Plant developed analytical profile of *Stereospermum suaveolens* in Indian Traditional Knowledge

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*Stereospermum suaveolens* (Roxb.) DC. is used in folk medicine for the treatment of diabetes, pain, fever, inflammations and asthma. The major phytoconstituents found in the plant bark are lapachol and apigenin exhibit various pharmacological activities. To ensure the content of uniformity of biomarkers, the aim of the present study was to develop a robust RP-HPLC method for simultaneous estimation of lapachol and apigenin in the bark of *Stereospermum suaveolens*. The RP-HPLC method was carried out in reverse phase C18 column using methanol and water (1% glacial acetic acid) as mobile phase in the ratio of 80:20 (v/v), at the flow rate of 1.0 ml/min and the λmax was set at 270 nm. The calibration range of lapachol and apigenin was found to be 10-100 and 1-80 µg/ml, with the linear equation Y= 26513X + 62826 and Y= 23838X + 58264 with coefficient of determinants (r²) of 0.996 and 0.993 respectively. The LOD and LOQ were found to be 0.268±0.520, 0.878±0.183 ng ml⁻¹ (for lapachol) and 0.031±0.36, 0.079±0.85 ng ml⁻¹ (for apigenin). The % RSD of precision and recovery of lapachol and apigenin was < 2.0%. The developed method was accurate, specific, precise and reproducible.

**Keywords:** RP-HPLC, *Stereospermum suaveolens* (Roxb.) DC., Lapachol, Apigenin, Ayurveda

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*Stereospermum suaveolens* (Roxb.) DC. (Family: Bignoniaceae) is commonly known as *Trumpet*. Various parts of the plant are used in folk medicine for the treatment of diabetes, diuretic, pain, fever, inflammations and asthma¹. The flowers with honey mixtures are used for the control of hiccup. The fruit is useful for the treatment of leprosy. The root has an anticancer activity and also used in preparation of Ayurvedic formulation known as *Dashmula*¹⁻³. The plant bark contains sterekunthal B, stereochenols A and B, lapachol, dehydro-α-lapachone, apigenin and leaves contain scutellarein, stereolensin, dinatin (4,5,7-trihydroxy-6-methoxyflavon) and dinatin-7-glucuroniside¹⁴. The major constituents are lapachol (naphthaquinone) and apigenin (flavonoid) have several pharmacological activities, including antimetastatic, antimicrobial, antiviral, anti-inflammatory, antiparasitic, leishmanicidal, molluscicidal, antitumor, anti-abscess, anti-ulcer and anticancer⁵⁻⁷.

The development of marker profiling and standardization of medicinal plants are great importance for finding an optimum concentration of bioactive compounds present in herbal drugs and its formulation⁸. Herbal products and traditional formulation has major concern about quality related safety issue and quantification of bio-active constituents presents therein, several works in this context has been made from our laboratory⁹. However, lack of strict guidelines on the assessment of safety, efficacy and quality control of medicinal plant for their promotion and developments become difficult¹⁰. In this context, the HPLC is one of the most convenient and comprehensive separation technique for separating individual components in plant extracts which has a great importance in relation to authentication, fingerprinting, quantification, quality control in herbal industry¹¹. The chromatographic separation of active compounds mainly based on their structure, so the simultaneous estimation of lapachol and apigenin is highly obvious due to their structural resemblance¹². A thorough literature survey revealed that there is no report available for simultaneous estimation of lapachol and apigenin present in this plant. In *Stereospermum suaveolens* bark (SSB) contains many phytoconstituents, but among them lapachol is present in significant amount whereas the amount of apigenin...
is lesser. In this context, the aim of the present study is to develop a validated method for the simultaneous estimation of lapachol and apigenin from SSBME, through RP-HPLC.

Methodology

Extract preparation

*Stereospermum suaveolens* barks (SSB) were collected from the North Bengal region in 2013-2014, throughout the month of December and authenticated in School of Natural Product Studies, Jadavpur University, Kolkata vide voucher specimen number SNPS-1463 for future references. The barks were dried under shade and pulverized by using a mechanical grinder to make a coarse powder. Then powdered bark (970 gm) was extracted with 95% methanol at room temperature (25 ºC). The whole extract was collected, filtered and the solvent was evaporated to dryness under reduced pressure by using Eyela Rotary Evaporator (Japan) at 40-45 ºC. The concentrated methanol extract (yield 13.21% w/w) was transferred in an amber colored bottle and kept in a desiccator for further use.

Phytochemical analysis

The preliminary phytochemical analysis of the SSBME revealed that the presence of alkaloid, flavonoid, quinine and glycoside.

Chemicals and reagents

The HPLC solvents were using methanol (HPLC grade), water (Milli-Q water) and glacial acetic acid (HPLC grade). Other chemicals were purchased from Merck Ltd (Mumbai, India). Standard lapachol and apigenin were purchased from Sigma Aldrich (St. Louis, MO, USA).

Instrumentation

The HPLC system (Waters, Milford, MA, USA) used for the analysis was consisted of a 600 controller pump, a multiple-wavelength ultraviolet–visible (UV-Vis) detector equipped with an in-line degasser AF 2489 and a rhodyne 7725i injector equipped with a 20 µl loop. Quantitative estimation was performed with Empower 2 software programs using the external calibration method. A Milli-Q Academic water purification system (Bedford, MA, USA) equipped with 0.22 mm Millipak Express filter and Eyela (Tokyo, Japan) rotary vacuum evaporate were used. Membrane filters of 0.45 mm pore size (Millipore) were used for filtration of the mobile phase and Whatman’s syringe filters (NYL 0.45 mm) were used for the filtration of the sample.

Preparation of standard and sample solutions

A standard solution of lapachol or apigenin was prepared in methanol at the concentration of 1mg/ml. Calibration samples were prepared in the range of 10-100 and 1-80 µg/ml, for lapachol and apigenin, respectively. The sample solution was prepared by dissolving 10 mg of extract in 1ml. Both the standard and sample solutions were filtered through Whatman NYL 0.45 µm syringe filter. The responses were measured as peak areas and plotted against concentration.

Preparation of mobile phase

Mobile phase was prepared by using methanol as solvent A and water as solvent B in the ratio of 80:20 (v/v). The pH of solvent B was adjusted with 1% (v/v) glacial acetic acid and then both solvent was filtered through 0.45 mm pore size (Millipore) membrane filter followed by ultra-sonication to de-gas the solvent.

Method validation

The developed method was validated for linearity, specificity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision according to ICH guidelines.

Linearity

The linearity and calibration range of lapachol and apigenin was analyzing (n = 6) of the mixed standard solutions containing of lapachol 10-100 µg/ml and apigenin 1-80 µg/ml, in the optimized chromatographic conditions. The calibration curve was made by plotting the main peak area (Y-axis) against the concentration (X-axis) and linearity was determined by the linear regression analysis.

Specificity

The specificity of the method was determined by comparing the retention time of both standard and test samples. This mainly ensures the identity and the purity of the analyte and to minimize the error due to the contamination of the sample.

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

The Limit of Detection (LOD) and Limit of quantification (LOQ) was calculated based on the ICH guideline by determining the SD of the response (σ) and the slope of the linear equation (S). The LOD
and LOQ were calculated by the following equation
\[ \text{LOD} = 3.3 \frac{\sigma}{S}, \quad \text{LOQ} = 10 \frac{\sigma}{S}. \]

**Accuracy and precision**

Intra-day and inter-day assay accuracy and precision for each analyte were determined at LQC (low quality control), MQC (medium quality control) and HQC (high quality control). Intra-day and inter-day assay, both data were assessed by comparing data from within one run (n=6). Accuracy of the method was determined by standard addition technique and expressed in terms of % RSD for mean recovery of the theoretical concentration. The samples were spiked with three different amounts of standard compounds in triplicate and analyzed under the previously established optimal condition. The precision of the analytical method was assessed by performing intra-day and inter-day variation, assessed by injecting six replicates at three different concentrations of the reference compounds. Values were represented as % RSD of intra-day and inter-day runs.

**System suitability parameters**

System suitability testing was performed by using six replicates of test concentrations. Variations in number of theoretical plates, capacity factor and tailing factor were calculated as average of six replicates.

**Robustness**

Robustness study was performed by changing different mobile phase composition, flow rate and detection of wave length to determine their influence on the retention time.

Statistical analysis was performed using the Graph Pad Prism Version 5.0. The results are represented as the mean ± % RSD.

**Results and discussion**

**Optimization of chromatographic conditions**

The chromatographic separation was determined through reversed-phase C18 column (Waters Spherisorb 5 mm ODS2, 250 mm × 4.6 mm, 5 µm particle size) at ambient (25 ºC) conditions. The mobile phase consisted of methanol and water (1% glacial acetic acid) in the ratio of 80:20 (v/v) at 1.0 ml/min flow rate and the λ max was set at 270 nm.

**Linearity**

The calibration range of lapachol and apigenin was found to be 10-100 and 1-80 µg/ml, with the linear equation \( Y = 26513X + 62826 \) and \( Y = 23838X + 58264 \) with coefficient of determinants \( (r^2) \) of 0.996 and 0.993 respectively.

**Specificity**

The specificity test, demonstrated that other constituents present in the SSBME does not interfere with the main peak of lapachol and apigenin. The well-shaped peak indicated that the specificity of the method (Figs. 1 & 2).

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

The LOD and LOQ was found to be 0.268±0.520, 0.878±0.183 ng ml\(^{-1}\) (for lapachol) and 0.031±0.36, 0.079±0.85 ng ml\(^{-1}\) (for apigenin) in Table 1S.

**Accuracy**

The accuracy of the method was evaluated by recovery study. The high recovery values for lapachol (94.98–98.96 %) and apigenin (96.76–99.74 %) indicated the accuracy of the method (Table 2S).

**Precision**

The % RSD of intra-day and inter-day precision was found to be < 2%, which confirms high repeatability of the method (Tables 3S & 4S).

Fig. 1—RP-HPLC chromatogram of lapachol and apigenin (standard).

Fig. 2—RP-HPLC Chromatogram of Stereospermum suaveolens bark methanol extract.
**System suitability parameters**

The number of theoretical plates, capacity factor and tailing factor were found to be 5175, 3561 (desirable > 2000); 5.38, 5.12 (desirable 2–10); 1.39, 1.26 (desirable < 1.5) for lapachol and apigenin, respectively from the mean of six determinations of test concentration (Table 5S).

**Robustness**

The robustness was evaluated by analyzing (n = 6) the standard solution of lapachol (60 µg/ml) and apigenin (40 µg/ml) under the small changes (± 2) in the optimum conditions such as column temperature, flow rate, detection of wavelength and pH set for this method. But no significant changes were observed in the retention time, peak area response and recovery study.

**Determination of lapachol and apigenin in Stereospermum suaveolens bark methanol extract**

The mean retention time was observed 7.96±0.06 min (for lapachol) and 6.34±0.06 (for apigenin) by comparing between standard (Fig. 1) and extract chromatograms (Fig. 2). The amount of lapachol and apigenin found in SSBME was 1.42% and 0.46% (w/w), respectively.

The present method determined two standards in the presence of other phytochemicals in S. suaveolens

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**Table 1 S**—Limit of detection and limit of quantification of lapachol and apigenin

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Linearity range (µg/ml)</th>
<th>LOQ (ng/ml)</th>
<th>LOD (ng/ml)</th>
<th>Correlation coefficient</th>
<th>Regression equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lapachol</td>
<td>10-100</td>
<td>0.878</td>
<td>0.268</td>
<td>&gt; 0.99</td>
<td>Y= 26513X + 62826</td>
</tr>
<tr>
<td>Apigenin</td>
<td>1-80</td>
<td>0.079</td>
<td>0.031</td>
<td>&gt; 0.99</td>
<td>Y= 23838X + 58264</td>
</tr>
</tbody>
</table>

The LOQ and LOD were calculated by the equation of LOD = 3.3 σ/S, LOQ = 10 σ/S, where σ is the standard deviation and S is the slope of the calibration curve. In regression equation Y= peak area and X= sample concentration.

**Table 2 S**—Recovery studies for determination of lapachol and apigenin in SSBME

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Amount added (µg/ml)</th>
<th>Sample concentration (µg/ml)</th>
<th>Theoretical concentration (µg/ml)</th>
<th>Actual concentration (µg/ml)</th>
<th>Percentage recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lapachol</td>
<td>20</td>
<td>1315.3</td>
<td>1335.3</td>
<td>1268.5</td>
<td>94.98</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1315.3</td>
<td>1375.3</td>
<td>1328.8</td>
<td>96.61</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1315.3</td>
<td>1415.3</td>
<td>1400.7</td>
<td>98.96</td>
</tr>
<tr>
<td>Apigenin</td>
<td>10</td>
<td>1232.8</td>
<td>1242.8</td>
<td>1202.6</td>
<td>96.76</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>1232.8</td>
<td>1272.8</td>
<td>1259.3</td>
<td>98.33</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>1232.8</td>
<td>1312.8</td>
<td>1309.5</td>
<td>99.74</td>
</tr>
</tbody>
</table>

The above three concentrations are expressed as µg/ml. The percentage recovery was calculated by the Actual concentration/ Theoretical concentration × 100.

**Table 3 S**—Intra-day and inter-day precision of lapachol by using HPLC method

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Intra-day (n=6)</th>
<th>Inter-day (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>% RSD</td>
</tr>
<tr>
<td>7.35</td>
<td>Mean</td>
<td>1.19</td>
</tr>
<tr>
<td>7.56</td>
<td>1.46</td>
<td>23132</td>
</tr>
<tr>
<td>7.96</td>
<td>0.85</td>
<td>20478</td>
</tr>
</tbody>
</table>

**Table 4 S**—Intra-day and inter-day precision of apigenin by using HPLC method

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Intra-day (n=6)</th>
<th>Inter-day (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>% RSD</td>
</tr>
<tr>
<td>6.83</td>
<td>Mean</td>
<td>1.26</td>
</tr>
<tr>
<td>6.34</td>
<td>1.18</td>
<td>24029</td>
</tr>
<tr>
<td>6.66</td>
<td>1.06</td>
<td>22182</td>
</tr>
</tbody>
</table>

In both tables (3S and 4S), percentage of RSD were calculated by (SD/mean) × 100, where SD= standard deviation.
which indicates that the method is specific. This validated method is suitable for qualitative and quantitative analysis of lapachol and apigenin in the methanolic extract of *S. suaveolens* bark.

This developed method for the simultaneous estimation of lapachol and apigenin was rapid, simple, accurate, specific and precise and reproduced with a narrow linearity range as well as it also reduce the time of analysis. Thus, this method can be commercialized at industrial level for wide scope for separation as well as quality assessment of pharmaceutical preparation.

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**References**