

Determination of Ursolic Acid and Ursolic Acid Lactone in the Leaves of *Eucalyptus tereticornis* by HPLC

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Um método simples, isocrático, de HPLC foi desenvolvido para quantificação simultânea de dois triterpenos bioativos, ácido ursólico e lactona do ácido ursólico em folhas de *E. tereticornis*. As amostras foram analisadas em coluna RP-18 (4,6 × 250 mm, 5 µm) com metanol e água acidificada a pH 3,5 com TFA (88:12) a 210 nm. O método foi validado e aplicado para a quantificação simultânea dos triterpenos individuais em extrato de *E. tereticornis*. As curvas de calibração foram lineares no intervalo de concentração de 0,05 a 0,3 mg mL⁻¹ (r = 0,999 e 0,998, respectivamente). Os limites de detecção e quantificação foram 0,190 e 0,644 µg para ácido ursólico e 0,176 e 0,587 µg para lactona do ácido ursólico, enquanto as porcentagens de recuperação foram 97,32 e 96,23% para ácido ursólico e lactona do ácido ursólico, respectivamente. Este é o primeiro relato sobre o método HPLC de lactona do ácido ursólico com alta precisão e exatidão.

A simple isocratic HPLC method has been developed for the simultaneous quantification of two bioactive triterpenes, ursolic acid and ursolic acid lactone in *E. tereticornis* leaves. Samples were analyzed on RP-18 (4.6 × 250 mm, 5 µm) column with methanol and water acidified to pH 3.5 with TFA (88:12) at 210 nm. The method was validated and applied for the simultaneous quantification of the individual triterpenes in *E. tereticornis* extract. The calibration curves were linear over a concentration range of 0.05-0.3 mg mL⁻¹ (r = 0.999 and 0.998, respectively). The limits of detection and quantification were 0.190 and 0.644 µg for ursolic acid, and 0.176 and 0.587 µg for ursolic acid lactone, while the percentage recoveries were 97.32 and 96.23% for ursolic acid and ursolic acid lactone, respectively. This is the first report on the HPLC method of ursolic acid lactone with high precision and accuracy.

Keywords: *Eucalyptus tereticornis*, HPLC, triterpenoids, ursolic acid, ursolic acid lactone

Introduction

Eucalyptus tereticornis Smith (Myrtaceae, Mysore Gum) extensively grown in various states of India.¹ It is a valuable quick growing species grown on large scale for afforestation programmes throughout India for its use as a raw material for pulp wood industry. *Eucalyptus* is a popular medicine for abscess, arthritis, asthma, boils, bronchitis, burns, cancer, catarrh, cold, cough, croup, cystitis, diabetes, diphtheria, dysentery, dyspepsia, fever, flu, gripe, inflammation, laryngitis, leprosy, malaria, miasma, phthisis, rhinitis, sores, sore throat, spasms, tuberculosis, tumors, vaginitis, wounds, and worms.²⁻⁵

The leaves are a good source of essential oil,⁶ and in Ayurveda its oil is used as an antiseptic and for respiratory tract infections. Apart from the essential oil, a number of phytochemicals have been isolated from the various

parts of *E. tereticornis* such as, flavanoids,⁷ euglobal T1,⁸ triterpenoids as betulonic acid, tereticornate A, tereticornate B,⁷ betulonic acid,⁹ 2 α -hydroxy ursolic acid, ursolic acid, ursolic acid lactone¹⁰ and β -sitosterol.⁹ After essential oil isolation, the leaves of *E. tereticornis* can be further utilized for large scale isolation of anticancer agent, ursolic acid (UA, **1**) and ursolic acid lactone (UAL, **2**)¹¹ (Figure 1).

Ursolic acid has shown many important pharmacological activities, analgesic, anti-tumor,¹² anti-bacterial,¹³ diuretic, anti-diabetic, anti-oxidant, COX-2 inhibitor, anti-inflammatory,¹⁴ hepatoprotective,¹⁵ anti-HIV¹⁶ and anti-cancer against various human cancer cell lines.^{17,18} Because of its anticancer activities, ursolic acid has been the subject of interest for preclinical anti-cancer research. On the other hand ursolic acid lactone has also shown antiproliferative activity against human gastric adenocarcinoma (MK1), uterine carcinoma (HeLa) and murine melanoma (B16F10)¹⁹ along with substantial anti-inflammatory activity.²⁰

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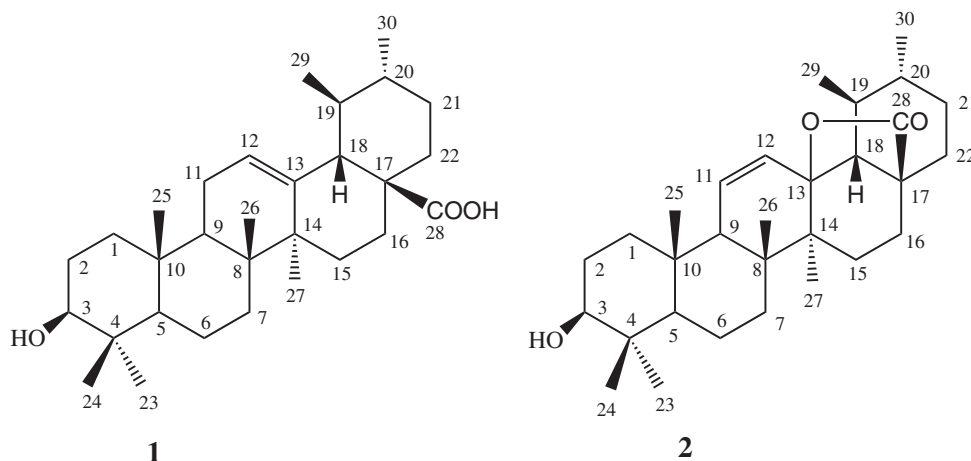


Figure 1. Chemical structures of ursolic acid (**1**) and ursolic acid lactone (**2**).

Although there are several HPLC analytical methods for ursolic acid from different plants,²¹⁻²³ there is no HPLC method for the analysis of bioactive ursolic acid lactone as well as for UA (**1**) and UAL (**2**) in the leaves of *E. tereticornis*. Hence the objective of present work is to develop simple, rapid, precise and accurate method for determination of bioactive UA (**1**) and UAL (**2**) in the leaves of *E. tereticornis* for better evaluation and medicinal utilization of leaves in the future.

Experimental

Plant material

The leaves of *E. tereticornis* were collected from the medicinal farm of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, Uttar Pradesh, India during the month of January, 2008. Voucher specimen (CIMAP N.12470) is deposited in the Botany and Pharmacognosy Department of the institute.

Isolation and characterization of reference compounds

The reference marker compounds, ursolic acid (**1**) and ursolic acid lactone (**2**) were isolated in $\geq 95\%$ purity by fast centrifugal partition chromatography (FCPC)¹¹ and characterized on the basis of their spectroscopic data (see Supplementary Information).^{7,24}

Sample preparation

The dried leaves of *E. tereticornis* (100 mg) were extracted three times with ethyl acetate (5 mL) in an ultrasonic extractor (Micro clean -109 bath; Oscar India). All the extracts were combined and evaporated to dryness

at 35 °C. The residue was dissolved in 1 mL methanol and filtered in 0.45 μm membrane filtered through (Millipore).

Chemicals and standards

All the reagents and solvents used were of HPLC grade (E. Merck Ltd., Mumbai, India), while the reference marker compounds, ursolic acid (**1**) and ursolic acid lactone (**2**) were isolated in $\geq 95\%$ purity as discussed above.

Apparatus and chromatographic conditions

A sonicator (Microclean 109, Oscar Ultrasonic, Mumbai, India) was used for sample preparation, The 300 MHz NMR (Avance, Bruker, Switzerland) was used to record ¹H and ¹³C NMR.

A HPLC pump (Shimadzu, Tokyo, Japan), LC-10A equipped with Shimadzu SPD-M10 Avp (PDA set at 210 nm) detector and Rheodyne injection valve with a 20 μL loop was used. Ursolic acid (**1**) and ursolic acid lactone (**2**) were analyzed on a Water symmetry shield RP-18 column (4.6 \times 250 mm i.d., 5 μm , Waters). The mobile phase used was a mixture of methanol-water acidified to pH-3.5 with TFA (88:12 v/v) with 1 mL min⁻¹ flow rate. The HPLC system was operated at room temperature 25 \pm 1 °C.

Preparation of standard solution

The stock solution of 1 mg mL⁻¹ was prepared in methanol for standard markers UA (**1**) and UAL (**2**). A serial dilution was made for each stock solutions at concentrations of 50, 100, 200 300 $\mu\text{g mL}^{-1}$, by adding methanol and 20 μL of each was used for plotting the standard curve for UA (**1**) and UAL (**2**), respectively.

Validation of HPLC method

Linearity (calibration curve)

The linearity of the method for each standard was established by injection of 20 μL of standard solution of different concentration. The linearity of UA (**1**) and UAL (**2**) were analyzed in triplicate. The calibration curves were constructed by plotting peak areas against analyte concentration. The linearity was assessed by calculating the slope, y-intercept and coefficient of determination (r^2) using least squares regression equation.

Limit of detection (LOD) and limit of quantification (LOQ)

The linear regression equation of calibration curve was used to determine the LOD and LOQ. Under the chromatographic conditions employed in the current study, LOD ($S/N = 3$) and LOQ ($S/N = 10$) were determined for UA (**1**) and UAL (**2**).

Precision and accuracy

The repeatability (intra-day precision), the intermediate precision (inter-day precision) and the accuracy of the method were determined at three different concentrations. Samples were prepared by spiking stock standard solutions with ethyl acetate extract of *E. tereticornis* leaves. The intra-day and inter-day precision of the method for UA (**1**) and UAL (**2**) were measured by peak area determination of triterpenoids in triplicate.

The precision is expressed as % RSD and the accuracy is expressed as the percentage recovery of the added triterpenoids.

Recovery

To determine the recovery percentage, a measured amount of 0.8 mg each of UA (**1**) and UAL (**2**) were added to the ethyl acetate extract of *E. tereticornis* leaves and was analyzed according to the following formula,

$$\text{Recovery (\%)} = (A - B)/C \times 100\%$$

where A is the amount detected, B is the amount of sample without standards and C is the spiked amount of the standards.

All the statistical analysis was performed on the GraphPad Prism-5 (Graph Pad software, Inc., USA, 2008).

Results and Discussion

Triterpenoids show poor UV absorption due to lack of chromophore moieties in their chemical structures. This is the major limitation in the analysis of this class

of compounds using UV detection. However, there are some reports on triterpenoids and saponins detection at low wavelength range^{25,26} in other plants. In some cases derivatization²⁶ has also been used for the detection at higher wavelengths, but this additional step may result in significant error in the method. As discussed earlier, although there are several HPLC analytical methods for ursolic acid from different plants,²¹⁻²³ there is no HPLC method for the analysis of bioactive ursolic acid lactone, hence this work was designed to develop a simple, rapid, precise and accurate method for UA (**1**) and UAL (**2**) determination in the leaves of *E. tereticornis*.

It was observed that the mobile phases used in earlier HPLC methods for ursolic and oleanolic acid determination were composed of three solvents,²¹ acetic acid,²¹ H_3PO_4 , and buffers.²³ It has been common observation that frequent use of buffer in mobile phase reduces the life of column, HPLC pump, detector flow cell and creates high pressure. Therefore, use of TFA in HPLC methods to control pH and no ion pairing in acid is preferred in comparison to buffers.²⁷

Different types of column and mobile phase compositions were carefully tested in order to determine the optimal chromatographic conditions. It was found that better separation and shapes peak were achieved with Waters Symmetry Shield RP-18 (4.6×250 mm, $5 \mu\text{m}$) column at column temperature 25 ± 1 °C. For the mobile phase, separation using only methanol or water was unsatisfactory, but methanol as an organic modifier with an aqueous-TFA solution (pH-3.5) performed well. In this case addition of TFA improved separation and peak shapes by controlling pH without any ion pairing for acidic analytes.

An isocratic system added simplicity, precision, accuracy, and minimized the variation at baseline. The above series of investigation led us to arrive at an optimal mobile phase for the simultaneous determination of UA (**1**) and UAL (**2**) in *E. tereticornis* leaf extract, namely methanol-water acidified to pH-3.5 with TFA (88:12 v/v) with a flow rate 1 mL min^{-1} . Figure 2 (a, b and c) shows the HPLC chromatogram detected at 210 nm.

For validation of analytical methods, the guidelines of International Conference on the Harmonization [ICH-Q2 (R1)²⁸] recommend accomplishment of accuracy tests, precision and linearity of the method. The type of method and its respective use determine the parameter to be evaluated, especially, when the samples are complex biological matrices, as the case of herbal extracts UA (**1**) and UAL (**2**) presented retention time of 9.7 and 16.4 min. The calibration curves for UA and UAL were linear in the range of 0.05-0.3 mg mL^{-1} . The representative linear equations between peak area (y) against concentration (x)

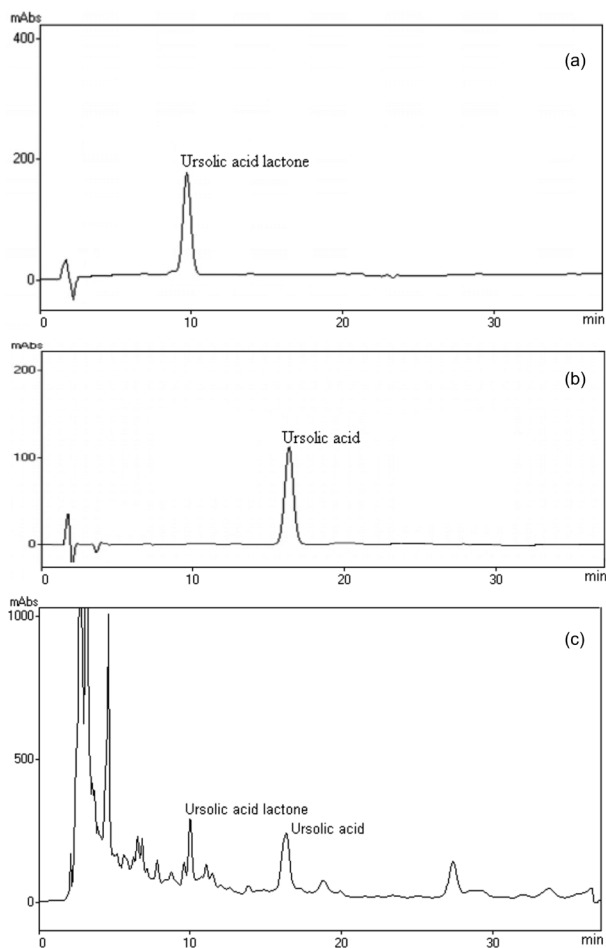


Figure 2. HPLC chromatograms of standards (a) UAL (r.t. = 9.8 min), (b) UA (r.t. = 16.3 min), and (c) ethyl acetate extract of *E. tereticornis* leaf (UAL: r.t. = 9.7 min, UA: r.t. = 16.4 min) detection at 210 nm.

were $Y = 394113x + 513665$ ($r^2 = 0.999$) and $Y = 353923x + 450929$ ($r^2 = 0.998$). The correlation coefficients were greater than 0.99 ($r^2 > 0.998$) indicating high degree of correlation and good linearity of the method.

The limit of detection (LOD), which can be detected but not necessary quantified under the stated experimental condition, were 0.190 and 0.176 μg for UA (**1**) and UAL (**2**) respectively. The limit of quantification, which can be determined with acceptable precision and accuracy, were 0.644 and 0.587 μg for UA (**1**) and UAL (**2**), respectively. These results indicate that the method provided adequate sensitivity. The intra-day and inter-day precision of the method for UA (**1**) and UAL (**2**) were measured by peak area determination of triterpenoids in triplicate. The low value of %RSD ($< 2\%$) reflect the high precision of the method. The percentage recoveries for the intra-day accuracy were 97.32-98.45 and 96.25-97.51 and those for inter-day accuracy were 97.51-98.27 and 96.31-97.15 for UA (**1**) and UAL (**2**) respectively. Both percentage recoveries were within 96.3-98.5 indicating good accuracy

of the method. The method was used to quantify the amount of UA (**1**) and UAL (**2**) in 5 samples of ethyl acetate extract of *E. tereticornis* leaves. The percentages of UA (**1**) and UAL (**2**) in the *E. tereticornis* ethyl acetate extract were $1.7 \pm 0.15\%$ and $0.8 \pm 0.04\%$ (m/m), respectively. In our method UA has been detected at 16.3 min, while in a previous method it was detected at 19.5 min. In this way our method has a time advantage of 16.4% over the previous method. Another advantage of our method is the low acidic pH (3.5) of mobile phase in comparison to the high acidic pH (2.8) in the previous method.²³

Conclusions

In this study, we developed a simple, precise, and accurate HPLC method for the simultaneous quantification of ursolic acid (**1**) and ursolic acid lactone (**2**) in leaf of *E. tereticornis*. The experimental conditions, including the mobile phase composition, column temperature and flow rate were optimized to provide high resolution and reproducible peaks. From preliminary experiments, the best results were obtained using a mobile phase of methanol: water acidified to pH 3.5 with TFA (88:12, v/v). A flow rate of 1 mL min^{-1} was found to be appropriate for shortening the run time without compromising the peak resolution. A controlled column temperature of $25 \pm 1 \text{ }^\circ\text{C}$ was required to obtain reproducible results. The method was validated in compliance with the International Conference on Harmonization (ICH) guideline [ICH-Q2 (R1)]²⁸ and is suitable for the simultaneous determination of individual triterpenoids in the leaf extract of *E. tereticornis* with excellent precision, accuracy, and linearity. The method is isocratic with an uncomplicated mobile phase, and the sample preparation and assay procedures are simple and rapid. This is for the first time, a HPLC method for the quantitative determination of ursolic acid lactone (UAL, **2**) has been developed with high precision and accuracy. Therefore we suggest that this method can be routinely used for the analysis of UA (**1**) and UAL (**2**) in the extracts and formulations containing *E. tereticornis* leaves.

Supplementary Information

Supplementary data are available free of charge at <http://jbc.sbj.org.br> as PDF file.

Acknowledgments

Financial support for this research by CSIR Network project NWP 09 is gratefully acknowledged. We also thank all of our colleagues for their excellent assistance.

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Submitted: May 13, 2011

Published online: January 24, 2012