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ORIGINAL ARTICLE

Quantification and enrichment of sinensetin in the leaves of *Orthosiphon stamineus*

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KEYWORDS

Enrichment;
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Densitometer

Abstract Sinensetin was identified in the leaves of *Orthosiphon stamineus* by thin-layer chromatography, GC coupled with mass spectrometry (MS), UV, IR and ^{1}H-NMR spectroscopy. Enrichment of sinensetin by using different mixture solvent system and the quantification analysis by thin-layer chromatography-imaging densitometric method using as external standard method showed the highest concentration of 0.32% of sinensetin in the mixture solvent of acetone–water (70:30) when compared to methanol–water (1:1) of only 0.15% in the leaves of *Orthosiphon stamineus*. The TLC densitometer, although yields slightly higher values than the other analytical methods, is preferred due to its simplicity, ease and low cost.

1. Introduction

Over 4000 chemically unique flavonoids have been identified in plant sources. These low-molecular-mass substances, found in all vascular plants, are phenylbenzopyrones with an assortment of basic structures (Harborne, 1993). On average, the daily western diet contains approximately 1 g of mixed flavonoids in fruits, vegetables, nuts, seeds, stems, flowers, as well as tea and wine (Kuhnau, 1976). This quantity can provide pharmacologically significant concentrations in body fluids and tissues. Flavonoids may have existed in nature for over one billion years (Swain, 1975). Methoxyflavones have important effects in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors, precursors of toxic substances and have long been recognized to possess antiallergic, anti-inflammatory, antiviral, antiproliferative and anticarcinogenic activities as well as to affect some aspects of mammalian metabolism (Gabor, 1986; Farkas et al., 1986; Cody et al., 1988; Das, 1989; Havsteen, 1984; Welton et al., 1988; Selwayse, 1986; Kupchan et al., 1965). Sinensetin is a very important polymethoxyflavone as well as a bioactive natural product that has a wide range of biological activities such as antibacterial, antifungal, antitumor, anticancer, prostaglandin binding and insect antifeedant (Hossain and Ismail, 2004).

In this paper we describe the enrichment and quantification of sinensetin in the leaves of *Orthosiphon stamineus* using different solvent systems by high performance thin-layer chromatography-imaging densitometric method.

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2. Experimental

2.1. Materials and reagents

Methanol (analytical-reagent grade), ethyl acetate (analytical-reagent grade) and chloroform (analytical-reagent grade) were purchased from Merck (Germany). Water purified by a Nan-Pure-unit (Barnstead, Boston, MA, USA) was used. Standard sinensetin was isolated and purified by us in our laboratory. Silica gel for thin-layer chromatography was purchased from E-Merck. All other solvents and chemicals were purchased from E-Merck.

2.2. Thin-layer chromatography plates

Silica gel GF_{254} was suspended in water, homogenized with an electric stirrer and spread on glass plates (20×10 cm) with Camag applicator. The tuff was previously sieved and the fraction with particle size < 40 μm was used for the layer preparation. The thickness of the wet layer was 0.5 mm. Preparative TLC plates (20×10 cm glass plates precoated with thickness 1 mm silica gel GF_{254}) were also purchased from Merck, Germany.

2.3. Instrumentation

The CAMAG analyzer, a reflectance spectrometer equipped with an IBM computer; monitoring range 190–700 nm was used for analyses of the mixture solvents extract. Data acquisition and processing were performed using the software winCATS.

2.4. Plant material

O. stamineus Benth (Lamiaceae) leaves were collected from Penang, Malaysia. The plant was identified and the voucher specimen was deposited in the herbarium of the School of Biology, University Sains Malaysia. It was dried at room temperature or dried in an oven below 40 °C. The dried leaves were pulverized and sifted through a sieve of mesh size 500 μm.

2.5. Extraction

Approximately 5 g of dried leaves powder of *O. stamineus* was accurately weighed and added to 50% methanol (75 ml). After ultrasonication for 30 min in an ultrasonic cleaning bath, the extract was filtered and evaporated to dryness using rotatory evaporator and dried by vacuum pump. The above dry powder methanol extract (1 g) was dissolved in 70% acetone–water (7 ml) and heated below 40 °C with ultrasonication for 30 min (Fig. 1). The extract was filtered and dried by the same procedure as described under methanol extract.

2.6. Preparation of samples

Standard solution was prepared from crystalline pure substance of sinensetin for the construction of calibration curves and quality control of the samples. Sinensetin standard solution was prepared in methanol at a concentration of 0.010, 0.0125, 0.0150, 0.0175 and 0.0200 mg/ml, respectively. Sample of sinensetin was prepared as follows. 70% Acetone extract (5 mg) was dissolved in a 1 ml methanol solution, and used to quantify from the standard curve constructed from standard solutions.

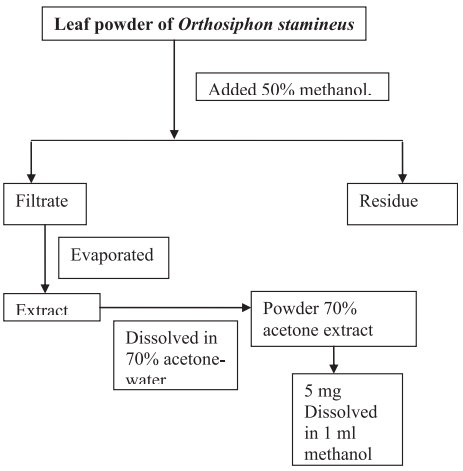

Figure 1 Sample preparation procedure for the HPTLC determination of sinensetin in the leaves of *Orthosiphon stamineus*.

2.7. Chromatographic conditions

Standard and samples were applied to the plates by means of the micropipette (Switzerland) equipped with 100 μl tip, the diameter of the spot is 20 mm, the application volume was 10 μl. Spots per plate were applied 15 mm from the bottom edge, 20.1 mm apart from each other. The plate was developed at room temperature in an unsaturated glass twin through a chamber in solvent system chloroform–ethyl acetate (60:40), the ascending migration of the mobile phase over a distance of 8.5 cm. After separation, the plate was dried in a stream of warm air. The plate was removed, dried and the spots were visualized under UV lamp at 365 nm. The chromatogram was scanned with the spectrodensitometer at the maximum of each sample.

3. Results and discussion

A number of lipophilic flavonoids are present in *O. stamineus* leaves, sinensetin and TMF being the most abundant. In addition to these two components, orange peel contains nobiletin, 3′,4′,3,5,6,7,8-heptamethoxyflavone and tangeretin. It should be noted that polymethoxylated flavonoids differ only

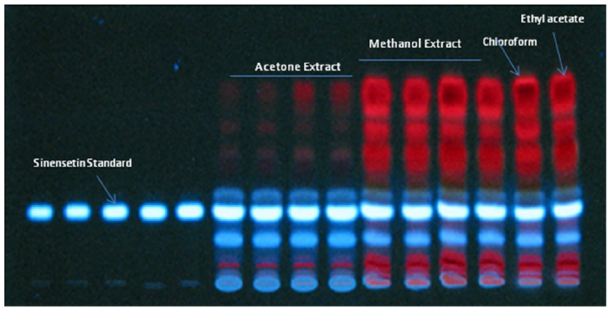

Figure 2 The chromatograms of the samples and the standard without any spray reagent, in UV light at 365 nm.

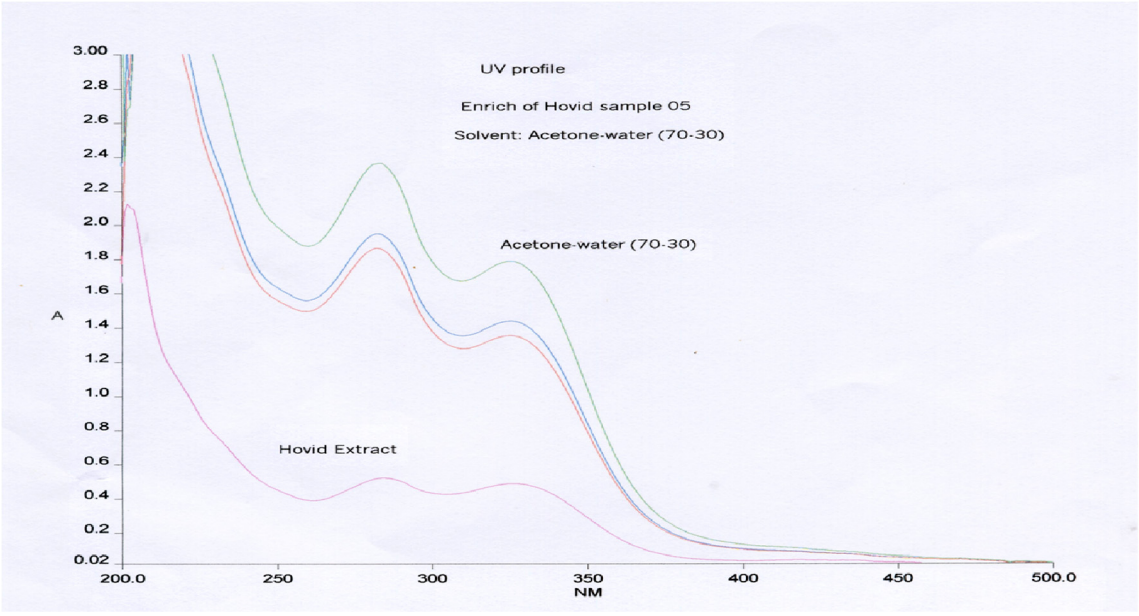

Figure 3 UV spectra of 70% acetone and 50% methanol extract of leaves of *Orthosiphon stamineus*.

in the position and the number of methoxy groups owing to the hydrophobic nature of these compounds and the small difference in polarity. Thin-layer chromatography (TLC)-densitometry is the current method for the quantitation of some polymethoxylated flavonoids in pharmaceutical formulations. Quantitative TLC in situ scanning densitometry is rapidly gaining wide acceptance in pharmaceutical analysis (Cody et al., 1988; Das, 1989; Havsteen, 1984). This is because of its simplicity, accuracy, cost effectiveness and the possibility of simultaneous determination of a number of samples on a single TLC plate. The HPTLC allows the identification and the quantification of more than 20 samples in the same chromatographic run. The analysis of the samples requires 15–30 min compared with more than 2 h using a typical HPLC method. Moreover, there is no need for conditioning steps, as with HPLC, and each analysis by HPTLC is less expensive. However, we describe the quantitative determination of sinensetin from the different solvent extracts of *O. stamineus* by using densitometer at 365 nm.

The chromatograms of the samples were visualized in UV light at 365 nm. The plate was without sprayed reagent and the chromatograms were visualized in UV light at 365 nm. The chromatograms of the samples show the presence of the spots with the same color and at the same R_f values as of the standard. Fig. 2 shows the chromatograms of the samples and the standard at 365 nm without any sprayed reagent.

From the UV spectra we can observe that the absorption of 70% acetone is higher than the 50% methanol extract, which shows that enrichment with acetone–water (70:30) has increased the flavonoid sinensetin (Fig. 3).

The calculated R_f values (Table 1) for the standard and the separated components from samples demonstrates the presence of sinensetin in this acetone enriched extract.

The quantitative determination was performed by thin-layer chromatography-densitometry using the calibration curve method. Standard curves for sinensetin generated by plotting the area of five spots vs. the concentration, gave high correlation coefficients (Fig. 4). Linear response was achieved

Table 1 The calculated R_f and % sinensetin for the standard and for the separated spots from samples.

Samples/standard (sinensetin)	Concentration	R_f values	% Of Sinensetin
Standard 1	100 ppm (10 µl)	0.49	–
Standard 2	125 ppm (10 µl)	0.49	–
Standard 3	150 ppm (10 µl)	0.49	–
Standard 4	175 ppm (10 µl)	0.49	–
70% Acetone extract	10 µl	0.49	0.32
50% Acetone extract	10 µl	0.49	0.19
100% Acetone extract	10 µl	0.49	0.16
100% Methanol extract	10 µl	0.49	0.13
50% Methanol extract	10 µl	0.49	0.15
100% Chloroform extract	10 µl	0.49	0.18
100% Ethyl acetate extract	10 µl	0.49	0.21
50:50% Chloroform–ethyl acetate extract	10 µl	0.49	0.22

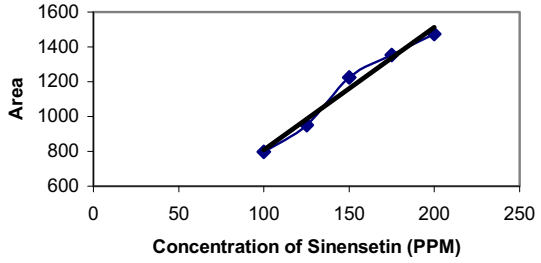

Figure 4 Calibration curve for sinensetin.

for sinensetin in the concentration range 18.76 μg/10 μl. Over this concentration range, the linear regression analysis of peak areas (y) in function of concentration (x), calculated by least square method, leads to the following equations: $y = 278 + 143.78x$ ($r = 0.997$, $n = 3$) for sinensetin.

The equations of this curve are:

$$y = 278 + 143.78x\,(r = 0.997, n = 3)$$

where y is the peak area and x is the applied volume in a spot.

The concentration was obtained with the formula:

$$C\%\,\mathrm{g/g} = V_e C_{et}/10m$$

where $C\%$ (g/g) is the concentration, V_e is the corresponding volume from the standard, C_{et} is the concentration of the standard solution, 10 is the quantity of samples in μl, and m is the weight of the plant used for extraction.

The amount of sinensetin in methanol calculated from the standard curve by densitometric method is 0.15%. When the methanol extract was enriched with acetone–water (70:30) the amount of sinensetin increased to 0.32% showing a considerable increase in sinensetin by the enrichment procedure.

The results obtained from the densitometer are compared with those of HPLC and found to be almost similar.

So the flavonoid sinensetin extraction can be improved by enriching with acetone–water (70:30) solvent system. And a high content of the flavonoid was found after enrichment.

4. Conclusion

Sinensetin was determined quantitatively by densitometric method and confirmed by chromatographic and spectral methods. This analytical procedure permits a fast and reliable determination of these drugs in pharmaceutical dosage forms and can be used for routine analysis. However, the scanning densitometry is superior in terms of speed, simplicity and cost.

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