ABSTRACT

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**Mimusops elengi** Linn., commonly called as bakul, is found all over India. It is a famous plant for its fragrant flowers. Each part of this plant possesses medicinal properties. A simple and reverse phase high performance liquid chromatographic (HPLC) method has been developed for the quantitative determination of betulinic acid and ursolic acid which are triterpene acids present in stem bark of *Mimusops elengi* Linn. The developed HPLC method was validated using International Conference on Harmonization (ICH) guidelines. The developed HPLC method was found to be rapid, simple and precise and provided good resolution of betulinic acid and ursolic acid from other phytoconstituents present in the dried stem bark powder of *Mimusops elengi* Linn.

**Keywords:** *Mimusops elengi* Linn., betulinic acid, ursolic acid, triterpene acids, High performance liquid chromatography, Sapotaceae

INTRODUCTION

*Mimusops elengi* Linn., is a medicinal plant belonging to family Sapotaceae. It is a small to large evergreen tree which grows up to 15 m in height and has many medicinal properties. The stem bark of *Mimusops elengi* Linn. is reported to have wound healing\(^1\)\(^,\)\(^2\) anti-oxidant\(^3\)\(^,\)\(^4\), anti-hyperglycaemic\(^5\)\(^,\)\(^6\) anti-urolithiatic\(^7\) and antibacterial activity\(^8\). The stem bark is reported to contain many phytochemicals such as β-amyrin, baccic acid, betulinic acid, lupeol, taraxerone, taraxerol, ursolic acid, α-spinasterol, β-D-glucoside of β-sitosterol and quercetol\(^9\). Betulinic acid and Ursolic acid are triterpene acids. Betulinic acid is useful for its pharmacological properties, especially in the treatment of diarrhoea, dysentery and cholera\(^9\). Ursolic acid has several important biological activities like anti-inflammatory and antioxidant properties. It is effective for in vitro reduction of growth of a variety of cancer cells\(^11\). It also possesses hepatoprotective, anti-ulcer, hypolipidemic and anti-atherosclerotic potential\(^12\). It is significant anti-tumourigenesis and antioxidant agent\(^13\). A reverse phase HPLC method has been developed for separation of three triterpene acids betulinic acid, ursolic acid and oleic acid\(^13\). The separation was carried on C18 silica Lichrosopher 100RP-18 column (125mm x 4mm, 5μm). The detection was done at \(λ= 225\) nm. Another HPLC method has been reported for identification of betulinic acid and ursolic acid from apple\(^15\). The separation was achieved on Develosil RP aqueous column (250mm x 4.6mm, 5μmPhenomenex). The detection was done at \(λ=210\) nm. However, no HPLC method is reported for the simultaneous quantitation of betulinic acid and ursolic acid from stem bark of *Mimusops elengi* Linn. Considering medicinal properties of betulinic acid and ursolic acid, in the present research work, a high performance liquid chromatographic method was developed for separation and quantitation of betulinic acid and ursolic acid. Thus, precise and accurate HPLC method has been developed and validated using International Conference on Harmonization (ICH) guidelines for simultaneous determination and quantitation of betulinic acid and ursolic acid from dried stem bark powder of *Mimusops elengi* Linn.

MATERIALS AND METHODS

**Experimental Reagents**

All the solvents used in the analysis were of HPLC grade. Methanol (purity- 99.7 %), acetonitrile (purity- 99.8 %) and distilled water used were procured from LiChrosolv Merck, India.

**Reference Standards**

The reference standards betulinic acid (purity \(\geq 98.0\%\) HPLC Grade) and ursolic acid (purity \(\geq 90.0\%\) HPLC Grade) were purchased from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinheim, Germany).

**Plant Material**

The stem bark of *Mimusops elengi* Linn., was collected from Keshav Srushti, Mumbai, India. Herbarium of the plant was prepared and authenticated from Botanical Survey of India (BSI), Pune, India. The herbarium voucher specimen number was DHMMIE3. The stem barks of *Mimusops elengi* Linn., were washed with water to remove soil particles, dried at 45 ± 2°C\(^16\), powdered and then sieved through BSS mesh no. 85 and stored in an airtight container at room temperature (25 ± 2°C).
Preparation of Solutions
Preparation of stock standard solutions of betulinic acid and ursolic acid
Stock solutions of betulinic acid and ursolic acid with concentration of 1000.0 µg/mL were prepared.

Preparation of sample solution
About 1.0g of finely powdered stem bark powder of *Mimusops elengi* Linn., was extracted in 10mL of methanol. The extract was passed through 0.45 µm nylon filters (Millipore) before the analysis.

HPLC Conditions
HPLC analysis was performed using Shimadzu UFLC Prominence chromatograph, equipped with binary gradient pump (LC-20AD) and fitted with auto sampler (SIL-20 AC HT) and oven (CTO-20 AC). A reversed phase, phenomex RP C18 (250mmx 4.6mm, 5µm) column was used for the chromatographic separation. The detection was done using PDA detector (SPD-M20A). LC solution chromatographic software was used for data acquisition. The mobile phase used is water and acetonitrile with the volume ratio of 88:12. The injection volume was 10µL. The detection was done using PDA detector (SPD-M20A) at λ = 210 nm.

Method Validation
Linearity
Preparation of calibration curve of betulinic acid and ursolic acid
Each standard solutions of betulinic acid in the concentration range of 1.0µg/mL to 750.0 µg/mL and 10.0 µL of each standard solution of ursolic acid in the concentration range of 1.0 µg/mL to 500.0 µg/mL were injected in triplicates into the chromatographic system in triplicate, under the optimized chromatographic conditions. The peak areas were recorded for each injected concentration of betulinic acid solution. The calibration curves of both betulinic acid and ursolic acid were obtained by plotting graphs of mean peak areas vs. corresponding concentrations. The results listed in Table 1.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)
The limit of detection (LOD) and limit of quantitation (LOQ) were determined at signal to noise ratios of 3:1 and 10:1, respectively. The LOD and LOQ values obtained for both the components are listed in Table 1.

System Suitability
System suitability was carried out to verify that resolution and reproducibility of the system were acceptable for the analysis. Mixture of standard solution of betulinic acid with concentration of 500.0 µg /mL and ursolic acid with concentration of 100.0 µg /mL was injected in six replicates under optimized chromatographic conditions. The values of percent relative standard deviations of peak area and retention time of standards were taken as an indicator of system suitability and are less than 2, indicating that the method is suitable for analysis.

Precision
The method was validated in terms of repeatability and intermediate precision. The repeatability was evaluated by triplicate analysis of three sample solutions prepared separately. The intermediate precision of the method was evaluated by analyzing three sample solutions in triplicate on three different days, under the optimized chromatographic conditions. The peak areas of betulinic acid and ursolic acid were recorded. The precision results were expressed as percentage relative standard deviations of peak areas of betulinic acid and ursolic acid and are listed in Table 1. The results indicate that the proposed method is precise and reproducible.

Robustness
The robustness of the method is a measure of method’s capacity to remain unaffected by small deliberate variations in the method parameters and provides an indication of reliability of the method during normal usage. The mobile phase composition was altered to acetonitrile: water (89.0:11.0/v/v) and acetonitrile: water (88.0:12.0/v/v) and flow rate was changed to 0.9 mL/min and 1.1 mL/min. The resolution between betulinic acid and ursolic acid and tailing factors of both components from sample solution did not change much due to alteration in the methods. The amounts of betulinic acid and ursolic acid from dried stem bark powder of *Mimusops elengi* Linn. obtained by altered method and that obtained by normal method was found to be similar. The modifications did not affect the system suitability criteria. However, slight variation in the retention time was observed, which was due to changes made in the mobile phase composition and flow rate. From the observations, it was concluded that the method is robust as the above mentioned deliberate changes made in the method did not affect the results.

Assay procedure
The developed and validated HPLC method was used for quantitation of betulinic acid and ursolic acid from the methanolic extract of dried stem bark powder of *Mimusops elengi* Linn. Amounts of betulinic acid and ursolic acid present in the sample solution were determined from the calibration curve, by using the peak area of betulinic acid and ursolic acid in the sample.

Recovery
The accuracy of the method was established by performing recovery experiment by using standard addition method at three different levels. The results of accuracy are listed in Table 2.

RESULTS AND DISCUSSION
Different mobile phases were tried for simultaneous HPLC separation of betulinic acid and ursolic acid from other components of the dried stem bark powder of *Mimusops elengi* Linn. and good separation was achieved by using water: acetonitrile (88.0:12.0 v/v) as mobile phase. Detection was carried out at λ = 210 nm as both betulinic acid and ursolic acid showed maximum response at this wavelength.
Table 1: Method validation data for simultaneous quantitation of betulinic acid and ursolic acid

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear range (n=3) (µg/mL)</td>
<td>Betulinic acid</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.999</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>0.30</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>1.0</td>
</tr>
<tr>
<td>System suitability (%R.S.D.)</td>
<td>Less than 2</td>
</tr>
<tr>
<td>Repeatability (% R.S.D.) (n=3)</td>
<td>0.88</td>
</tr>
<tr>
<td>Intermediate precision (% R.S.D.) (n=9)</td>
<td>0.96</td>
</tr>
<tr>
<td>Assay (mg/g)</td>
<td>4.6523</td>
</tr>
</tbody>
</table>

Table 2: Results of recovery study for simultaneous HPLC quantitation of betulinic acid and ursolic acid from methanolic extract of dried stem bark powder of *Mimusops elengi* Linn.

<table>
<thead>
<tr>
<th>Level</th>
<th>Amount of sample (g)</th>
<th>Amount of standard added to sample (mg)</th>
<th>Mean amount of standard found (mg) ±S.D.</th>
<th>Percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.005</td>
<td>4.6365 ± 0.0488</td>
<td>97.71</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.003</td>
<td>4.7331 ± 0.0325</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.002</td>
<td>4.8258 ± 0.0305</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.005</td>
<td>4.9313 ± 0.0356</td>
<td></td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>0</td>
<td>1.005</td>
<td>1.3179 ± 0.01051</td>
<td>98.65</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.003</td>
<td>1.4126 ± 0.01067</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.002</td>
<td>1.5204 ± 0.01051</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.005</td>
<td>1.6108 ± 0.01256</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ±S.D. (n=7)

Figure 1: HPLC chromatograms obtained for mixture of standard betulinic acid and standard ursolic acid

Figure 2: HPLC chromatograms obtained for methanolic extract of dry stem bark powder of *Mimusops elengi* Linn.
The developed HPLC technique is precise, specific and accurate and can be used for the routine quality control analysis and simultaneous quantitative determination of betulinic acid and ursolic acid from the dried stem bark powder of *Mimusops elengi* Linn.

ACKNOWLEDGMENTS
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REFERENCES

CONCLUSION
The developed HPLC technique is precise, specific and accurate and can be used for the routine quality control analysis and simultaneous quantitative determination of betulinic acid and ursolic acid from the dried stem bark powder of *Mimusops elengi* Linn.

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