Development of HPLC Fingerprint Analysis of Traditional Diabetes Herbal Jamu Diabetes Plant Materials

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1.0 INTRODUCTION

Diabetes mellitus is a multifactorial disease. It is an endocrine and metabolic disorder characterized by chronic hyperglycemia [1]. Multiple biochemical impairments associate with micro- and macro-vascular complications, which is a major cause of morbidity and death in Diabetes mellitus [2]. With the numbers of people affected by diabetes multiplying worldwide, the disease is taking an ever-increasing proportion of national and international health care strategies [3]. It is projected to become one of the world’s main disablers and killers within the next 25 years [4, 5]. Traditional herbal medicines have been used throughout the world for a range of diabetes [6]. Herbal preparations used for the treatment of diseases and its knowledge are handed down from generation to generation and has been used in healthcare delivery in many parts of the world. The traditional herb medicines showed, since centuries, beneficial effects on health promotion, out of side effects, as compared with synthetic drugs [7, 8]. Herbal medicines have received great attention as alternative medicines in recent years in Malaysia and are sold as a dietary supplement [9]. Jamu is
a traditionally formulated herbs being used as traditional medicines for years to treat illnesses in the region of Indonesia and Malaysia. The plant materials mixture of *Andrographis paniculata*, *Cinnamomum zeylanicum*, *Curcuma xanthorrhiza*, *Eugenia polyantha* and *Orthosiphon stamineus* are used in the formulation of traditional JD. *Andrographis paniculata* is a plant that has been effectively used in traditional Asian medicines for centuries. It has been used for several purposes, primarily preventing diabetes mellitus [10]. *Curcuma xanthorrhiza* is a low growing plant with a root and reported to be useful for the treatment of diabetes, rheumatism, anticancer, hypertension and heart disorders [11]. *Eugenia polyantha* has been known since long time ago as a spice that can be used for therapy. Empirically, *Eugenia polyantha* can be used for hypertension, diabetic, diarrhea, gastritis and skin diseases [12]. *Orthosiphon stamineus* is the herb that traditionally used in Malaysia to treat kidney problem, gout, and diabetes mellitus [9].

Recently, chromatographic fingerprint technique, as a more meaningful quality control method of herbal samples, has been attracting more and more people's attention, because the fingerprint technique emphasizes on the integral characterization of compositions of samples with a quantitative degree of reliability and focus on identifying and assessing the stability of the plants. Chromatographic fingerprint is a kind of method to show chemical information of medicines with chromatograms, spectograms and other graphs by analytical techniques. To date, varieties of chromatographic techniques involving fingerprint include TLC, gas chromatography, HPLC, etc. [13]. Because of its advantages and popularization, HPLC fingerprint analysis has been regarded as the first choice. However, fingerprint analysis shows only the result of similarity calculated based on the relative value; retention time, with the selected marker compound as reference standard, and does not display the absolute quantity. Obviously, quantitative determination of some marker components is necessary.

Number of studies concerning chromatographic fingerprint analysis on different plant materials have been conducted [14, 15] and nothing has been reported on the HPLC fingerprint analysis on the herbal formulation JD plant materials. Consequently, the objective of this research was to develop the fingerprint method for ground plant materials of Jamu Diabetes (JD) by HPLC determination using rosmarinic acid, curcumin, cinnamaldehyde and andrographolide as standards, which are important constituents present in the formulation.

### 2.0 MATERIALS AND METHODS

#### 2.1 Plant Materials and Chemicals

The ground plant materials of JD was supplied by Naturemedic Supply, Johor, Malaysia. HPLC grade methanol, acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used in the present study were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

#### 2.2 Preparation of Plant Extracts

The ground plant materials of JD (100 g) was macerated with a 1 L solution of 80% methanol and 20% distilled water for 2 days in a tightly sealed vessel at room temperature, and stirred during the maceration. For water extraction, the plant material (500 g) was macerated with 1.5 L distilled water and the mixture was boiled for one hour. For both cases, the maceration was repeated until a colorless liquid was obtained. The resultant extracts were combined and combined extract was filtered, concentrated at reduced pressure on rotary evaporator (Laboratora 4000, Heidelberg, Germany) or by freeze dryer (Heto PowerDry LL1500, Thermo Electron Corporation, UK). Afterword the sample was diluted with 2 mL of methanol and the solution was centrifuged (Kubota KR-20000T, Kubota Medical Appliance Supply, Japan) at 4000 rpm for 10 min at 25°C. After centrifugation the supernatant was filtered through a 0.45-µm PTFE membrane filter (Sartoriusstedium biotrich, Aubagne Cedex, France) prior to HPLC analysis.

#### 2.3 Preparation of Standards

Stock solutions of each compound were prepared by dissolving weighed quantities of standard compounds in HPLC grade methanol and diluting to the desired concentration. By using the stock solutions, a series of mixed working standard solutions were prepared with different concentration ranges and all the solutions were stored under refrigeration.

#### 2.4 Development of HPLC Fingerprint

A Perkin Elmer Series 200 liquid chromatograph system furnished with automated gradient controller, SPD-M20A Photo Diode Array UV-Vis detector and a reversed-phase C18 analytical column (150 × 4.6 mm ID, ZORBAX Eclipse Plus) was used. The column was maintained at 37°C and 0.50 µL of sample was injected for development of the chromatogram. The flow-rate was maintained at 1 mL min⁻¹. The mobile phase was optimized to give the best separation between the chromatographic peaks. Mobile phase consisted of acetonitrile (15 mL), de-ionized water (310 mL), methanol (170 mL) and acetic acid (5 mL) to set the pH around 3.5 was used for both water extract and water extract concentrated by vacuum rotary evaporator. On the other hand, the mobile phases consisted of acetonitrile (10 mL), de-ionized water (90 mL) and formic acid (5 mL) (A) and de-ionized water (10 mL), acetonitrile (90 mL) and formic acid (5 mL) (B) were used for methanol/water extract concentrated by vacuum rotary evaporator whereas the mobile phases consisted of de-ionized water (90 mL), methanol (10 mL) and formic acid (0.5 mL) (A) and de-ionized water (10 mL), methanol (90 mL) and formic acid (0.5 mL) (B) were used for freeze dried methanol/water extract. The reference compounds at different concentrations used were rosmarinic acid, curcumin, andrographolide and cinnamaldehyde for generating HPLC standard fingerprints. The fingerprint of extract was analyzed by interpolating data from fingerprint of extract and standard markers.

### 3.0 RESULTS AND DISCUSSION

#### 3.1 Standardization of Fingerprint

The objective of the works was to establish the best way to have appropriate fingerprints for all the main phytochemicals present in the samples. The relationship between the fingerprints was notified. For traditional quality control system, rosmarinic acid, curcumin, andrographolide and cinnamaldehyde are used as the marker substances to evaluate the extract of JD plant materials. The contents of these compounds present in the sample were determined by linear regression analysis. To determine the linearity equations and linear scope for the analysts, a series of standard solutions at different concentrations were tested. All results indicated that the conditions for the fingerprint analysis were satisfactory.
3.2 Sample Analysis by HPLC

HPLC analysis was performed using the different standard markers (Figures 1-4) dissolved in methanol at different concentrations. Each standard fingerprint in different concentrations was obtained and compared with the HPLC methanolic extract chromatogram data. Figure 5 shows two dominant peaks of water extract of JD plant materials which were not separated and others were not traceable. Then the concentrated water extract by rotary vacuum evaporator was prepared to raise the percentage of the components in the sample for better resolution. The curves still not separated and the components were not traceable with standard markers (Figure 6). As shown in Figure 7, freeze dried methanolic extract displayed 4 separated peaks; then it was decided to use the same mobile phase for HPLC test with standard markers to identify the different phytochemicals existed in the methanol extract.

The rosmarinic acid standard curve was established by plotting concentrations (mg mL\(^{-1}\)) versus area (UV*sec). The equation 1 represents this data plotting by using microsoft excel.

\[ y = 2 \times 10^{-7} x - 242.47 \]

Where \( y \) is area and \( x \) is concentration. The area 1,937,501 (UV*sec) at 2.32 min from the fingerprint of methanolic extract of JD plant materials was considered and by putting this value in the above equation, the 0.0968 mg mL\(^{-1}\) was computed for rosmarinic acid present in the sample. Similarly, by using standard curves of other markers, the concentrations of curcumin, andrographolide and cinnamaldehyde from the sample were calculated and tabulated accordingly in Table 1. As shown in Table 1, it can be generalized that the major constituents in JD extract are curcumin, rosmarinic acid, andrographolide and cinnamaldehyde. Therefore, HPLC fingerprint analysis was performed based on the relative retention time. The retention time was 2.10, 2.32, 2.67 and 3.22 min for curcumin, rosmarinic acid, andrographolide and cinnamaldehyde respectively. Methanol extract contained the higher amount of cinnamaldehyde and lower amount of curcumin.
Figure 3 HPLC Chromatogram of Andrographolide standard marker

Figure 4 HPLC Chromatogram of Cinnamaldehyde standard marker

Figure 5 HPLC Chromatogram of water extract of JD plant materials
Figure 6 HPLC Chromatogram of concentrated water extract of JD plant materials

Figure 7 HPLC Chromatogram of the methanolic extract of JD plant materials. 1-unknown chemical, 2- curcumin, 3- rosmarinic acid, 4- Andrographolide, 5- cinnamaldehyde

Table 1 The amounts of curcumin, rosmarinic acid, andrographolide and cinnamaldehyde present in the sample

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Compounds</th>
<th>Regression equation</th>
<th>Area (uV*sec)</th>
<th>Concentration (mg mL⁻¹)</th>
<th>Concentration (mg per 500 mg plant materials)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.15</td>
<td>Unknown</td>
<td>-</td>
<td>2157</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.10</td>
<td>Curcumin</td>
<td>$y = 2 \times 10^{-7} x - 57448$</td>
<td>924661</td>
<td>0.0434</td>
<td>0.8672</td>
</tr>
<tr>
<td>2.32</td>
<td>Rosmarinic Acid</td>
<td>$y = 2 \times 10^{-7} x - 242.47$</td>
<td>1937501</td>
<td>0.0968</td>
<td>1.9360</td>
</tr>
<tr>
<td>2.67</td>
<td>Andrographolide</td>
<td>$y = 2 \times 10^{-7} x - 21377$</td>
<td>3905579</td>
<td>0.1942</td>
<td>3.8840</td>
</tr>
<tr>
<td>3.22</td>
<td>Cinnamaldehyde</td>
<td>$y = 2 \times 10^{-7} x - 333271$</td>
<td>5819776</td>
<td>0.2743</td>
<td>5.4860</td>
</tr>
</tbody>
</table>

4.0 CONCLUSION

A HPLC method was successfully developed for fingerprint analysis of JD plant materials. The HPLC fingerprint of rosmarinic acid, curcumin, andrographolide and cinnamaldehyde represent the characteristic markers of this herb's constituents for the first time. The fingerprint obtained by using our established method provides a good repeatability in separation pattern which demonstrated that the fingerprint presented in this paper is a rapid, reliable and effective method suitable for either qualitatively or quantitatively determination of the constituents present in JD plant materials.
Reference


