCM extracts from *E. globulus* are recorded 10 % and 47 % (w/w) respectively. The lipophilic fractions of plants were shown to be mainly composed of triterpenic compounds.[4]

Total terpenoids estimation by calorimetric method (method 1) shows that methanolic and DCM extracts contain 45±5% (450 mg/gm) and 80± 5% (795 mg/gm) terpenoids respectively.

Total terpenoid estimation from extract by (method 2) shows that methanolic and DCM extracts contain 52±5% (0.52 gm/gm) and 84±5 (0.84 gm/gm) terpenoids respectively.

Drugs were characterized by determination of Melting point of ursolic acid and oleanolic acid, that are showing 288±2˚C and 298±2˚C respectively.

UA and OA both show reasonably good absorbance at 345 nm. Therefore 345 nm was selected as detection wavelength for both standards for HPTLC.

The mobile phase petroleum ether: ethyl acetate: acetone (7.8:2.2:0.2 v/v/v) gave good separation, compact spot, good resolution with $R_f$ 0.24 and 0.40 for UA and OA, respectively.

With optimized mobile phase composition and saturation time, the drugs Ursolic acid and Oleanolic acid showed Rf value 0.24 and 0.40, respectively.

## Simultaneouse estimation of UA and OA in DCM and Methanolic extracts

Densitogram of ursolic acid [(200ng/spot); (Rf = 0.24)], oleanolic acid (200 ng/spot); (Rf=0.40)] and the mixture of both standards in DCM and methanolic extract [(200 ng/spot) ( Rf = 0.24 for UA and 0.40 for OA)] were shown in Figure 2 A, B and C).

Densitogram of DCM extract was shown in figure 2D, having six peaks, the fourth peak Rf value (0.25) and fifth peak Rf value (0.39) were coinciding with standard Rf values of ursolic acid and oleanolic acid respectively. The concentration of ursolic acid and oleanolic in DCM extract of *E. globulus* were found to be 174.57 ng/spot and 146.30 ng/spot.

Densitogram of methanolic extract was shown in Figure 2 E having four peaks, Rf (0.25) value of fourth peak was coinciding with standard Rf value of ursolic acid. The concentration of ursolic acid in methanolic extract of *E. globulus* was found to be 97.59 ng/spot.

## Method validation

The presented method was validated for linearity, specificity, precision, accuracy and Calibration curve was prepared using mixed working standard solution in the range of 100-500 ng/spot for both Ursolic acid and Oleanolic acid. The 3D chromatogram is shown in Figure 3. The other component present in extract did not interfere in the separation and resolution of UA and OA. Both UA and OA were found to be linear in the above-mentioned range with correlation coefficient of 0.9954 and 0.9937, respectively. The average linear regression equations for calibration curves were y=3.397x + 586.18 and y = 2.8602x – 199.5 for UA and OA, respectively. Linearity data and its summary are depicted in Table 1.

Comparison of each spectrum scanned at peak start (s), peak apex (m) and peak end (e) positions of bands in samples showed a high degree of correlation (above 0.99), confirmed the purity of the bands are presented in Figure 4 which confirm that the method is specific.

For precision of method, repeatability of sample application and measurement and interday and intraday precision was measured. The data for repeatability and Intermediate precision of measurement and

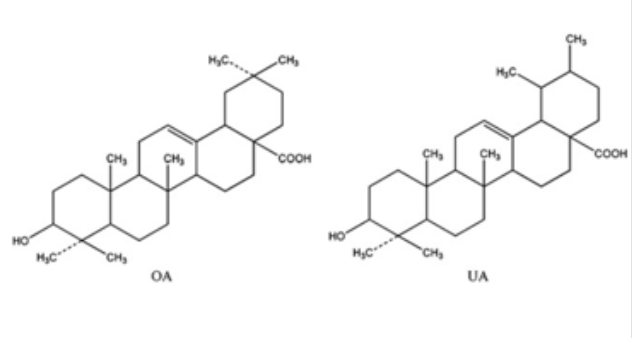


**Figure 1:** Chemical structures of oleanolic acid and ursolic acid.

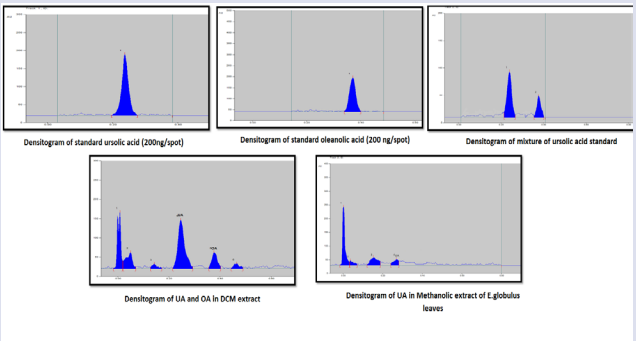


**Figure 2:** Densitogram of (A) Standard ursolic acid, (B) Standard oleanolic acid, (C) Mixture of standard ursolic and oleanolic acid, (D) DCM extract and (E) Methanolic extract.

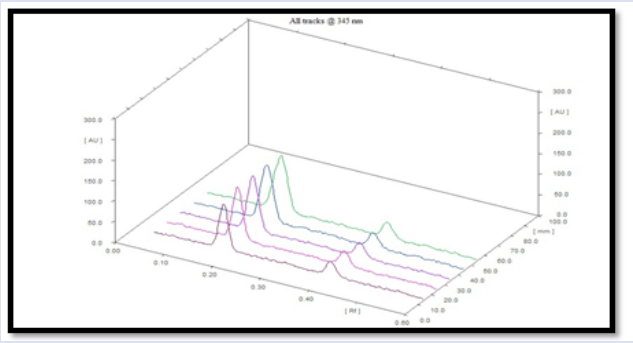

**Figure 3:** 3D densitogram of ursolic and oleanolic acid (100-500 ng/spot).

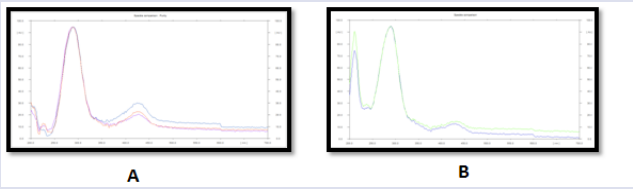


**Figure 4:** An Overlain spectra of standard ursolic acid and ursolic acid present in DCM and methanolic extracts, B Overlain spectra of standard oleanolic acid and oleanolic acid present in DCM extract.

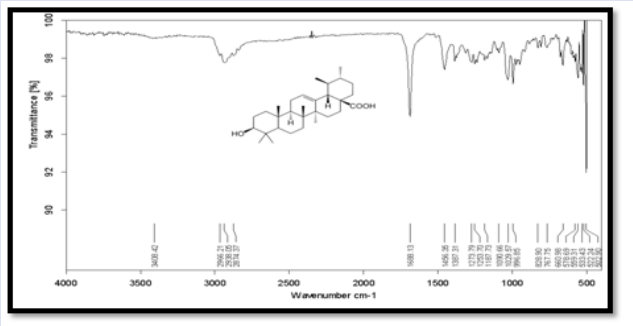


**Figure 5:** Reported FT-IR spectra of standard ursolic acid.

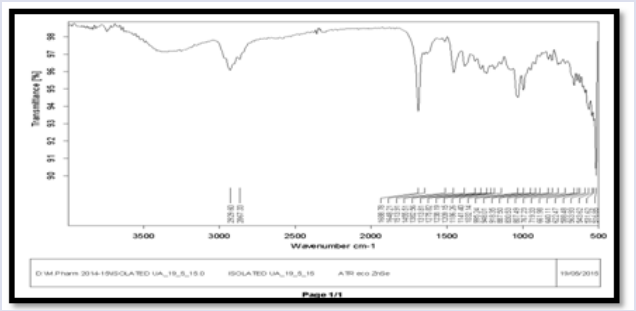

**Figure 6:** Observed FT-IR spectra of isolated ursolic acid.

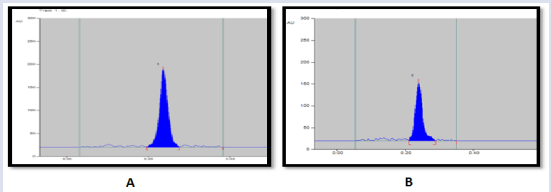

**Figure 7:** Densitogram of (a) standard ursolic acid and (b) isolated ursolic acid.

**Table 1: Linearity of the method**

| Standard | ng/spot | Mean ± SD | %RSD |
|---|---|---|---|
| UA | 100 | 1234.3 ± 15.2695 | 1.23 |
| | 200 | 1645.9 ± 8.3321 | 0.5 |
| | 300 | 1969.1 ±24.1119 | 1.22 |
| | 400 | 2239.5±16.2333 | 0.72 |
| | 500 | 2635.96 ±29.5585 | 1.12 |
| | | Y=3.397x + 586.18; $R^2$=0.9954 | |
| OA | 100 | 391.7 ± 5.7515 | 1.46 |
| | 200 | 663.0 ±11.0779 | 1.65 |
| | 300 | 923.4 ±12.9309 | 1.40 |
| | 400 | 1182.9 ±17.4372 | 1.47 |
| | 500 | 1561.82 ±6.5716 | 0.42 |
| | | Y=2.8602x - 199.5; $R^2$=0.9937 | |

**Table 2: Repeatability of sample application and measurment**

| Parameters | UA (300 ng/spot) | | OA (300 ng/spot) | |
|---|---|---|---|---|
| | Area ± SD | RSD | Area ± SD | RSD |
| Repeatability of measurement (n=7) | 1989.44 ± 7.85 | 0.39 | 988.98 ± 9.80 | 0.99 |
| Repeatability of sample application (n=7) | 1981.46 ± 8.69 | 0.43 | 980.05 ± 11.21 | 1.14 |

sample application of UA and OA are depicted in Table 2 and table 3 respectively.

Accuracy of the method was determined by recovery study from standard mixture of ursolic acid and oleanolic acid at 80%, 100% and 120% level by standard addition method. The results for accuracy are depicted in table 4.

**Table 3: Intermediate precision**

| Standard | Conc (ng/spot) | Intraday precision | | Interday Precision | |
|---|---|---|---|---|---|
| | | AREA MEAN ± SD | %RSD | AREA MEAN ± SD | %RSD |
| UA | 300 | 1647.23 ±7.85 | 0.47 | 1647.91 ±10.88 | 0.66 |
| | 400 | 1968.40 ±9.56 | 0.49 | 1979.83 ±17.85 | 0.90 |
| | 500 | 2231.13 ±12.68 | 0.56 | 2241.80 ±24.55 | 1.09 |
| OA | 300 | 667.23 ±12.23 | 1.83 | 663.60±13.17 | 1.98 |
| | 400 | 972.70±10.94 | 1.12 | 978.66±11.80 | 1.20 |
| | 500 | 1186.86 ±11.60 | 0.97 | 1182.30±22.29 | 1.88 |

The other component present in extract did not interfere in the separation and resolution of UA and OA. Comparison of each spectrum scanned at peak start (s), peak apex (m) and peak end (e) positions of bands in samples showed a high degree of correlation (above 0.99), confirmed the purity of the bands. This shows specificity of method.

Limit of detection and limit of quantitation was determined by using equation method. The LOD for UA and OA were found to be 21.15 and 6.09 ng/spot, respectively. The LOQ for UA and OA were found to be 64.10 and 18.46, respectively. Summary of validation parameters are depicted in table 5.

## Quantitation of ursolic acid and oleanolic acid in *E. globulus* leaves

Prepared extracts were analysed using developed method. Both DCM and methanolic extract was weighed 10 mg and diluted up to 10 ml (100µg/ml) and filtered through Whatman filter paper no. 41.A volume of 1 ml filtered solutions were further diluted upto 10 ml with methanol (100 µg/ml). 20 µl of both extracts solutions were spotted along with mix standard of UA and OA (20 µg/ml). The concentration of UA was found in DCM (100 µg/ml) (8.72 % w/w), methanolic (100 µg/ml) 4.87 % w/w and methanolic (10,000 µg/ml) (8.49 %w/w) and the concentration of OA was found in DCM (100 µg/ml) 7.31 % w/w and methanolic (10,000 µg/ml) 1.92 % w/w.

## Isolation and identification of ursolic acid from *E. globulus* leaves

Yield obtained from leaves was 0.06%. Identification of isolated ursolic acid was carried out by peak interpretation of FT-IR and comparison of densitogram and Rf value by HPTLC.

## Identification from FT-IR

FT-IR spectra of isolated UA was compared with FT-IR spectra of standard UA are shown in figure 5 and 6. Peak interpretation is depicted in table 6.

## Identification from HPTLC

Densitogram of isolated ursolic acid show $R_f$ value 0.26 which is nearer to the $R_f$ value of standard ursolic acid i.e. 0.24 figure 7. Results of FT-IR interpretation and densitogram comparison shows that compound isolated from *E. globulus* leaves was ursolic acid.

**Table 4: Accuracy of method**

| Standard | Amount of standard from pre-analysed sample (ng/spot) | Amount of standard spiked (ng/spot) | Total amount spotted (ng/spot) | Area | Mean of spiked amount recovered ± SD (ng/spot) (n=3) | % recovery |
|---|---|---|---|---|---|---|
| UA | 200 | 0 | 200 | 1276.86 | - | - |
| | 200 | 100 | 300 | 1610.37 | 101.61 ± 0.68 | 101.50% |
| | 200 | 200 | 400 | 1950.27 | 201.41 ± 0.53 | 100.78% |
| | 200 | 300 | 500 | 2287.90 | 300.74 ± 0.71 | 100.31% |
| OA | 200 | 0 | 200 | 364.19 | - | - |
| | 200 | 100 | 300 | 661.04 | 100.70 ± 1.22 | 100.87% |
| | 200 | 200 | 400 | 944.46 | 199.80 ± 0.97 | 99.98% |
| | 200 | 300 | 500 | 1227.5 | 298.74 ± 1.03 | 99.65% |

**Table 5: Summery of validation parameters**

| Parameters | UA | OA |
|---|---|---|
| Linearity range (ng/spot) | 200-600 | 200-600 |
| Correlation coefficient ($R^2$) | 0.9954 | 0.9937 |
| Precision | | |
| Repeatability | | |
| Repeatability of measurement (n =7) | 0.39 | 0.99 |
| Repeatability of sample application (n = 7) Intermediate precision | 0.43 | 1.14 |
| Intra-day precision (n = 3) | | |
| Inter-day precision (n = 3) | 0.47-0.56% | 0.97-1.83% |
| | 0.66-1.33% | 1.20-1.98% |
| % Recovery | 100.31% - 101.50% | 99.65% - 100.87% |
| Limit of Detection (LOD) (ng/spot) | 21.15 | 6.09 |
| Limit of Quantitation (LOQ) (ng/spot) | 64.10 | 18.46 |

**Table 6: Interpretation of FT- IR spectra**

| Observed Wave number ($cm^{-1}$) | Indicating group |
|---|---|
| Bonded –OH | 2929.60 |
| Bond C=O | 1648.2 |
| Aromatic | 1513.91 |
| C=C stretch aromatic | 1455.51 |
| -C-OH deformation vibrations | 1382.56,1313.29 |
| -C-OH stretching vibrations | 1186.26,1141.60 |
| 9H-C– H out-of-plane bending | 807.49 |

## CONCLUSION

A validated HPTLC method for separation and determintion of ursolic acid and oleanolic acid in DCM extract of *E.globulus* has been developed. The HPTLC method is specific, accurate and reproducible and can be used for the separation and simultaneous estimation of the two active components. The developed method offers a cost-effective alternative to the HPLC method for the separation and quantitation of two components. Isolation and identification of isolated compound by HPTLC and FT-IR was carried out. Determination of total triterpenoid present in *E. globulus* leaf extract was carried out and was found to contain 40-60% total triterpenoid. Therefore, in order to ensure and improve the therapeutic benefits, it is necessary to quantify each of the major bioactive components in the leaves of *Eucalyptus globulous* derived extracts and phytomedicines.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

Authors declare no conflict of interest.

## ABBREVIATION USED

UA: Ursolic acid; OA: oleanolic acid; DCM: dichloromethane; AIDS: acquired immune deficiency syndrome; HIV: Human immunodeficiency virus infection; MECC: micellar electrokinetic capillary chromatography; FTIR: Fourier transform infrared spectroscopy (FTIR); RP-HPLC: Reversed phase High-performance liquid chromatography; HPLC: High-performance liquid chromatography; HPTLC: High performance thin layer chromatography.

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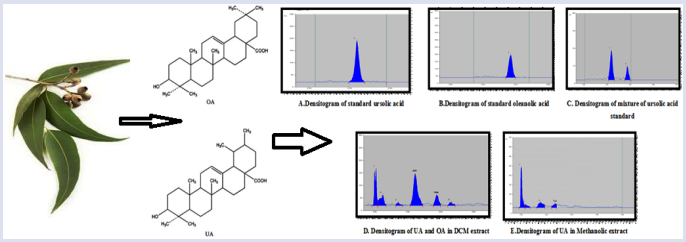
## GRAPHICAL ABSTRACT




## SUMMARY


- HPTLC method for simultaneously separation of ursolic acid and oleanolic acid in Eucalyptus globulus leaf extract was developed.
- Ursolic acid was also isolated from Eucalyptus globulus leaves.
- The linearity range for UA was found to be 200-600 ng/spot with correlation coefficient 0.9954 and for OA the linearity range was 200-600 ng/spot with correlation coefficient 0.9937.
- The method was found to be accurate, precise and specific.
- The developed HPTLC method was successfully validated.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