Standardization of an Herbal Formulation (DIA-2) Containing Allium sativum and Lagerstroemia speciosa extracts using HPTLC-UV densitometry

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ABSTRACT

The bulbs of Allium sativum (ASE) and leaves of Lagerstroemia speciosa (LSE) are traditionally used to treat a variety of health disorders in the southern part of India. DIA-2 is a herbal formulation developed to contain fixed ratio (1:1 w/w) of ASE and LSE. In the present study, the phytochemical analysis of ASE and LSE was carried out following standard protocols. Also, a simple, rapid and reproducible high performance thin-layer chromatographic (HPTLC) fingerprint of DIA-2 and its ingredients was developed. A characteristic HPTLC fingerprint has been generated for ASE, LSE and DIA-2 using appropriate mobile phase and precoated plates with silica gel 60 F	extsubscript{254} as stationary phase. Densitometric scanning of LSE and DIA-2 were performed at 366 nm and ASE at 515 nm after derivatization with 0.2% ninhydrin (in acetone). The HPTLC fingerprint of DIA-2 was found to be reproducible and revealed nine peaks at the selected wavelength. This study provides a simple, rapid, reproducible and economic chromatographic technique for qualitative standardisation of DIA-2 and its ingredients.

Key words: HPTLC fingerprint, UV-densitometry, Allium sativum, Garlic, Lagerstroemia speciosa, banana, DIA-2

INTRODUCTION

India has been recognized for its wide variety of spices and medicinal plants1. Herbal medicines are generally prepared as a complex mixture using medicinal plants2. Herbal medicines have drawn increased attention, since they have easy access without the need for laborious pharmaceutical synthesis like modern medicines3. Due to their complex nature, herbal formulations pose enormous analytical challenges for quality control4. They lack effective quality control methods right from the selection of raw materials till the finished products5. In the modern era, development of modern scientific knowledge has taken forward to develop analytical technique for the quality evaluation and standardization of herbal based formulations6. High Performance Thin Layer Chromatographic (HPTLC) technique may be used as a rapid analytical method to control the quality of herbal and their formulations7. HPTLC fingerprint may also be used as a chemical fingerprint of the herb(s)/formulations for quality control8. It may also serve to access the uniformity of the active ingredients and to identify batch-to-batch variation during formulation9. HPTLC fingerprint developed with or without marker facilitates to authenticate and to access the quality of herbal drugs10 and provides the concept of phytoequivalence11. HPTLC chromatogram can also be used as an ideal tool for conducting stability studies to predict the shelf life, monitor adulterations if any and extraction processes of herbal medicines12. Compared to pharmaceuticals, stability studies of herbal medicines is difficult to conduct because of the presence of mixture of various types of phytoconstituents. Preliminary phytochemical analysis of herbal drug also provides necessary information pertaining to the nature of phytoconstituents present in the herbal drug and helps in devising the mobile phase of different polarity to establish a HPTLC fingerprint profile of good resolution13. The complexity of herbal formulation, due to inclusion of multiple interactive ingredients hinders the standardization of these drugs. Herbal preparations standardized with one or more standardised ingredients can form the basis for developing quality control methods for herbal formulations.

The bulbs of Allium sativum (ASE) and leaves of Lagerstroemia speciosa (LSE) are used as a folk remedy for treating diabetes mellitus14-15. DIA-2 is an herbal mixture formulated with minimum number of standardised ingredients of ASE (1.1 % w/w alliin) and LSE (1.28 % w/w corosolic acid) in a fixed ratio of (1:1 w/w). It was developed with an objective to manage hyperglycemia, obesity and oxidative stress. The present study aims to subject the ingredients of DIA-2 for qualitative phytochemical analysis and to develop HPTLC fingerprint densitogram of DIA-2 and its ingredients.

MATERIALS AND METHODS

Chemicals and Reagents

Authentic standardized extract of Allium sativum (ASE) and Lagerstroemia speciosa (LSE) were obtained from M/s. Amravati HPLC Pvt Ltd, Indore, India and K. Patel Phyto Extractions Ltd., Mumbai, India, respectively. ASE is aqueous extract of dried bulbs Allium sativum and LSE is 40% methanolic extract of dried leaves of Lagerstroemia speciosa. Both ASE and LSE were supplied in the powder form by the dealer and claimed to contain 1.1 % alliin w/w and 1.28 % w/w corosolic acid, respectively.

Preparation of DIA-2

DIA-2 is a fixed combination (1:1 w/w) of standardized extracts from ASE and LSE. Individual powder extracts ASE and LSE were mixed in equal proportion (1:1) and triturated using a mortar and pestle to yield a consistent homogeneity.

Qualitative phytochemical analysis of ASE and LSE

The test solution of extracts were prepared by dissolving by 1mg of extract in 100ml of 80% methanol and used for various phytochemical analysis described herein. The different chemical constituents tested for ASE and LSE includes flavonoids, phenolic compounds, tannins, reducing sugars, steroids and triterpenoid glycosides, anthroquinone glycosides, saponin glycosides and alkaloids

Test for flavonoids14

Test solution was treated with 1 ml of 10 % sodium hydroxide. The appearance of a yellow colour which turns into colourless on addition of a few drops of dilute hydrochloric acid. This indicates the presence of flavonoids.

Test for Phenolic compounds14

To the test solution few drops of alcoholic ferric chloride solution were added a bluish green or bluish black precipitate indicates the presence of phenols.
Test for Tannins\textsuperscript{14}
To the test solution, a few drops of 1% lead acetate solution were added. Formation of yellow precipitate indicates the presence of tannins.

Test for Reducing Sugars\textsuperscript{15}
Test solution was heated with equal volumes of Fehling’s solution A and B. Formation of a red precipitate of cuprous oxide indicates the presence of reducing sugars.

Test for Steroids & Triterpenoid Glycosides\textsuperscript{15}
To the test solution, a few drops of 1% lead acetate solution were added. Formation of a red precipitate of cuprous oxide indicates the presence of steroid moiety.

Test for Anthroquinone Glycosides\textsuperscript{15}
To the test solution, 5 ml of dilute hydrochloric acid was added and boiled on a water bath for 10 min and filtered. Filtrate was extracted with benzene and equal amount of ammonia solution was added and shaken well. Formation of pink or red colour in ammonia layer indicated the presence of anthraquione moiety.

Test for Saponin Glycosides\textsuperscript{15}
To the test solution, 20 ml of water was added and shaken for few minutes, formation frothing which persist for 60 s indicated the presence of saponins.

Test for Alkaloids\textsuperscript{15}
To the test solution, Dragendorff’s reagent (Potassium bismuth iodide) was added and the formation of orangish red colour indicates the presence of alkaloid.

HPTLC Fingerprint Analysis of ASE, LSE and DIA-2

Sample Preparation
Prepare 80% v/v methanol (MeOH) solution. Weigh accurately 100 mg of test substance and transfer into a 10 ml volumetric flask. Dissolve the contents with 5 ml of 80% MeOH, and sonicate until its dissolved. Make up the volume to 10 ml with 80% MeOH. Sonicate the contents for another 10 minutes and filter using whatman filter paper and this solution was stored in 5 ml glass vials and used for HPTLC analysis.

HPTLC-Photodensitometry conditions and instrumentation
 Instrument : CAMAG HPTLC (Switzerland)  
Applicator : CAMAG linomat V  
Syringe : Hamilton syringe (100 µl)  
Scanner : CAMAG TLC scanner 3  
Photo-documentation : CAMAG Reprostar 3 (winCATS software version 1.3.4.)

Layer
Stationary phase : Pre coated silica gel 60 F\textsubscript{254} (KGAa, Darmstadt, Germany)  
Plate thickness : 0.2 mm  
Plate size : 100 x 100 mm

Mobile phase
ASE : chloroform: methanol: acetic acid: water (64:32:12:8)  
LSE : chloroform: ethanol: acetic acid: water (60:30:10:5)  
DIA-2 : chloroform: methanol: acetonitrile (80:10:10)  
Solvent ratio : Vol. / Vol (v/v)

Environmental Condition
Temperature : 25±2°C  
Relative Humidity : 55–65%.

Sample application
Application rate : 10 s µl–1  
Table speed : 10 mm s–1  
Distance from starting : 15 mm  
Distance from bottom : 10 mm  
Volume applied : 2.5 – 10 µl  
Band length : 10 mm  
Distance between tracks : 10 mm

Development
Developing chamber : Twin trough glass chamber (20 x10 cm)  
Developing solvent : 5 ml mobile phase/through

Chamber saturation time : 1 h  
Developing mode : Ascending mode  
Development distance : 80 mm  
Detection reagent : Nil  
Plate Drying : hair dryer for 5 min

Detection
Scanning wavelength : 254 nm and 366 nm  
Slit dimensions : 5 mm x 0.45 mm

Documentation
Calculation of R\textsubscript{f} = Distance travelled by solute component 

Detection Reagent Preparation (0.2% Ninyhydrin Solution)
200 mg of ninhydrin was weighed and dissolved in a small volume (~1 mL) of acetone; to this, 1 mL of pyridine was added, and the volume was made up to 100 mL with acetone\textsuperscript{16}.

RESULTS AND DISCUSSION
Standardization of herbal preparations is meant to assure the minimal quality to be in compliance with national and international regulations\textsuperscript{17}. Phytochemical analysis is one of the tools to access the quality of herbal drugs, which include preliminary phytochemical screening, chemoprofiling using modern analytical techniques. High-performance thin-layer chromatography (HPTLC) has been emerged as an important analytical tool for characterization of the phytoconstituents in herbal drugs. This involves developing TLC fingerprinting profiles. The concept of phytoequivalence was developed to ensure consistency of herbal products, where a chromatographic fingerprint of an herbal drug was constructed and compared with the profile of a reference product\textsuperscript{18}. The concept of phytoequivalence could be utilized for addressing the problem in quality control of herbal drugs\textsuperscript{19}.

The analysis of a single plant drug represents an enormous challenge in setting standards for quality control and it finds more difficult to deal with are multitherbal preparations\textsuperscript{20}. DIA-2 is an herbal mixture (1:1 w/w) of ASE and LSE, formulated with an intention to manage the metabolic disorders hyperlipidemia, obesity and obesity associated diabetes with well experimented, documented and clinically proven herbs. The distinct features of DIA-2 to that of other herbal formulations were they contain minimum number of ingredients and contain standardized extracts of ASE and LSE instead of crude herbal extracts. The presence of minimum number ingredients facilitates in setting the parameters during quality control.

The phytochemical analysis of the individual ingredients of DIA-2 was analysed and results indicated in table 1 show the presence and absence of the phytochemical present. Both the extracts showed large quantity of phenolic compounds and saponin Glycosides. Alkaloids were found rich in ASE whereas tannins and anthroquione glycosides were found abundant in LSE. The number of positive signs indicates the intensity of the phytoconstituent concentration present in each extract.

Table: 1- Qualitative phytochemical analysis of ASE and LSE

<table>
<thead>
<tr>
<th>Chemical Groups</th>
<th>Test</th>
<th>Inference ASE LSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>Sodium hydroxide Test</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>Feric Chloride Test</td>
<td>+++</td>
</tr>
<tr>
<td>Tannins</td>
<td>Lead Acetate Test</td>
<td>+++</td>
</tr>
<tr>
<td>Reducing Sugars</td>
<td>Fehling’s Test</td>
<td>-</td>
</tr>
<tr>
<td>Steroids &amp; Triterpenoid Glycosides</td>
<td>Liebermann-Burchard test</td>
<td>-</td>
</tr>
<tr>
<td>Anthroquinone Glycosides</td>
<td>Bierganger’s test</td>
<td>+</td>
</tr>
<tr>
<td>Saponin Glycosides</td>
<td>Frothing Test</td>
<td>+++</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Dragendorff’s Test</td>
<td>+++</td>
</tr>
</tbody>
</table>

Faintly: (+); Moderately: (+++); Highly: (+++); Absent: (–).

Table: 2- Maximum Rf Values and Area % of ASE, LSE and DIA-2 HPTLC chromatogram

<table>
<thead>
<tr>
<th>Peak</th>
<th>ASE Max Rf</th>
<th>Area %</th>
<th>LSE Max Rf</th>
<th>Area %</th>
<th>DIA-2 Max Rf</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.36</td>
<td>10.88</td>
<td>0.08</td>
<td>2.43</td>
<td>0.14</td>
<td>2.96</td>
</tr>
<tr>
<td>2</td>
<td>0.21</td>
<td>26.12</td>
<td>0.17</td>
<td>13.84</td>
<td>0.20</td>
<td>26.76</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
<td>33.93</td>
<td>0.27</td>
<td>3.79</td>
<td>0.31</td>
<td>7.08</td>
</tr>
<tr>
<td>4</td>
<td>0.36</td>
<td>5.26</td>
<td>0.33</td>
<td>9.24</td>
<td>0.36</td>
<td>2.49</td>
</tr>
<tr>
<td>5</td>
<td>0.48</td>
<td>23.81</td>
<td>0.47</td>
<td>35.18</td>
<td>0.53</td>
<td>1.55</td>
</tr>
<tr>
<td>6</td>
<td>0.55</td>
<td>7.82</td>
<td>0.58</td>
<td>28.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.70</td>
<td>6.54</td>
<td>0.67</td>
<td>3.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.85</td>
<td>21.17</td>
<td>0.84</td>
<td>15.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.90</td>
<td></td>
<td>12.37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{14} K.S. Kesavanarayanan et al. / Journal of Pharmacy Research 2011, 4(11), 3910-3914

\textsuperscript{15} Measurement of phytoequivalence could be utilized for addressing the problem in quality control of herbal drugs\textsuperscript{19}.

\textsuperscript{16} The concept of phytoequivalence could be utilized for addressing the problem in quality control of herbal drugs\textsuperscript{19}.

\textsuperscript{17} The concept of phytoequivalence could be utilized for addressing the problem in quality control of herbal drugs\textsuperscript{19}.

\textsuperscript{18} The concept of phytoequivalence could be utilized for addressing the problem in quality control of herbal drugs\textsuperscript{19}.

\textsuperscript{19} The concept of phytoequivalence could be utilized for addressing the problem in quality control of herbal drugs\textsuperscript{19}.

\textsuperscript{20} The concept of phytoequivalence could be utilized for addressing the problem in quality control of herbal drugs\textsuperscript{19}.
HPTLC fingerprint analysis of ASE

![HPTLC fingerprint and densitogram of ASE at 515 nm](image1.png)

**Fig-1: HPTLC fingerprint and densitogram of ASE at 515 nm**

HPTLC fingerprint analysis of LSE

![HPTLC fingerprint and densitogram of LSE at 254 and 366 nm](image2.png)

**Fig-2: HPTLC fingerprint and densitogram of LSE at 254 and 366 nm**
The biomarkers used in standardization of ASE and LSE were allicin, alliin and corosolic acid respectively. There are some reports on the application of chromatographic techniques for the analysis of ASE and LSE, but attempts to apply these techniques for developing chromatographic fingerprints as an herbal formulations are not available.

Phytochemical markers – based standardization were being employed as an analytical tool in quality control of herbal medicines. This approach would only serve as an additional parameter in assessing the quality of the drug since they are difficult to obtain and often quite expensive. The chromatographic fingerprints are useful in monitoring the entire process of formulation. Regulatory agencies also recommend chromatographic fingerprint as the basis for proper identification and quality control of herbal medicinal products. Herbal drugs with similar chromatographic fingerprint have similar properties and a similarity in chromatographic fingerprint pattern has been a suitable analytical approach in quality control of multi-herb botanical drug products.

The present study was focused on developing a densitometric method to construct chromatographic fingerprint using the HPTLC. Several mobile phases with different solvent ratio covering wide polarity range were tried for developing HPTLC fingerprint with maximum separation of phytochemicals present in ASE, LSE and DIA-2. In the present experiment, different solvent systems with different polarity were tried based on trial and error method. The optimization of mobile phase and solvent selection was based on our earlier experience, on modification of published data on ASE and LSE and on the chemical nature of the compounds present therein. Better resolution was achieved with the mobile phase chloroform: methanol: acetic acid: water (64:32:12:8 v/v) for ASE, LSE and DIA-2 respectively. The chromatograms developed for ASE, LSE and DIA-2 were shown in Fig. 1-3 respectively. The chromatograms developed for ASE, LSE and DIA-2 were shown in Table 2. The maximum peak percentage area was found to be 33.93%, 35.18%, 28.45% at Rf values 0.30, 0.47, 0.58 for ASE, LSE and DIA-2 respectively (see Table 2).

Derivatization is mainly used in the post chromatographic mode for localization of the separated component zones on the layer. In our study, we attempted to develop chromatograms without derivatization that compromise the selectivity of the method by reacting with compounds that may not be resolved from the compound of interest. However, in our study, we were able to construct the chromatogram for ASE only after derivatization with 0.2% ninhydrin reagent, a reagent used for identification of compounds containing an amino group in their structure.

CONCLUSION
HPTLC fingerprints developed for ASE and LSE could be used to document the quality of the raw material used in the formulation of DIA-2. The chromatogram of DIA-2 depicts how the starting materials are being transferred into an herbal formulation and also reveals the composition of the constituents and whether the composition has been changed during batch-to-batch production. The HPTLC method developed for DIA-2 and its individual ingredients was found to be simple and may be used for the routine quality control analysis and of simultaneous determination of phytomedicines containing ASE and LSE can be performed using this method.

REFERENCES


12. Liu CT, Sheen LY, and Liu CK. Does garlic have a role as an antidiabetic agent? Mol Nutr Food Res. 2007; 51:1353-1364.


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