Application of modern RP-HPLC technique for the quantitation of betulinic acid from traditional drug *Symplocos racemosa* Roxb.

Sunita Shailajan¹*, Sasikumar Menon², Dipti Singh¹, Gauri Swar¹, Suhina Bhosale¹
¹Herbal Research Lab, Ramnarain Ruia College, Matunga, Mumbai 400 019, India.
²Institute for Advanced Training and Research in Interdisciplinary Sciences (IATRIS), Sion (East), Mumbai 400 022, Maharashtra, India.

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**ABSTRACT**

Objective: *Symplocos racemosa* Roxb. is traditionally used in various ailments like leprosy, diarrhoea, dysentery, menorrhagia and uterine disorders etc. The plant exhibits these therapeutic effects due to the presence of different biologically active phytoconstituents like ellagic acid, symplocoside, betulin, oleanolic acid, β-sitosterol, betulinic acid, etc. The bark has been reported to possess antiandrogenic, anticancer, antioxidant, antiulcer, hepatoprotective, angiogenic activity, in gynecological disorders etc. Therefore, the bark of *S. racemosa* has been an important ingredient of many traditional and Ayurvedic formulations like Rodhrasava, Pushyanug churna, Dashmularista, etc. The present study aims to develop and validate an efficient RP-HPLC method for the quantification of betulinic acid from the bark of *Symplocos racemosa* and an Ayurvedic formulation.

**Material and Methods**: Separation was achieved on C₁₈-column using acetonitrile:distilled water (85:15, v/v) as the mobile phase at a flow rate of 1 mL/min. The detection was carried out at 210 nm.

Result and Conclusion: The linearity range was 5-150 µg/mL. The content of betulinic acid in *Pushyanug churna* was found to be 2.55±0.039 mg/g. The validated method was found to be simple, sensitive, accurate, rugged and reproducible. The developed method can be recommended for marker-based standardization of traditional formulations containing bark of *S. racemosa*.

**Key words**: *Symplocos racemosa*, Lodhra, Betulinic acid, RP-HPLC, Validation.

**INTRODUCTION**

*Symplocos racemosa* Roxb. (Symplocaceae), is an evergreen tree found in the plains and lower hills throughout India (Jadhav et al., 2013). The bark of *S. racemosa* is traditionally used in treatment of liver and bowel complaints, tumors, uterine disorders, asthma, fever, snake-bite, gonorrhea and arthritis, gynaecological disorders, menorrhagia, leucorrhrea & other menstrual disorders. (Acharya et al., 2016; Singh et al., 2015; Bhusnar et al., 2014; Bhutani et al., 2004). Bark of *S. racemosa* is reported for anti-cancer, hepatoprotective, antioxidant, anti-androgenic, anti-inflammatory, wound healing, anti-microbial and anti-diabetic activity (Acharya et al., 2016; Menghani et al., 2011). The therapeutic use of the plant is principally attributed to the presence of biomarkers (Shailajan et al., 2015) of which betulinic acid, pentacyclic triterpenoid (Figure 1), is one of the bioactive phytoconstituents betulinic acid has a wide range of biological and medicinal properties, including anti-human immunodeficiency virus (HIV), antibacterial, antimalarial, anti-inflammatory, antihelmintic, antiinocceptive, anti-herpes simplex viruses-1 (HSV-1), immune-modulatory, antiangiogenic, and anticancer activity (Sook et al., 2015).

![Fig.1: Structure of betulinic acid.](image-url)
Literature survey reveals that various chromatographic methods are available for quantitative estimation of betulinic acid from different plants (Pai et al., 2011; Mukherjee et al., 2010). However, there are few HPTLC and rapid HPLC methods available for analysis of betulinic acid from *S. racemosa* bark and traditional formulations. Taking this into consideration, an attempt has been made to develop a simple, precise, rapid and effective HPLC method for the estimation of betulinic acid from the bark of *S. racemosa*.

As an application of developed method, betulinic acid has been quantified from bark of *S. racemosa* collected in different seasons and from a known Ayurvedic formulation *Pushyanug churna*.

**MATERIALS**

**Plant material**

The bark of *S. racemosa* was collected from Mahabaleshwar, Maharashtra and authenticated by Agharkar Research Institute, Pune (Authentication No. ARI 10-173) and a voucher specimen was deposited for further reference. Bark of *S. racemosa* was collected in two different seasons viz. summer and winter, to study seasonal variation in the content of betulinic acid. Samples were shade dried for 7 days, then dried at 37±2°C for 2 days, powdered in a mixer grinder, sieved through 85 mesh (BSS) and stored in air-tight containers.

**Reference standards and chemicals**

Betulinic acid (Purity ≥ 98%) was purchased from Sigma-Aldrich. HPLC grade solvents like methanol, acetonitrile and distilled water were procured from Merck Specialties Pvt. Ltd., Mumbai, India.

**Marketed sample**

The traditional Ayurvedic formulation, *Pushyanug churna* containing *S. racemosa* as one of the key ingredient (Baidyanath, Batch No. 150012), was purchased from Pharmacy in Mumbai.

**METHODS**

**Preparation of standard solution:**

10.0 mg of standard (Betulinic acid) was accurately weighed and transferred to 10.0 mL standard volumetric flask. The content was initially dissolved in minimum quantity of methanol, sonicated and then diluted up to the mark with methanol. The stock solution of 1000.0µg/mL was used to prepare working solutions of 100.0µg/mL, 10.0µg/mL and 1.0 µg/mL.

**Optimization of Extraction Conditions**

**Extraction condition for *S. racemosa***

Powder (2.0 g) of *S. racemosa* was extracted in methanol (10.0 mL) into stoppered conical flask. The mixture was vortexed for 1 min, sonicated for 5 min, kept on shaker for 6h, filtered through Whatman filter paper no.1 and then filtered through millipore filter (0.45 µm). The filtrates were used for further HPLC analysis.

**Extraction conditions for marketed formulation (*Pushyanug churna*)***

The marketed formulation *Pushyanug churna* was extracted in methanol in the ratio 1:10 (w/v). The mixture was vortexed for 1 min, kept standing overnight, sonicated for 5 min, filtered through Whatman filter paper no.1 followed by filtration through millipore filter (0.45 µm) and the filtrate was used for further HPLC analysis.

**INSTRUMENTATION AND OPTIMIZED CHROMATOGRAPHIC CONDITIONS**

**HPLC conditions**

HPLC analysis was performed at room temperature on JASCO’s HPLC system equipped with PU pumps (HG-1580-31) and a rhodyne injector. Separation was achieved on a Cosmosil C18 (150 X 4.6 mm, 5.0 µm) column with PDA detector (MD-1510). Samples were eluted using mobile phase of acetonitrile: distilled water (85: 15, v/v), delivered at a flow rate of 1.0 mL/min. Detection was carried out at 210 nm. The injection volume was 20 µL for all runs. Data acquisition and analysis were carried out using Jasco-Borwin Chromatography Software, version 1.5.

**Validation of the method**

The analytical method was validated for linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), specificity, and robustness, in accordance with ICH guidelines. Further statistical evaluations were performed.

**Selectivity and specificity**

During the experiment, using PDA detector, UV scan from 200-400 nm in the time window of the analytes was performed to reveal interfering compounds and to evaluate the selectivity of the method. Specificity of the intended method was established by comparing the HPLC retention time and absorption spectra of target peaks from the analyzed samples with that of the reference standard.

**System suitability**

System suitability experiment was assessed by injecting five consecutive injections of betulinic acid at a concentration of 10.0 µg/mL. Values with relative standard deviation (RSD) of ≤ 2% for peak areas and retention time (Rt) were accepted.

**Calibration curves**

Linearity of betulinic acid was determined at seven different equi-spaced concentrations in triplicate and plotted using linear regression of the mean peak area versus concentration. A
straight line fit was made through the data points by least-square regression analysis in order to obtain linear regression equation (y= mx+c).

**Sensitivity**

LOD and LOQ were estimated by measuring the signal-to-noise ratio (S/N). Stock solution of betulinic acid was serially diluted with methanol to prepare the series of samples with low concentrations and injected into the HPLC system. LOD and LOQ was considered at S/N of 3:1 and 10:1, respectively.

**Precision**

Intra-day precision was evaluated from replicate analysis (n=3) of the three quality control samples on the same day while inter-day precision was assessed by analyzing them on three consecutive days in triplicate (n=3). Accuracy values within the range of 85–115% and % RSD of ≤ 2 was the acceptance criteria.

**Stability**

Samples in triplicate were subjected to bench top and short term stability testing at 0.0 h and 2.0 h at RT, 0.0 h and 6.0 h at 4˚C, respectively. Long term stock solution stability of betulinic acid was tested at 4˚C and samples were analyzed in triplicate after 15 days. Values within a difference range of ±5% were accepted.

**Robustness**

Robustness of the method was assessed by deliberately modifying the experimental conditions in terms of four factors such as analyst (analyst 1 and 2), batch of column [columns of two different lot/batch from same manufacturer (Cosmosil, K64005 and K65113)], change in mobile phase composition (1.0 ± 0.1 mL) and flow rate (1.0 ± 0.1 mL/min). The chromatographic variations were evaluated by analyzing the effect on peak areas and R of the three QC samples of betulinic acid in triplicate. The results were expressed in terms of % mean difference. Values within a range of ±5% were accepted.

**Recovery**

The accuracy of the method was evaluated by measuring the recovery of betulinic acid using the standard additions method. Quality control samples at three concentrations were added to the known amount of S. racemosa bark, extracted and analyzed. Each set of additions was repeated thrice at each level. The results were calculated using the formula: Recovery (%) = [(amount found) / (original amount+ amount added)] × 100 and expressed as the percentage of analyte recovered.

**ASSAY AND METHOD APPLICATION**

The developed method was used to determine the content of betulinic acid from samples of S. racemosa collected from Mahabaleshwar in different seasons and from the marketed Ayurvedic formulation Pushyanug churna. Relative retention time and relative peak area of each characteristic peak from the samples of S. racemosa and its formulation related to the peak from betulinic acid were calculated using regression equation. The quantitative expression of the analyte in the chromatographic pattern of S. racemosa was derived.

Microsoft Excel was used to determine mean, standard deviation, relative standard deviation and mean difference during the analysis.

**RESULTS**

**Estimation of betulinic acid from the bark extract of S. racemosa collected from Mahabaleshwar in two different seasons**

The method was applied to evaluate the content of betulinic acid from the bark of S. racemosa collected from Mahabaleshwar (Maharashtra, India) in two different seasons viz summer and winter. The peak of betulinic acid from these samples was identified by comparing their retention times obtained from that of the peak of standard (Figure 3).

It was observed that the sample collected from Mahabaleshwar in summer showed maximum content of betulinic acid than the sample collected in winter. Results are summarized in (table 4)

![Fig. 2: Linearity plot for betulinic acid.](image)

**Estimation of betulinic acid from Pushyanug churna (Ayurvedic formulation)**

The method was further applied to an Ayurvedic formulation Pushyanug churna containing S. racemosa as one of the key ingredient. A single peak was observed at the same retention time in the sample of Pushyanug churna (Figure 3). There was no interaction between betulinic acid and other excipients present in the formulation of Pushyanug churna. The content of betulinic acid in Pushyanug churna was 2.5486 ± 0.03933 mg/g which was found to be less as per the label claim.
Table 1: Results of validation of betulinic acid using RP-HPLC technique.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Linearity (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration range (µg/mL)</td>
<td>5.0 - 150</td>
</tr>
<tr>
<td>Regression equation</td>
<td>y=3111.x -10285</td>
</tr>
<tr>
<td>Correlation coefficient ($r^2$)</td>
<td>0.998</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td></td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>2.5</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>System suitability (% RSD , n=5)</strong></td>
<td></td>
</tr>
<tr>
<td>Rt (min)</td>
<td>0.80</td>
</tr>
<tr>
<td>Area</td>
<td>1.44</td>
</tr>
<tr>
<td><strong>Precision (% RSD, n = 3)</strong></td>
<td></td>
</tr>
<tr>
<td>Parameters</td>
<td>Area</td>
</tr>
<tr>
<td>Intra-day</td>
<td>0.68-1.53</td>
</tr>
<tr>
<td>Inter-day</td>
<td>0.61-1.57</td>
</tr>
</tbody>
</table>

Table 2: Recovery of betulinic acid from the sample.

<table>
<thead>
<tr>
<th>Amount spiked (µg/mL)</th>
<th>Concentration after spiking (µg/mL)</th>
<th>Amount recovered (µg/mL)</th>
<th>% Recovery (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5 (LQC)</td>
<td>74.5</td>
<td>64</td>
<td>85.90</td>
</tr>
<tr>
<td>25 (MQC)</td>
<td>93.87</td>
<td>79</td>
<td>84.15</td>
</tr>
<tr>
<td>120 (HQC)</td>
<td>188.87</td>
<td>160</td>
<td>87.71</td>
</tr>
</tbody>
</table>

Table 3: Results of robustness for betulinic acid by variation in analyst, batch of column, flow rate and mobile phase composition.

<table>
<thead>
<tr>
<th>Parameter (n=3)</th>
<th>Change</th>
<th>$R_i$</th>
<th>% Mean difference</th>
<th>% RSD</th>
<th>% Mean difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyst</td>
<td>Analyst1</td>
<td>0.14-0.89</td>
<td>--</td>
<td>0.79-1.94</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Analyst 2</td>
<td>0.11-1.75</td>
<td>0.30-3.98</td>
<td>0.64-1.50</td>
<td>0.11-1.54</td>
</tr>
<tr>
<td>Column</td>
<td>Column 1</td>
<td>1.00-1.49</td>
<td>--</td>
<td>0.47-1.62</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Column 2</td>
<td>0.36-1.27</td>
<td>-3.86-1.79</td>
<td>0.78-1.08</td>
<td>-3.11-2.49</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.9 ml/min</td>
<td>1.26-3.91</td>
<td>-1.67-(-0.04)</td>
<td>0.15-1.47</td>
<td>-1.88-1.78</td>
</tr>
<tr>
<td></td>
<td>1.0 ml/min</td>
<td>0.38-1.60</td>
<td>--</td>
<td>1.40-1.73</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>1.1 ml/min</td>
<td>0.10-1.29</td>
<td>-0.41-0.61</td>
<td>1.06-1.93</td>
<td>-4.45-1.45</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>84:16 (v/v)</td>
<td>0.00-1.42</td>
<td>0.25-3.21</td>
<td>1.54-1.86</td>
<td>-3.22-(-0.85)</td>
</tr>
<tr>
<td></td>
<td>85:15 (v/v)</td>
<td>0.45-0.67</td>
<td>--</td>
<td>0.76-1.58</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>(ACN: D/W)</td>
<td>0.39-0.78</td>
<td>-2.22-(-0.16)</td>
<td>0.80-1.68</td>
<td>-2.69-(-1.15)</td>
</tr>
</tbody>
</table>

Table 4: Result of assay and method application for betulinic acid.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Content of betulinic acid (mg/g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symplocos racemosa (summer)</td>
<td>0.238 ± 0.0238</td>
</tr>
<tr>
<td>Symplocos racemosa (winter)</td>
<td>0.084 ± 0.0063</td>
</tr>
<tr>
<td>Marketed formulation Pushyanug churna</td>
<td>2.5486 ± 0.03933</td>
</tr>
</tbody>
</table>

* Mean ± SD, n=3.
DISCUSSION

Plant constituents vary considerably depending on several factors like temperature, light, drying, packing, storage etc. (Thakur et al., 2011). These variations impair not only the quality of phytotherapeutic agents but also their therapeutic value. The source and quality of raw materials play a pivotal role in ensuring the quality and stability of herbal formulations (Calixto, 2000). Thus, standardization and quality control of raw material and the final herbal formulations is of utmost importance (Gautam et al., 2010). In the development of the HPLC method for estimation and quantitation of betulinic acid, different chromatographic conditions were tried to improve the separation of marker (betulinic acid) from other peaks in suitable time. Betulinic acid was optimally detected and quantified by using RP-HPLC-PDA with acetonitrile and distilled water (85:15, v/v). The retention time (Rt) of betulinic acid was found to be 5.2 ± 0.002 min under optimized chromatographic conditions. The specificity of the intended method was established by comparing the retention time and absorption spectra of the target peaks from the analyzed samples with the reference compound. The limit of detection (LOD) and limit of quantification (LOQ) was found to be respectively 2.5 µg/mL and 5.0 µg/mL for betulinic acid, suggesting adequate sensitivity of the method. The percent coefficient of variations during the system suitability study was found to be 0.80 and 1.44% respectively for retention time and response of betulinic acid. The response for the marker was found to be linear in the range of 5.0 - 150 µg/mL with a coefficient of determination of 0.998. From the above results, it can be said that betulinic acid showed a broad range of linear detection. This resulted in a regression equation, y = 3111.8x - 10285 for betulinic acid. Inter and intra-day precision for the quality control samples of betulinic acid was within the acceptance limit of 85-115%. Mean recovery for the quality control samples of betulinic acid was found to be 85.92%. Betulinic acid was found to be stable for 6 h at ambient temperature and for 15 days below 8 °C. The method was found to be simple, rapid, specific, precise and rugged as the values obtained were within the acceptance limits. The developed and validated method was applied for the estimation and quantitation of betulinic acid from methanolic extract of S. racemosa samples collected from Mahabaleshwar in different seasons and from an Ayurvedic formulation, Pushyanug churna.

CONCLUSION

The developed RP- HPLC method represents a reliable procedure for detection, separation and quantitation of betulinic acid that has been validated as per ICH guidelines. The method has adequate accuracy, sensitivity, reproducibility and stability. Thus, the developed method can be successfully applied for routine quality check of the plant extracts, phyto-pharmaceuticals or multi-herbal combinations containing S. racemosa which will aid in their standardization. Since bark of other Symplocos species are commonly used as adulterant for Symplocos racemosa, it is very difficult to differentiate adulterants from the crude drug of Symplocos racemosa bark with the naked eye. Safety and quality are important parameters and there is a need for standardization; there is also a chance of contamination with heavy metals, microbial organisms, and other contaminant such as substitution or adulteration of other plant material (Sharma et al., 2010). The developed method can aid in identifying the presence of adulterants and confirming the authenticity of the plant material used. Although betulinic acid is not a plant specific marker, it was chosen for its proven therapeutic efficacy against various ailments and for the quality evaluation of S. racemosa bark. The impact of seasonal variation was clearly evident from the chromatographic analysis indicating that the climatic conditions have a direct effect on the content of chemical phytoconstituents. The variation observed in the formulation (Pushyanug churna) when compared with the label claim could be attributed to the amount of crude drug added or the quality of the material used by the industry. This method can be utilized as routine quality control tool for the quality evaluation of S. racemosa bark and polyherbal formulations. These methods can be applied to other plant raw materials containing the same phytochemical marker. Hence, HPLC is a powerful practical tool for comprehensive quality control of plant raw materials and their final formulations.

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REFERENCES


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