Development of High-performance Thin-layer Chromatography (HPTLC) Validated Method for Simultaneous Quantification of Eucalyptol and α-Pinene in Lamiaceae Plants

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Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim and Objective: Several plants from Lamiaceae family are used in the Saudi Arabia as a condiment and food preparation, and are generally used in the traditional preparation to treat various diseases, including anti-inflammatory, antioxidant, and microbial infections. Some of Lamiaceae species such as Mentha longifolia, Rosemarinus officinalis and Salvia officinalis having pharmacological active compounds such as α-pinene and eucalyptol. The aim of present study was to develop an accurate and precise chromatographic technique for quantification of α-pinene and eucalyptol in the Lamiaceae plants.

Methods: The high-performance thin layer chromatography (HPTLC) method was developed as per International Conference on Harmonization (ICH) guideline.

Results: Simultaneous determination of α-pinene and eucalyptol was achieved by developing a
densitometric analysis of high-performance thin layer chromatography (HPTLC). Silica gel 60 F254 glass-backed plates (E-Merck, Germany, 0.2 mm layers) as stationary phase and mixture n-hexane: ethyl acetate 8 : 2 (%, v/v) as mobile phase were used to produce a sharp, symmetrical and well-resolved peak at an Rf value of 0.19 ± 0.02 and 0.52 ± 0.04 for α-pinene and eucalyptol, respectively. Linearily range for α-pinene was 100–700 ng/spot (r² = 0.9988), whereas that for eucalyptol was 1000–7000 ng/spot (r² = 0.9987).

**Conclusion:** The developed method was found to be a simple, accurate, and precise, and it may be used to simultaneously analyses of many medicinal plants samples containing α-pinene and eucalyptol.

**Keywords:** Eucalyptol; α-pinene; Mentha longifolia; Rosemarinus officinalis; Salvia officinalis; HPTLC and ICH.

1. INTRODUCTION

The extracts and essential oils of *M. longifolia*, *S. officinalis* and *R. officinalis* are widely used as functional ingredients for therapeutics, flavoring, condiments, and food and nutraceutical industries [1,2]. Gas chromatography and mass spectroscopy (GC-MS) analysis have been already evaluated for the presence of both α-pinene and eucalyptol in the leaves of *M. longifolia* [3], *S. officinalis* [4] and *R. officinalis* [5].

A bicyclic monoterpane, (±)-α-pinene (2,6,6-triméthylbicyclo[3.1.1]hept-2-ène) (Fig. 1) is a common terpene found in many medicinally important aromatic dietary plants such as Mentha, holy basil, origanum, rosemary, thyme, ginger, and cardamom, etc. [6-8]. In the essential oils of conifer plants such *Abies concolor* and *Pinus roxburghii*, up to 40% α-pinene content were reported [9,10]. Isolated, α-pinene has been evaluated for pharmacological activities such as bronchodilator, antioxidant, hypoglycemic, antimicrobials, anti-inflammatory, sedative, and gastro protective activities [11]. Compound, α-pinene has been approved as a safe food additive by the U.S. FDA and it is widely used as a flavoring ingredient in several food products [12].

Eucalyptol (1,3,3-Trimethyl-2-oxabicyclo [2.2.2] octane) is a natural monoterpane and also known as 1,8-cineole (Fig. 2). It is found in a number of medicinally active aromatic plants such as mentha, cardamom, artemisia, coriander, origanum, rosemary, thyme, ginger, etc. [6,13-14]. In some of plants such as essential oil of leaves of *Cinnamomum longepaniculatum* and eucalyptus, up to 80% eucalyptol was reported [15]. Isolated, eucalyptol compound is known for several pharmacological activities such as mucolytic, antioxidant, spasmylytic, antiasthmatic, anti-inflammatory, antianxiety and various others respiratory and gastrointestinal diseases [16,17]. There are several reports on the safety of eucalyptol, and very low acute toxic was reported [18].

![Fig. 1. Chemical structure of α-pinene](image1)

High-performance thin liquid chromatography (HPTLC) involves the separation of molecules on the basis of affinity towards the adsorbent. Several instrumentation methods have been reported for the estimation of eucalyptol and α-pinene alone or in combination with other essential oils. HPTLC method developed for only eucalyptol for the essential oil of *Callistemon Citrinus* [19], *Amomum subulatum* [20], and in formulation [21] and in combination menthol and eucalyptol [22]. The other chromatographic (HPLC and GC-MS) methods have been also developed for the quantitative analysis of α-pinene and eucalyptol along with other essential oils [23,24].

![Fig. 2. Chemical structure of Eucalyptol](image2)
Extensive literature survey revealed that several methods were reported for the estimation of eucalyptol with alone and other ingredients by HPTLC but not with α-pinene. Hence, in the present study an attempt has been made to rapid development and validates a simple, accurate, and precise, concurrent HPTLC method for the quantification of α-pinene and eucalyptol in the methanol extract and essential oils of M. longifolia, S. officinalis and R. officinalis leaves.

2. METHODS

2.1 Plant Materials

The leaves of M. longifolia, S. officinalis and R. officinalis were obtained from the local market of Al-Kharj, Saudi Arabia and identified by Dr. Osman A. Elmakki Department of Pharmacognosy, College of Pharmacy, Prince Sattam Bin Abdulaziz University (PSAU), Al-Kharj, Saudi Arabia (SA). A specimen vouchers (M. longifolia: PSAU-02-CPH-2017, S. officinalis: PSAU-03-CPH-2017, Rosemary: PSAU-05-CPH-2017) were preserved in the herbarium, College of Pharmacy (Department of Pharmacognosy), PSAU, Al-Kharj, KSA.

2.2 TLC Plates, Apparatus, Standard and Solvents

Pre-coated, containing silica gel 60F254 backed HPTLC glass plates (10 × 10 cm, 0.2 mm thick) were obtained from E. Merck, (Darmstad, Germany). The extracts and essential oils were applied to the plates as band wise with the help of sample applicators (Camag, Linomat V). The other apparatus such as Camag twin-trough chamber, Camag TLC Scanner 4 and software (WinCATS integration) were used for the development of present method. The standard compounds, α-pinene (≥98%) and eucalyptol (99%) were procured from Sigma-Aldrich. Solvents of analytical grade (AR) were obtained from BDH (UK).

2.3 Extraction of Plant Materials

The preparation of M. longifolia, S. officinalis and R. officinalis leaves methanol extracts and essential oils were done by following our previous method projected for the estimation of linalool [25]. The powder, 100 g of the selected plants were sonicated using methanol to obtain methanol soluble extract and 200 g powder of plants were subjected to hydro-distillation using a Clevenger apparatus for 4h for the separation of essential oils.

2.4 Preparation Standard and Sample Solutions

Analytical grade (AR), methanol was used to prepare the stock solution of standard compounds α-pinene and eucalyptol (1 mg per 1 ml) and the stock solution further diluted to100 μg/ml. Extract and essential oil (10 mg) of each plants (M. longifolia, S. officinalis and R. officinalis) were separately added to 10 ml volumetric flask and the volume was made up to the mark with methanol.

2.5 Chromatographic Conditions, Derivatization and Densiometric Scanning

The quantitative analysis of α-pinene and eucalyptol was carried out on HPTLC plate. 20 ml of hexane: ethyl acetate (8: 2; v/v) was placed in the twin-trough glass chamber and saturated for 15 min at room temperature (25±2°C). The standards and samples were applied on TLC plates using Camag applicator. The TLC plates were transferred to the saturated chamber; Linear ascending development was performed up to 80 mm and thereafter, the plates were removed from the chamber and air-dried for 15 min before derivatization. For the visualization of compounds, the plates were derivatized by spraying anisaldehyde-sulfuric acid reagent after heated at 100°C for 5 min. The densitometric analysis of the separated α-pinene and eucalyptol was carried out by Camag TLC Scanner using deuterium lamps set at 500 nm. The slit dimension used was 4.00 × 0.45 mm, scanning speed was 20 mm/s and sensitivity was kept at auto-mode.

2.6 Preparation of Standard Calibration Curve

For the preparation of calibration, standard solution (1, 2, 3, 4, 5, 6 and 7 μL) of α-pinene was applied on TLC plate to have the concentration in the range of 100, 200, 300, 400, 500, 600 and 700 ng/band. Different volumes of working standard (10 to 70 μL) of eucalyptol were applied on TLC plate to have the concentration in the range of 1000 to 7000 ng/band. The calibration curve was plotted using data of peak areas against the corresponding amount per spot.
2.7 Method Validation

The developed method was validated as per ICH guidelines [26]. The linearity of the method for the analysed standard were checked between 100–700 ng/spot and 1000–7000 ng/spot for α-pinene and eucalyptol respectively. The standard curve was used to calculate the regression equation, correlation coefficient, slope and intercept of the curves.

2.8 Accuracy

Accuracy, as % recovery were performed by addition of α-pinene and eucalyptol at four different levels (0, 50, 100 and 150%) to a pre-analysed sample solution 200 ng/band and 2000 ng/band for α-pinene and eucalyptol respectively. All the experiments were determined in triplicate (n=3). Recovery (%) and relative standard deviation (RSD, %) were calculated for each concentration level.

2.9 Precision

Repeatability of method was obtained from relative standard deviation (RSD, %) value by repeating the assay three times in same day for intra-day precision and different day for inter-day. The intra-day and inter-day variation by HPTLC was carried out at three different concentration levels 300, 400 and 500 ng/spot of standard solution.

2.10 Robustness

The robustness of TLC densitometric method was aimed to determining the influence of small changes in the chromatographic conditions (ratio of the solvent system, distance development, and chamber saturation time) on Rf value and peak area of α-pinene and eucalyptol.

2.11 Limits of Detection and Quantification

Standard deviation (SD) method was used for the determination of limit of detection (LOD) and limit of quantification (LOQ). These were determined from the slope (S) of the calibration curve and standard deviation (SD) of the blank sample. The LOQ and LOD were calculated using Equations 1 and 2 below:

\[
\text{LOD} = 3.3 \times \text{SD}/S \quad (1)
\]

\[
\text{LOQ} = 10 \times \text{SD}/S \quad (2)
\]

2.12 Quantification of Eucalyptol and Alpha-pinene in the Methanol Extract and Essential Oils

The extracted methanol extracts and essential oils from M. longifolia, S. officinalis and R. officinalis and standards were simultaneously injected and chromatograms were obtained under the same conditions as for the analysis of α-pinene and eucalyptol. The peak area of the corresponding to the Rf value of the α-pinene and eucalyptol was recorded. The amount present in the tested extracts were calculated using regression equation obtained from the calibration plot.

3. RESULTS

3.1 Method Development

The mixture of solvents, n-hexane and ethyl acetate 80:20 (%, v/v) was selected as mobile phase and it was resulted in a symmetrical, sharp and well resolved peaks at Rf value of 0.19 and 0.52 for α-pinene and eucalyptol respectively (Fig. 3). Visible light spectra measured for the bands showed maximum absorbance at 500 nm for both α-pinene and eucalyptol respectively. The HPTLC densitograms of standard for α-pinene and eucalyptol respectively in the methanol extract and essential oil of M. longifolia, S. officinalis and R. officinalis were shown in Figs. 4–5.

3.2 Calibration Curve

The peak areas against the amount of α-pinene and eucalyptol were linear in the range of 100–700 ng/spot and 1000–7000 ng/spot respectively. Linear regression data for the plots are presented in Table 1 and the correlation coefficient (R²) was 0.9988 and 0.9987 respectively for α-pinene and eucalyptol which was highly significant (P<0.0001). The linear regression equation was \( y = 10.085x + 385.43 \) and \( y = 0.9283x + 1669.3 \) where \( y \) is response and \( x \) is amount of standard compounds.

3.3 Method Validation

3.3.1 Accuracy

The accuracy of the method, as % recovery, was performed in the triplicate mode and was presented in Table 2. The percentage recovery of α-pinene and eucalyptol were in the range of 97.50-99.17% and 98.84-99.58% respectively.
3.3.2 Precision

The range of percentage relative standard deviation (RSD, %) for intraday (repeatability) precision was 0.63-0.66% and 0.46-0.80% respectively for α-pinene and eucalyptol. Similarly, the RSD (%) range for inter-day precision was 0.76-0.81% and 0.55-0.88% respectively for α-pinene and eucalyptol. Results from determination of repeatability and intermediate precision, expressed as RSD (%), were shown in Table 3.

3.3.3 Robustness of the method

The range of RSD (%) obtained after introducing small deliberate change (robustness) into the densitometric HTLC procedure was 0.28-0.31% and 0.63-0.64% for α-pinene and eucalyptol and the results were reported in Table 4.

3.3.4 Limit of detection and limit of quantification

Results of LOD and LOQ of the proposed method were reported in Table 5.

3.3.5 Quantification of eucalyptol and alpha-pinene in methanol extract and essential oils from M. longifolia, S. officinalis and R. officinalis

Peaks of α-pinene and eucalyptol from methanol extract and essential oils of M. longifolia, S. officinalis and R. officinalis were identified by comparing their RF values with those obtained by chromatography of the standard (Eucalyptol and alpha-pinene) under the same conditions. The presence of α-pinene and eucalyptol content in the extracts and oils were quantified by using linear regression equation and the results were shown in Table 6.

---

**Table 1. Linear regression data for the calibration curve of eucalyptol and alpha-pinene (n=6)**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>α-Pinene</th>
<th>Eucalyptol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (ng/spot)</td>
<td>100-700</td>
<td>1000-7000</td>
</tr>
<tr>
<td>Regression equation</td>
<td>Y = 10.085x + 385.43</td>
<td>Y = 0.9283x + 1669.3</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9988</td>
<td>0.9987</td>
</tr>
<tr>
<td>Slope ± SD</td>
<td>10.085± 0.1721</td>
<td>0.9283 ± 0.1225</td>
</tr>
<tr>
<td>Intercept ± SD</td>
<td>385.43 ± 71.21</td>
<td>1669.3 ± 79.02</td>
</tr>
<tr>
<td>95% confidence interval of slope</td>
<td>4.543 to 5.143</td>
<td>9.1456 to 9.667</td>
</tr>
<tr>
<td>95% confidence interval of intercept</td>
<td>841 to 986.1</td>
<td>5051 to 5173</td>
</tr>
<tr>
<td>P value</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

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Fig. 3. HPTLC densitogram of standard α-pinene (a) and eucalyptol (b)
Table 2. Accuracy of the proposed method (n=3)

<table>
<thead>
<tr>
<th>Excess drug added to analyte (%)</th>
<th>Theoretical content (ng)</th>
<th>Conc. Found (ng) ± SD</th>
<th>% Recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2000</td>
<td>195.00 ± 2.45</td>
<td>97.50</td>
<td>1.26</td>
</tr>
<tr>
<td>50</td>
<td>3000</td>
<td>294.17 ± 3.37</td>
<td>98.06</td>
<td>1.15</td>
</tr>
<tr>
<td>100</td>
<td>4000</td>
<td>396.67 ± 2.16</td>
<td>99.17</td>
<td>0.54</td>
</tr>
<tr>
<td>150</td>
<td>5000</td>
<td>490.17 ± 5.81</td>
<td>98.03</td>
<td>1.19</td>
</tr>
</tbody>
</table>

**4. DISCUSSION**

Folklore or traditional uses of medicinal plants are very common in Saudi Arabia. The medicinal plants belong to the Lamiaceae family are either cultivated in Saudi Arabia or exported from the other parts of the world. The medicinal plants such as M. longifolia, S. officinalis, and R. officinalis are very common and easily available in the local market of Saudi Arabia. The present study was performed for the selected plants purchased from Al-Kharj city local market.
Table 3. Precision of the proposed method

<table>
<thead>
<tr>
<th>Conc. (ng/spot)</th>
<th>Precision</th>
<th>α-Pinene</th>
<th></th>
<th>Eucalyptol</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg. Conc. ± SD (n = 3)</td>
<td>Standard error (SE)</td>
<td>% RSD</td>
<td>Avg. Conc. ± SD (n = 3)</td>
<td>Standard error (SE)</td>
</tr>
<tr>
<td>300 Repeatability</td>
<td>3470.60 ± 2.46</td>
<td>9.17</td>
<td>0.65</td>
<td>3431.00 ± 27.03</td>
<td>11.04</td>
</tr>
<tr>
<td>400 Intraday precision</td>
<td>4537.40 ± 29.89</td>
<td>12.21</td>
<td>0.66</td>
<td>4556.00 ± 27.57</td>
<td>11.26</td>
</tr>
<tr>
<td>500 Intermediate</td>
<td>5448.40 ± 34.08</td>
<td>13.92</td>
<td>0.63</td>
<td>5297.00 ± 24.49</td>
<td>10.00</td>
</tr>
<tr>
<td>300 Intermediate precision</td>
<td>3440.60 ± 26.13</td>
<td>10.67</td>
<td>0.76</td>
<td>3423.00 ± 30.11</td>
<td>12.29</td>
</tr>
<tr>
<td>400 Inter-day precision</td>
<td>4541.40 ± 36.83</td>
<td>15.04</td>
<td>0.81</td>
<td>4558.00 ± 29.94</td>
<td>12.23</td>
</tr>
<tr>
<td>500 Inter-day</td>
<td>5444.40 ± 41.33</td>
<td>16.88</td>
<td>0.76</td>
<td>5299.00 ± 29.27</td>
<td>11.95</td>
</tr>
</tbody>
</table>

Table 4. Robustness of the proposed method

<table>
<thead>
<tr>
<th>Conc. (ng/spot)</th>
<th>Original* (Hexane: Ethyl acetate)</th>
<th>Used</th>
<th>Changed</th>
<th>α-Pinene</th>
<th></th>
<th>Eucalyptol</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area ± SD, (n = 3)</td>
<td>Rf</td>
<td>% RSD</td>
<td>Area ± SD, (n = 3)</td>
<td>Rf</td>
<td>% RSD</td>
<td></td>
</tr>
<tr>
<td>400 8:2</td>
<td>4519.40 ± 46.96</td>
<td>0.18</td>
<td>0.28</td>
<td>5299.00 ± 31.25</td>
<td>0.59</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>8:1.9</td>
<td>4517.40 ± 27.86</td>
<td>0.19</td>
<td>0.30</td>
<td>5295.00 ± 25.63</td>
<td>0.48</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>8:2</td>
<td>4511.40 ± 48.54</td>
<td>0.20</td>
<td>0.31</td>
<td>5293 ± 27.33</td>
<td>0.52</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>8:2.1</td>
<td>+0.1, -0.1</td>
<td>0.20</td>
<td>0.31</td>
<td>5293 ± 27.33</td>
<td>0.52</td>
<td>0.53</td>
<td></td>
</tr>
</tbody>
</table>

*Hexane:Ethyl acetate.
Fig. 5. HPTLC densitogram of α-Pinene and Eucalyptol in essential oils (EOs): (a) *M. longifolia*, (b) *S. officinalis*, (c) *R. officinalis*

Table 5. LOD and LOQ of the proposed method

<table>
<thead>
<tr>
<th>Standard</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>6.28</td>
<td>21.08</td>
</tr>
<tr>
<td>Eucalyptol</td>
<td>8.57</td>
<td>26.89</td>
</tr>
</tbody>
</table>

Table 6. Contents (% w/w) of eucalyptol and alpha-pinene in its methanol extracts (ME) and essential oils (EO)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Eucalyptol</th>
<th>α-Pinene</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. longifolia ME</em></td>
<td>0.017</td>
<td>0.008</td>
</tr>
<tr>
<td><em>S. officinalis ME</em></td>
<td>0.01</td>
<td>0.018</td>
</tr>
<tr>
<td><em>R. officinalis ME</em></td>
<td>0.022</td>
<td>0.012</td>
</tr>
<tr>
<td><em>M. longifolia EO</em></td>
<td>0.26</td>
<td>0.33</td>
</tr>
<tr>
<td><em>S. officinalis EO</em></td>
<td>0.19</td>
<td>0.23</td>
</tr>
<tr>
<td><em>R. officinalis EO</em></td>
<td>0.45</td>
<td>0.34</td>
</tr>
</tbody>
</table>

indicated that the amount of α-pinene and eucalyptol are indifferent range compared with literature reports [3-5]. The developed TLC procedure was optimized with a view to quantifying the presence of α-pinene and eucalyptol in the extracts and essential oils. The current method was developed and validated using ICH guidelines (ICH, 2010). The mobile phase containing n-hexane and ethyl acetate resulted in a sharp, symmetrical, and well-resolved peak at obtained *Rf* value, hence it was optimized for the separation of α-pinene and eucalyptol. In present finding the content of eucalyptol and α-pinene were found higher in the essential oil of *R. officinalis* as compared to *M. longifolia* and *S. officinalis*. Guetat and coworker (2014), reported that the essential oils from *R. officinalis* contents both 1,8-Cineole (23.16%) and α-pinene (19.48%) [27]. Takayama and coworker (2016), reported the essential oil of *R. officinalis* contains cineole (28.5%), and α-pinene (21.3%) [28]. *M. longifolia* and *S. officinalis* have been also reported to have both
eucalyptol and α-pinene but comparatively lower percentage content than R. officinalis [29-31]. In the present report, the content of eucalyptol and α-pinene in the essential oil extracted from R. officinalis is higher than the M. longifolia and S. officinalis. The method explored in the present study could be used for the quality control of products of any herbs which contain α-pinene and eucalyptol. In the previous study, the HPTLC of eucalyptol in different family such as Zingiberaceae [20], Myrtaceae [19], and Lamiaceae [32] have been reported but not for α-pinene. The developed HPTLC method is accurate, precise and sensitive and applicable to the analysis of several aromatic plants containing monoterpenes α-pinene and eucalyptol. It is a simple method to analyze the efficacy of the medicinal plants containing α-pinene and eucalyptol and may help the manufacturer for quality control and standardization of crude essential oil, plants, and herbal formulations. This method will be used for the simultaneous quantification of α-pinene and eucalyptol in the extracts and essential oils of plants.

5. CONCLUSION

The proposed HPTLC method developed for simultaneous quantitative analysis of α-pinene and eucalyptol in the crude extracts of Lamiaceae family plants without any interference. The proposed developed and validated method was found to be accurate, simple, sensitive, and reproducible and is applicable to the analysis of a wide variety of α-pinene and eucalyptol containing products. The present developed method may use for the standardization and quality control of commercial aromatic plants and herbal formulations, food and pharmaceutical products having α-pinene and eucalyptol as the active ingredients.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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