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HPLC-TOF/MS profile and nitric oxide scavenging activity of *Orthosiphon stamineus* leaf extracts

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ABSTRACT

Objective: The aim of the present study is to develop liquid chromatography (LC)/Time-of-flight mass spectrometry (TOF/MS) profile for methanol and water extracts of *Orthosiphon stamineus* leaf using SEN and RA as flavonoid and non-flavonoid polyphenolic markers in the extracts. The study also evaluates *in vitro* nitric oxide radical scavenging effect of the extracts. **Method:** Orthogonal Time of Flight Mass Spectrometer equipped with HPLC separation module was used in the analyses of the extract. The in vitro nitric oxide scavenging activity of the extracts was measured according to the method described by Rao. **Results:** The qualitative analysis of the extracts performed with HPLC-TOF/MS confirmed the presence sinensitin (SEN) and rosmarinic acid (RA) in the extracts. The extracts showed *in vitro* nitric oxide scavenging activities. **Conclusions:** The HPLC-TOF/MS method could be employed for quality determination of herbal medicinal products and formulations containing *O. stamineus*. The extracts may play a significant role in prevention of degenerate disease due to its ability to scavenge nitric oxide radical.

1. Introduction

Orthosiphon Stamineus Benth (Lamiaceae), is a medicinal plant native to tropical Asia. It has been used to treat urinary lithiasis, edema, eruptive fever, influenza, rheumatism, hepatitis and jaundice[1]. The therapeutic effects of *O. stamineus* leaf extracts are attributed mainly to its polyphenol content. The leaf extracts of the plant was reported to contain chemically active polyphenols including eupatorin (EUP), sinensetin (SEN) and rosmarinic acid (RA) [2]. Sinensetin was reported to have antiproliferative activity in MDA-MB-468 human breast adenocarcinoma and MCF-10A normal breast cell lines [3]. RA is also a multi-active substance used in cosmetics to maintain healthy skin due to its antioxidant qualities which is superior to that of vitamin E [4].

Time of flight analyzers separate ions based on their different travel times (in microseconds) over a known distance in a flight tube caused by applying a uniform electromagnetic field. Lighter ions travel faster and arrive at the detector first, and flight time is converted to mass using known kinetic energy relationships between mass and velocity. The HPLC-TOF/MS technique gives more information on structures of eluting polyphenols including accurate mass values and wide mass range [5]. The aim of the present study is to develop liquid chromatography (LC)/Time-of-flight mass spectrometry (TOF/MS) profile for methanol and water extracts of O. stamineus leaf using SEN and RA as markers. The study also evaluates *in vitro* nitric oxide radical scavenging effect of the extracts.

2. Materials and methods

2.1. Chemicals

Rosmarinic acid (RA), and sinensetin (SEN), were purchased from Indofine Chemical Co. (Hillsborough, USA). Caffeic acid (CA) and formic acid were obtained from Sigma (USA). Methanol, tetrahydofuran (THF), formic acid and water were obtained from Merck (Darmstadt, Germany). All others chemicals were of analytical or HPLC grade.

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2.2. Plant material

Plants were grown from cuttings using standard agronomic practices at Kepala Batas, Penang, Malaysia. The leaves were collected from 30-45-day-old white-flowered plants. The specimen was labeled, numbered and annotated with the date of collection and locality. Voucher specimen of the plant material was deposited at Bilik Herba, School of Pharmaceutical Sciences, Universiti Sains Malaysia.

2.3. Preparation of leaf samples

Plant leaves were ground to a homogeneous powder after drying in an oven (35 ℃). The dried powdered leaves were extracted with methanol and water respectively, using soxhlet apparatus. The crude extracts were filtered and evaporated under reduced pressure.

2.4. HPLC-TOF/MS analysis

2.4.1. Preparation of the leaf extract for HPLC-TOF/MS analysis

A weighed quantity of the methanol extract was initially reconstituted in methanol: water: tetrahydofuran (THF): formic Acid (90:10:10:1) to a concentration of 1 mg/mL and then diluted into methanol:water (1:1) to a concentration of 50 μg/mL for analysis.

2.4.2. HPLC-MS conditions

Micromass LCT (Orthogonal Time of Flight Mass spectrometer) equipped with a Waters 2795 HPLC separation module (Waters Corporation, Mass Spectrometry Technologies Centre, Manchester, UK) was used for LC-MS analysis. Waters Symmetry C18 column, 2.1 x 100 mm, 3.5 μm particle size was used. The mobile phase consisted of; [A] water + 0.1% formic acid and [B] methanol + 0.1% formic acid , applying the following gradient: 2% to 98% B in 20 min, hold to 25 min then re-equilibrate to 2% B at 30 min. The flow rate was 300 μL/min direct into source (no split). Injection volume was 20 μL and the UV detector was set at wavelength of 330 nm. The MS conditions were, electrospray positive ion mode; Acquisition range of 200-800 Da; Data format using real-time exact mass centroid at 1 spectrum/second; 6000 FWHM resolution and sampling cone 30 V.

2.4.3. HPLC-MS calibration

The mass range, 100-1000 Da, was initially calibrated externally using the known reference mass clusters from sodium formate infused directly into the source. Subsequently, data was acquired in a real-time exact mass (centroid) mode using a single point reference (lockmass) infused into the reference sprayer of the Lockspray interface at 10 μL/min. The reference mass used was Leucine-Enkephalin, m/z 556.2771 Da, sampled at 1 spectrum/second at 5 seconds intervals and stored in a second data file for continuous pseudo-internal mass adjustment of the analyte spectra.

2.4.5. Nitric oxide radical scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH gives rise to nitric oxide which under aerobic conditions react with oxygen to produce nitrite ions that can be determined using Griess reagent [6]. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. The scavenging effect of the extracts on nitric oxide was measured according to the method of Rao with some modifications [7]. Curcumin was used as positive reference compound. All tests were performed six times. The percentage inhibition of nitric oxide radical generation was calculated.

3. Results

3.1. HPLC-TOF/MS analysis

Typical HPLC-TOF/MS base peak intensity (BPI) and UV diode array chromatograms of the methanol and water extracts are shown in Figure 1. The markers (RA and SEN) were identified on bases of comparison of retention times with the retention times of the authentic standard and confirmed by extracted mass spectra. RA was eluted at retention time of 13.43 min and SEN was eluted at retention time of 16.51 min in both extracts. Figure 2 shows the HPLC-MS base peak intensity (BPI) chromatogram of SEN and RA from the leaf extracts. Figure 3 shows the extracted mass spectra of SEN (Molecular mass = 373) and RA (M = 360) from the leaf extracts. Mass spectrum of SEN shows exact molecular masse at m/z 373 Da. The mass spectrum of RA shows a molecular ion at m/z 383 Da (as M + Na).

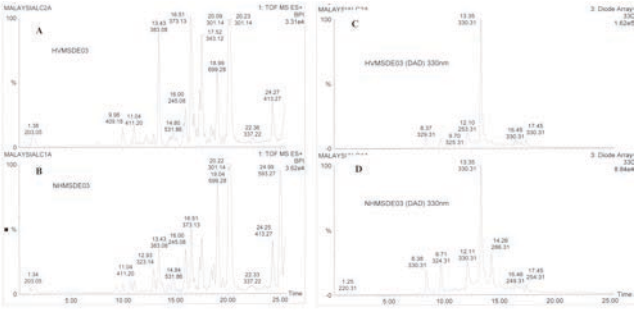

Figure 1. HPLC-TOF/MS and UV diode array chromatograms of leaf extracts of Orthosiphon stamineus; (A) Base peak intensity of methanol extract (B) Base peak intensity of water extract (C) Diode array detection of methanol extract (D) Diode array detection of water extract . See experimental section for analytical protocol.

3.2. Nitric oxide radical scavenging activity

The extracts showed considerable scavenging of nitric oxide. The IC_{50} values for the Curcumin (positive reference

compound), methanol and water extracts were 33.28±4.56 μg/mL, 36.45±4.12 μg/mL, and 40.83±4.20 μg/mL, respectively.

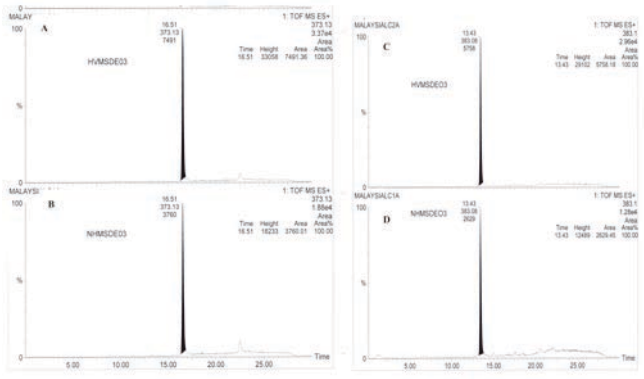

Figure 2. HPLC-MS base peak intensity of (A) SEN from the methanol extract; (B) SEN from the water extract; (C) RA from the methanol extract; (D) RA from the water extract. See experimental section for analytical protocol.

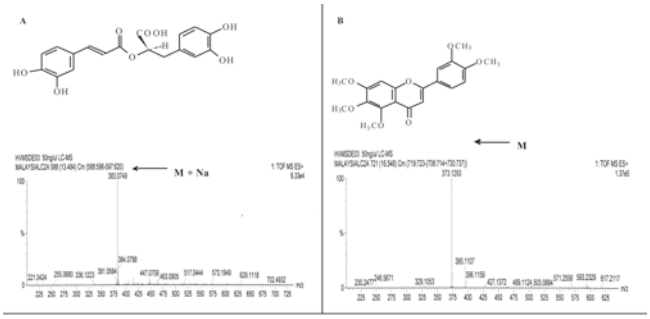

Figure 3. Chemical structure and HPLC-MS spectra of (A) RA (Molecular mass = 360) (B) SEN (Molecular mass = 373) from leaf extracts of *Orthosiphon stamineus*. See experimental section for analytical protocol. M = molecular mass.

4. Discussion

4.1. HPLC-TOF/MS analysis

The coupling of high-performance liquid chromatography (HPLC) with time of flight mass spectrometers (TOF/MS) instrumentation is a technique which gives more structural information on eluting polyphenolic mixtures such as herb and vegetable extracts. Similar HPLC-TOF/MS and UV diode array fingerprints of the methanol and water extracts were obtained. The HPLC-TOF/MS method established the presence of the SEN and RA in the methanol and water extracts. The technique provided information on the structures and accurate mass values of the markers. SEN and RA were selected for this preliminary study based on our earlier report on HPLC analysis of the leaf extracts which showed the presence of SEN and RA as major flavonoid and non-flavonoid polyphenolic compounds in the extracts, respectively [2]. The exact mass of SEN and RA components observed in the samples were similar to the expected masses which authenticate their identity (Figure 2). The positive identification by mass spectral fingerprint of SEN and RA provides evidence of their presence in the extracts.

4.2. Nitric oxide radical scavenging activity

Nitric oxide can act as free radical, electrophile and an oxidising agent. Despite its relative stability as free radical nitric oxide reacts with other free radicals such as oxygen and superoxide that are widely distributed in mammals [8]. Nitric oxide reacts with oxygen to form nitrogen dioxide which at biological systems can initiate auto-oxidation of fatty acids in lipid membranes, resulting in membrane damage due to its ability to abstract hydrogen from unsaturated fatty acids [9]. The nitric oxide scavenging activity of the extracts was comparable to that of the positive reference compound curcumin. Curcumin is naturally occurring polyphenolic compound abundant in the rhizome of the plant Curcuma longa Linn. It has been reported to inhibit nitrite formation during nitric oxide (NO) oxidation in solution [10].

Polyphenolic compounds comprising of flavonoids and caffeic acid derivatives were identified and quantified by HPLC in 80% aqueous methanolic extracts of *O. stamineus* [11]. Flavonoids are reported to be scavengers of nitric oxide radical [12]. Flavonoids suppress NO production and also scavenge NO in an acellular system using sodium nitroprusside under physiological conditions at a micromolar range [13]. Shoekes et al. demonstrated that flavonoid reduce ischemia-reperfusion injury by interfering with inducible nitric-oxide synthase activity [14].

RA which was used as non-flavonoid polyphenolic marker in this study is a caffeic acid derivative. RA from ethanolic extract of Prunella vulgaris was reported to inhibit lipopolysaccharide-induced prostaglandin E2 and nitric oxide in mouse macrophages [15]. Sinensetin which was used as the flavonoid marker in this study is a polymethoxyflavone. Polymethoxyflavones have been shown to exhibit a broad spectrum of pharmacological activities including anti-cancer, anti- atherosclerosis, anti-hypertension, and anti-inflammatory, and anti-carcinogenic properties due to their ability to scavenge free radicals [16]. A recent study showed that the following methoxyflavones, 5,7-dimethoxyflavone, trimethylapigenin, and tetramethylluteolin, markedly inhibited the production of NO in lipopolysaccharide (LPS)-activated RAW264.7 cells [17].

The present study established a suitable HPLC-TOF/MS method for the separation of polyphenols in the water and methanol extracts of *O. stamineus*. The qualitative HPLC-TOF/MS analysis confirmed the presence of EUP and SEN in the complex polyphenolic mixture *O. stamineus* extracts. The HPLC-TOF/MS method could be employed for quality determination of herbal medicinal products and formulations containing *O. stamineus*. Nitric oxide radicals

play significant roles in Oxidative stress related ailments. The extracts of *O. stamineus* may play a significant role in prevention of degenerate disease due to its ability to scavenge nitric oxide radical. Characterization and quantification of all the polyphenolic composition of the extract observed in the HPLC-UV-MS spectra is being studied.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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