COMPARATIVE TLC AND RP-HPLC FINGERPRINTING ANALYSIS OF THREE SOURCES OF MAYURASIKHA: AN AYURVEDIC DRUG

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ABSTRACT

A simple TLC and precise RP-HPLC method was developed and validated to standardize the three ambiguous botanical sources of Mayurasikha with the help of two active markers (stigmasterol and oleic acid) compounds. TLC study of the three plant extracts showed spots with different RI Values. In HPLC analysis, separation was through a Phenomenex Luna C18 column with mobile phase consisting of acetonitrile: water 87:13 (V/V) at 210 nm. The retention times of stigmasterol and oleic acid were about 2.36 and 7.55 min respectively and the developed method showed good sensitivity, significant linearity ($r^2 \geq 0.9992$) over the concentration ranges 2–12 and 50 – 500 $\mu$g/mL. The mean percentage recovery of stigmasterol was found to be 97.46 – 100.31% and for oleic acid 99.32 – 99.64%. Hence, the combination of TLC and RP-HPLC fingerprinting and quantitative analysis can be handy in identification and quality evaluation of the three ambiguous sources of Mayurasikha.

Keywords: Adiantum caudatum, Celosia argentea, Actiniopteris dichotoma, RP-HPLC and Mayurasikha

INTRODUCTION

Ancient Indian literature assimilates a remarkably expansive description about the medicinal plants and their health-giving substances. According to Ayurveda and other traditional system of medicine, more than 1250 Indian medicinal plants are used as remedies for different ailments. Plants are the reservoirs of important medicinal compounds, which produce definite physiological effects on humans and animals for these reasons, plants are used as the origin for traditional medicine system in many countries since ancient time. This plant based medicinal system is playing a vital role in the present synthetic era. In recent years, plant derived products are increasingly being sought out as medicinal products, nutraceuticals and cosmetics. They are available in health food outlets and pharmacies over the counter as self-medication. Though a number of scientific documentations are available on crude drugs, promoting these herbal drugs in national and international market is turning out to be more challenging task. Reason is, lack of reproducible biological reports and pharmacological data on the majority of herbal drugs. Another major problem is the selection of wrong plants or identification of the correct botanical source.

In Ayurveda and other Sanskrit literatures, one common vernacular name was used for two or more completely different plants. This difference of opinion may be due to different languages and description of the plant with more number of synonyms in ancient literature. Apart from that, people still depend on various tribal and folklore medicines. Hence, there is a need to establish a precise source of the plant to achieve the therapeutic uses mentioned in the classical Ayurvedic texts and to avoid the adulteration of herbal formulations. This becomes an important task to generate parameters for identification as well as differentiation among many sources having similar name. Ayurvedic drug Mayurasikha possess a wide variety of biological activities including Prameha (diabetes), Atisara (diarrhea), Pravahika, Jvara (fever), kustha (skin diseases), antibacterial, anti diarrhoeal and against jaundice. However, this is having three ambiguous botanical sources, Adiantum caudatum L. (Family: Pteridaceae), Celosia argentea L. (Family: Amaranthaceae) and Actiniopteris dichotoma Mitt. (Family: Pteridaceae) in ancient Ayurvedic literature. The three sources with the similar common name further leads to biological confusion and adulteration. Hence, the present proposed study was carried out, to resolve the ambiguity with the help of simultaneous TLC and RP-HPLC fingerprinting and quantity estimation with two active marker compounds. In addition to this, there is no validated RP-HPLC method for simultaneous determination of stigmasterol and oleic acid. This work reports a simple, optimized, and validated RP-HPLC method, for the qualitative and quantitative estimation of stigmasterol and oleic acid in three ambiguous botanical source of Mayurasikha.

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INDIAN DRUGS 53 (04) APRIL 2016
MATERIALS AND METHODS

Plant material

Adiantum caudatum and Celosia argentea were collected from adjoining areas of Visvesvaraya Technological University (VTU), Belagavi, Karnataka. Actinopyeris dichotoma was collected during the month of August 2013 from Karadi Guddi village, Belagavi, Karnataka. All plant materials were identified and authenticated by Dr. Harsha Hegde, Scientist C, RMRC, Belagavi. The herbaria were prepared and deposited at RMRC, ICMR, Belagavi, Karnataka. The voucher specimen numbers allotted for the above said plants are RMRC-985, RMRC-986, and RMRC-987 respectively.

Chemicals

Stigmasterol and oleanolic acid were purchased from Sigma Aldrich Chemicals Pvt.Ltd. Bangalore. Acetonitrile (HPLC grade) was purchased from Fisher Chemicals, USA. Millipore water and other reagents were of HPLC analytical grade.

Extraction

Dried powdered (500 g) material was placed in 1000 mL conical flask and then subjected to cold maceration technique with 70% V/V ethanol for 24 h at room temperature (27±2°C). Extract was collected and filtered, and the marc was further subjected for Soxhlation for 8 h with ethanol as a solvent. Filtrates of both maceration and Soxhlation were combined and concentrated by using a rotary evaporator (IKA RV 10) at 40°C under reduced pressure. Similar procedure were used for extraction of all the three plants.

TLC Fingerprinting

Preparation of solutions of extracts

Solutions of the extracts of Adiantum caudatum, Celosia argentea and Actinopyeris dichotoma were prepared by dissolving 50 mg of each of the extracts in 5 mL of methanol.

TLC conditions

Thin layer chromatographic technique (TLC) was used to separate the constituents present in the drugs. The optimized mobile phase were developed by trying various solvent system to separate the maximum number of constituents in three sources simultaneously. Three plant extracts were applied 1 cm above the edge of the chromatographic plates (silica gel 60 F 254 plates, Merck) and developed in chamber already saturated with solvent system (10 mL) toluene: ethyl acetate (7.5:2.5 V/V).

After development, plates were dried with a hair dryer and then derivatization of the chromatogram was performed by Camag glass reagent spray by spraying plate with anisaldehyde-sulphuric acid reagent (AS) Reagent, heated at 105°C for 5 min. and the differences and similarities between three plants were documented by its Rf values.

\[
R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent front}}
\]

Instrumentation and chromatographic conditions

A SHIMADZU HPLC system, consisting of LC-20AD a quaternary pump, an online degasser model DGU-20A5. Photo diode array detector (PDA) SPD-M20A, auto sampler SII20ACHT, rheodyne injection valve with 20µL loop and column oven CTO10ASVF was used for acquiring chromatographs. LC solution software version 1.25 was used for integration and processing of chromatograms. A reverse phased Phenomenex Luna C18 column (150mmx4.6mm, 5µ) equipped with Phenomenex guard column was used for chromatographic analysis. The analysis was carried out at ambient temperature (25°C). The mobile phase was a mixture of ACN: Water (87:13 V/V) with flow rate of 1 mL/min. Mobile phase was filtered through a PVDF syringe filters of 0.45µm (SPINCOTECH) and degassed before use. The injection volume was 10µL and the detection wavelength was 210 nm.

Preparation of standard solution

An accurately weighed amount of stigmasterol and oleanolic acid were dissolved in chloroform and methanol to obtain a concentration of 1 mg/mL. The above solutions were diluted with methanol to obtain appropriate concentration of 2-12µg/mL (SS) and 50-500 µg/mL (OA).

Sample preparation

An accurately weighed extracts (25mg) was taken into 10 mL volumetric flask. Chloroform and methanol (1:1) was added to the extracts and sonicated in an ultrasonic bath (BARNSON, USA) for 10 min with intermittent shaking. The solution was filtered through 0.45 µm PVDF syringe filter before injecting into HPLC system for analysis.

Method validation

The present RP-HPLC analytical method was validated for the quantification of stigmasterol, Oleanolic
acid in according to the ICH guideline (International Conference Harmonization, 2005) using analytical parameters like, system suitability, linearity, LOD & LOQ, accuracy, precision and robustness.

**System suitability**

System suitability testing is an integral part of an analytical procedure. The system suitability of the developed method was calculated by six replicate injections of standard solution of both markers compounds (SS & OA). This system suitability test was generally performed to assess the system performance based on the various factors like theoretical plates (column efficiency), peak tailing, retention time, and peak area.

**Linearity**

The linearity of an analytical method is the fitness (within a given range), to get test results that are directly proportional to the concentration of the analyte in the sample. The linearity between peak area and concentration was executed by analysis of six different concentrations of both marker (SS & OA) in a range of 2–12 & 50–500 μg/mL. Each concentration was injected and measured in triplicate.

**Limits of detection and quantification**

The limit of detection (LOD) and the limit of quantification (LOQ) were determined by using standard calibration curve. S/N ratio of 3:1 is considered as standard for valuing LOD whereas for LOQ S/N ratio of 10:1 is considered as standard, at which analyte can be readily quantified with accuracy and precision.

\[ \text{The LOD and LOQ may be expressed as} \]
\[ \text{LOD} = 3.3 \times \sigma / S \ \text{and} \ \text{LOQ} = 10 \times \sigma / S. \]
\[ \sigma = \text{standard deviation of the response} \]
\[ S = \text{slope of calibration curve} \]

**Recovery**

The closeness of agreement between accepted conventional true values and the values found was known as accuracy. The accuracy of the method was determined by recovery studies using the standard addition method. Three known standard concentrations of both markers were spiked with three different levels (50, 100, and 150%) in triplicate. The mean percent recoveries were calculated.

**Precision**

The precision of the method was carried out by performing repeatability and intermediate precision. Repeatability or Intra-day precision was performed by applying three different concentrations of mixture of standards in triplicate three times a day. Inter-day precision was performed by applying three different concentrations of mixture of standards in triplicate on three different days, respectively. For each analysis, the percent relative standard deviation (% RSD) of the peak area were calculated.

**Robustness**

Robustness was done by making minor changes in the analytical method at a single concentration level. Robustness of the proposed method was determined in four different ways, by making deliberate changes in the flow rate, mobile phase composition, temperature, and wavelength of the analysis. Effects of the selected factors were assessed over a series of conditions by determining the peak area, retention time, theoretical plates and tailing factor of the SS and OA peaks.

**RESULTS AND DISCUSSION**

Herbal drugs standardization should consist three basic elements like authenticity, purity, and assay. Authenticity relates to correct information about the starting material and purity means there is no substitution and adulteration of the original plant material. Assay is the part of standardization and chemical profiling with markers compounds. These basic elements of standardization will play a key role in quality evaluation, safety, and efficacy of herbal drugs. WHO established official guidelines for the assessment of herbal medicines to meet the global standards. US FDA made rules for standardization of alternative & complimentary medicines and their botanical extracts. In recent years, there has been more prominence to standardization of therapeutically potential medicinal plants, because of the intensive growth in the market potential of Ayurvedic and traditional medicines. Therefore, establishing correct identity of the plant drug would be the primary step in the standardization of Ayurvedic and herbal drugs. This can explore the long history of the safe and effective usage of Ayurvedic and herbal drugs. Hence, the present work aimed to standardize the Ayurvedic drug Mayurasikha, which is having three different botanical source. Morphologically all the three plants were different in their nature and they can be easily identified. However, raw material, powder drug and extracts were trading moreover by common name, which further needs the authenticity. Hence, present
work intended for the standardization of three plants, even if they supplied in the form of extract and powder drug. Therefore, an attempt was made by establishing TLC separation and RP-HPLC fingerprinting methods to identify and define the quantity of bioactive compounds (SS and OA) in the three ambiguous source of Mayurasikha. TLC chromatogram of three plant extracts are shown in Fig. 1. Alcoholic extracts of A. caudatum, C. argentea and A. dichotoma showed 5, 7 and 4 spots, respectively, after derivatization. Notable differences were observed between the RI values of three species and similar band at Rf 0.14 (Table I) was observed in A. caudatum and C. argentea. The spots with common Rf values, may be due to similar components of the extracts.

A variety of mobile phases were investigated for the development of the simultaneous RP- HPLC method for analysis of stigmasterol and oleanolic acid in three sources of Mayurasikha. It was observed that acetonitrile and water 87:13 (% V/V) produces a sharp and symmetric peak of stigmasterol and oleanolic acid at retention time of 2.36 and 7.53 min.

**Validation parameters**

The developed method was validated according to ICH guidelines to determine the parameters like system suitability, linearity, LOD & LOQ, accuracy, precision and robustness. System suitability means, ensuring the system performance. The retention time of peaks were

![Chemical structure of Stigmasterol](image)

**Fig. 1: Chemical structure of Stigmasterol**

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Name of the Drug</th>
<th>Mobile Phase</th>
<th>Rf values After derivatization with Anisaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Adiantum caudatum</td>
<td></td>
<td>0.14, 0.19, 0.36, 0.61, 0.72</td>
</tr>
<tr>
<td>2.</td>
<td>Celosias argentea</td>
<td>Toluene: Ethyl acetate (7.5:2.5 V/V)</td>
<td>0.10, 0.14, 0.17, 0.32, 0.47, 0.56</td>
</tr>
<tr>
<td>3.</td>
<td>Actinopterygis dichotoma</td>
<td></td>
<td>0.74, 0.17, 0.27, 0.39, 0.47</td>
</tr>
</tbody>
</table>

**Table II: System suitability parameters of stigmasterol and oleanolic acid**

<table>
<thead>
<tr>
<th>Injection</th>
<th>Retention time SS</th>
<th>Retention time OA</th>
<th>Peak area SS</th>
<th>Peak area OA</th>
<th>Theoretical plates SS</th>
<th>Theoretical plates OA</th>
<th>Tailing factor SS</th>
<th>Tailing factor OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.36</td>
<td>7.468</td>
<td>1483855</td>
<td>1111672</td>
<td>5245.316</td>
<td>10233</td>
<td>1.381</td>
<td>0.994</td>
</tr>
<tr>
<td>2</td>
<td>2.369</td>
<td>7.515</td>
<td>1481425</td>
<td>1114886</td>
<td>4915.65</td>
<td>10252.77</td>
<td>1.385</td>
<td>0.998</td>
</tr>
<tr>
<td>3</td>
<td>2.379</td>
<td>7.506</td>
<td>1487276</td>
<td>1112264</td>
<td>4978.951</td>
<td>10320.88</td>
<td>1.4</td>
<td>0.997</td>
</tr>
<tr>
<td>4</td>
<td>2.377</td>
<td>7.533</td>
<td>1486547</td>
<td>1110892</td>
<td>4902.034</td>
<td>10344.72</td>
<td>1.383</td>
<td>0.995</td>
</tr>
<tr>
<td>5</td>
<td>2.378</td>
<td>7.546</td>
<td>1487323</td>
<td>1112912</td>
<td>5047.56</td>
<td>10451.15</td>
<td>1.399</td>
<td>0.994</td>
</tr>
<tr>
<td>6</td>
<td>2.373</td>
<td>7.624</td>
<td>1497748</td>
<td>1111093</td>
<td>5393.931</td>
<td>10480.09</td>
<td>1.373</td>
<td>0.985</td>
</tr>
<tr>
<td>Mean</td>
<td>2.371</td>
<td>7.532</td>
<td>1487362</td>
<td>1112287</td>
<td>5080.574</td>
<td>10347</td>
<td>1.386</td>
<td>0.993</td>
</tr>
<tr>
<td>Standard deviation (SD)</td>
<td>0.0063</td>
<td>0.0520</td>
<td>5587.29</td>
<td>1476.2</td>
<td>197.9</td>
<td>101.11</td>
<td>0.010</td>
<td>0.004</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.267</td>
<td>0.69</td>
<td>0.3756</td>
<td>0.1327</td>
<td>3.85</td>
<td>0.977</td>
<td>0.766</td>
<td>0.490</td>
</tr>
</tbody>
</table>

**Table III: Linearity range, LOD and LOQ of marker compounds**

<table>
<thead>
<tr>
<th>Marker compounds</th>
<th>Regression equation</th>
<th>r²</th>
<th>LOD µg/mL</th>
<th>LOQ µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stigmasterol</td>
<td>y = 297565 x + 85517</td>
<td>0.9992</td>
<td>0.385</td>
<td>1.16</td>
</tr>
<tr>
<td>Oleanolic acid</td>
<td>y = 5473.6 x + 3998.3</td>
<td>0.9997</td>
<td>11.47</td>
<td>34.78</td>
</tr>
</tbody>
</table>

r² is the correlation coefficient of the equation  
y is the peak area  
x is the concentration of injected compound  

**INDIAN DRUGS 53 (04) APRIL 2016**
Table IV: Accuracy of the Stigmasterol and Oleanolic Acid (n=3)

<table>
<thead>
<tr>
<th>Spike level (%)</th>
<th>Amount added in (µg/mL)</th>
<th>Amount found in (µg/mL)</th>
<th>% Mean recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>OA</td>
<td>SS</td>
</tr>
<tr>
<td>50</td>
<td>3</td>
<td>75</td>
<td>2.92</td>
</tr>
<tr>
<td>100</td>
<td>4</td>
<td>100</td>
<td>3.91</td>
</tr>
<tr>
<td>150</td>
<td>7</td>
<td>175</td>
<td>7.022</td>
</tr>
</tbody>
</table>

Table V: Precision values for RP-HPLC analysis of Stigmasterol and Oleanolic acid (n=3)

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Stigmasterol (% RSD)</th>
<th>Oleanolic acid (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>SS</td>
<td>OA</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>0.6861</td>
</tr>
<tr>
<td>8</td>
<td>300</td>
<td>0.1064</td>
</tr>
<tr>
<td>12</td>
<td>500</td>
<td>0.5989</td>
</tr>
</tbody>
</table>

Table VI: Results of Robustness for Stigmasterol and Oleanolic Acid

<table>
<thead>
<tr>
<th>System suitability parameters (variations)</th>
<th>% RSD of Peak area (n=6)</th>
<th>% RSD of retention time (n=6)</th>
<th>Mean tailing factor (n=6)</th>
<th>Mean theoretical plates (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>OA</td>
<td>SS</td>
<td>OA</td>
</tr>
<tr>
<td>Change in mobile phase +2</td>
<td>0.311</td>
<td>0.048</td>
<td>0.231</td>
<td>0.115</td>
</tr>
<tr>
<td>- 2</td>
<td>0.193</td>
<td>0.068</td>
<td>0.145</td>
<td>0.091</td>
</tr>
<tr>
<td>Change in flow rate + 0.2</td>
<td>0.394</td>
<td>0.079</td>
<td>0.104</td>
<td>0.323</td>
</tr>
<tr>
<td>- 0.2</td>
<td>0.095</td>
<td>0.129</td>
<td>0.140</td>
<td>0.083</td>
</tr>
<tr>
<td>Change in temp. +5</td>
<td>0.711</td>
<td>0.378</td>
<td>0.189</td>
<td>0.463</td>
</tr>
<tr>
<td>- 5</td>
<td>0.499</td>
<td>0.100</td>
<td>0.285</td>
<td>0.965</td>
</tr>
<tr>
<td>Change in wavelength +5</td>
<td>0.239</td>
<td>0.172</td>
<td>0.289</td>
<td>0.727</td>
</tr>
<tr>
<td>-5</td>
<td>0.291</td>
<td>0.253</td>
<td>0.297</td>
<td>0.656</td>
</tr>
</tbody>
</table>

found to be 2.37 & 7.53, theoretical plate count for SS and OA found to be 5123.88 and 10347.1, tailing factors were obtained as 1.38 for stigmasterol and 0.99 for oleanolic acid respectively (Table II). The higher theoretical plate count (>5000) and low tailing factor (< 1.4) of SS and OA, indicate the good column efficiency. The linearity of the method was calculated by analyzing six different concentration of the both markers. The calibration curves shown good linearity and with excellent correlation coefficient values (R²) 0.9992 & 0.9997 respectively (Table III). The LOD and LOQ of SS were found to be 0.38 & 1.1 µg/mL whereas OA were found to be 11.47 & 34.78 µg/ml respectively (Table III). The accuracy was determined by comparing actual and measured concentrations. The mean percentage recovery values of SS was in the range of 97.46 – 100.31% and for OA it was found to be 99.32- 99.64% respectively (Table IV). Which indicates that developed method can be used for accurate determination of SS & OA in all the three plants. The results of intraday precision % RSD values of SS were ranged from 0.1- 0.6% and 0.04 – 0.2% for OA.

Whereas % RSD of Interday precision values of SS & OA were ranged from 0.05 – 0.16 % and 0.01–0.04% respectively (Table V). The low % RSD (<1%) values of the intraday and interday variations was confirmed that, the developed method has excellent precision. Robustness was checked to ensure that developed RP-HPLC is unaffected to slight changes in method parameters. Deliberate changes in chromatographic conditions have not shown significant changes in system suitability parameters like theoretical plates and tailing factor (Table VI), which indicates that, the developed method was found to be robust according ICH guidelines. Therefore, validation results specified that, the conditions used for the quantitative determination of markers and extracts were reliable and acceptable.

Quantification of the active marker compounds in extracts

The developed RP-HPLC method was successfully applied for identification and quantification of marker
compounds in all the three plant extracts. The chromatographic fingerprint profiles of the three plant extracts were shown in fig. 2. Based on the retention times of the standard compounds, the fingerprint analysis revealed that SS was present in all the three plant extracts. Whereas OA was present in A. caudatum and A. dichotoma. The contents of SS & OA in extracts were quantified by using the calibration curve of standard marker compounds. SS was present highest amount in A. caudatum (3.88 μg/mg), followed by C. argentea (3.70 μg/mg) and A. dichotoma (3.60 μg/mg). Whereas OA was found to be highest in A. caudatum (1.09 μg/mg), followed by A. dichotoma (0.72 μg/mg) and it was absent in C. argentea. Hence, all of these plants are showing different chromatographic fingerprint profiles, which clearly distinguish that the all the three plants would be different in chemical nature. The quantitative estimation of Stigmasterol in all the three plants and oleanolic acid content in A. caudatum and A. dichotoma may be helpful in the identification and standardization of the individual plant. Therefore, the combination of chromatographic fingerprinting and quantitative estimation of marker compounds helpful in identification and standardization of plant extracts, which may resolve the ambiguity associated with Mayurasikha.
CONCLUSION

This is the first report on the development and validation of a specific and reliable RP-HPLC fingerprinting analysis for simultaneous evaluation of these three ambiguous sources of Mayurasikha with the two active marker compounds. This simple and optimized TLC and HPLC methods were in all cases informative about similarities in their chemical characterization and differences in contents of active marker compounds. Therefore, the validated method was specific and accurate, which can be helpful for routine quality control of raw materials and formulations having Mayurasikha.

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INDIAN DRUGS 53 (04) APRIL 2016