Modeling of supercritical fluid extraction of flavonoids from *Calycopteris floribunda* leaves

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The aim of this study was to obtain flavonoid extracts from *Calycopteris floribunda* leaves using supercritical fluid extraction (SFE) with CO$_2$ and a co-solvent. Pachypodol, a potential anticancer drug lead compound separated from the extracts, was examined. Classical organic solvent extraction (CE) with ethanol was performed to evaluate the high pressure method. HPLC analysis was introduced to interpret the differences between SFE and CE extracts in terms of antioxidant activity and the concentration of pachypodol. SFE kinetics and mathematical modeling of the overall extraction curves (OEC) were investigated. Evaluation of the models against experimental data showed that the Sovová model performs the best. The supercritical fluid extraction process was optimized using a central composite design (CCD), where temperature and pressure were adjusted. The optimal conditions of SFE were: pressure of 30 MPa and temperature of 35°C.

**Keywords**: *Calycopteris floribunda*, supercritical fluid extraction, mathematical modeling, HPLC, antioxidants

**Introduction**

*Calycopteris floribunda* Lam. (Combretaceae) is a large woody climbing shrub (5–10 m long) native to Bangladesh and India and also found in many other parts of south-east Asia (Yusuf et al., 1994). The leaves of *Calycopteris floribunda* as recorded in the Asian traditional medicine systems are used in colic, malaria, and diarrhea (Kirthiika & Basu, 2001). More recent pharmacological studies have demonstrated that the leaves extracts from *Calycopteris floribunda* exhibit potential bioactivity.

In order to utilize this plant material, many significant works were carried out and several flavonoid constituents were separated from different low pressure extracts of the leaves (Mayer, 1999, 2004; Wall et al., 1994). Wang et al. (2008) described a method to determine total flavonoids in the *Calycopteris floribunda* leaves. The results showed the amount of total flavonoids to be 10.8 mass %. Some researches extracted volatile oil from the leaves of *Calycopteris floribunda* and reported it to exhibit high antimicrobial activity (Liu et al., 2009; Wang et al., 2009).

Flavonoids can act as effective free-radical scavengers and are treated as effective antioxidants and anti-inflammatory agents with numerous health benefits. Flavonoids from *Calycopteris floribunda* were reported to have an inhibitory effect on the activity of ABCG2, which can increase the effectiveness of chemotherapeutic treatment in cancer patients (Krujitzer et al., 2002; Pick et al., 2011; Versiani et al., 2011). Some flavonoids of *Calycopteris floribunda* were used as novel lead compounds. The main flavonoid, calycopterin (Rodriguez et al., 1972), separated from *Calycopteris floribunda* has anthelmintic, antiviral (in vitro inhibition of polio virus) (Mayer, 1999), and anticancer activities (antiproliferative and anti-aromatase) (Wall et al., 1994). Lewin et al. (2011) used calycopterin to synthesize many flavones displaying high antiproliferative activity. Pachypodol, a po-

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tential anticancer drug lead compound from the leaves of *Calycopteris floribunda*, can inhibit the growth of Caco-2 colon cancer cell line in vitro. However, this compound extracted by classical organic solvent extraction required repeated purification by column chromatography on silica gel. From 1.7 kg of dried *Calycopteris floribunda* leaves, only 56.1 mg of pachypodol were obtained (Ali et al., 2008). In order to extract and isolate pachypodol effectively, a highly selective extraction method should be investigated. Supercritical fluid extraction (SFE) is highly selective and it has many advantages over conventional methods. It is a promising process for the extraction of valuable compounds from natural plant material, especially for food and pharmaceutical products (Leitão et al., 2013). The addition of modifiers or co-solvents to CO₂ can also improve the extraction efficiency and it has been widely used in bioactive compounds extraction (Pereira et al., 2004; Yang et al., 2002). Ethanol has been largely used as a co-solvent in the extraction of bioactive compounds due to its low toxicity compared to other options (Chafer et al., 2004; Chiu et al., 2002; Lucas et al., 2007).

Considering the importance of this plant material and the lack of reports on the supercritical fluid extraction of the leaves, the aim of this work was to illustrate the difference between the supercritical extraction and the ethanol extract of *Calycopteris floribunda* leaves. Three models were employed to interpret the experimental data. Also, an effective method for the extraction of the lead compound pachypodol was investigated.

**Experimental**

**Materials and methods**

The leaves of *Calycopteris floribunda* were collected from the Yingjiang County, Yunnan Province, the People’s Republic of China, in June 2010, and the sample was authenticated by professor Liu at the Central South University. The plant leaves were washed and dried at 45°C for 16 h, grounded by a universal grinder, and the particle size was classified by sieves. The fraction formed by the size between 150 μm and 600 μm was used in the extraction procedures. The grounded material was stored at −10°C in a domestic refrigerator before the extraction procedures were performed.

A supercritical fluid extraction (SFE) unit HA121-50-01 (Nantong Huaan, China) was used to extract flavonoids from the *Calycopteris floribunda* leaves. SFE was carried out using (90.0 ± 0.1) g of raw leaf material in a cell column of approximately 477 mL (length of 30.0 cm, inner diameter of 4.5 cm). In this study, the extraction process was optimized using a central composite design (CCD), where temperature and pressure were adjusted (Bimakr et al., 2011). The factorial design was investigated by varying the temperature from 30°C to 50°C and the pressure from 10 MPa to 30 MPa. The choice of the temperature and pressure range used in the factorial design were based on literature (Pereira & Meireles, 2010). The extractions were carried out for approximately 300 min, while ethanol (Sinopharm Chemical Reagent Co., China) was used as the co-solvent in the concentration of 5 mass % at the CO₂ flow rate of 1.5 kg h⁻¹.

Classical organic solvent extraction (CE) with ethanol (Sinopharm Chemical Reagent Co., China) was employed to obtain flavonoids extracts from the *Calycopteris floribunda* leaves. The optimum extraction conditions by CE were ensured using a central composite design (CCD). The single factor experiments were designed in terms of solid solvent ratio, extraction time, extraction temperatures, and ethanol concentration. Based on the experiments, the optimum extraction was performed with 100 g of dried raw leaf material soaked in a 40 mass % ethanol solution for 8 h, the temperature was 70°C and the solid mass to solvent volume ratio was 1 : 55. The extract was then filtered and concentrated under reduced pressure to remove ethanol and to obtain the crude extract.

The extract was washed with petroleum ether (Sinopharm Chemical Reagent Co., China) to remove the lipid fraction; then, the water layer was extracted with ethyl acetate (EtOAc) (Sinopharm Chemical Reagent Co., China). The organic layer was concentrated under reduced pressure to dryness to obtain crude flavonoids, which were then extracted by CE, separated by column chromatography on silica gel repeatedly using a mixture of petroleum ether–EtOAc. Pachypodol was obtained in low yield. Crude flavonoids obtained by SFE were also separated by column chromatography on silica gel, with the polarity of the eluent increasing; pachypodol was crystallized as yellow needle-shaped crystals directly from the solvent (petroleum ether with ethyl acetate at the volume ratio of 20 : 1). Compared with pachypodol separated from the classical solvent extracts, purification of the SFE extract with column chromatography was simpler and the yield of pachypodol was higher. The structure of pachypodol was elucidated by a combination of UV, MS, and NMR spectroscopic analyses and also by comparison with published data (Ali et al., 2008).

HPLC analysis was performed to quantify the compound pachypodol in the *Calycopteris floribunda* leaves extracts obtained by CE and SFE. The extracts were analyzed using LC-2010A (Shimadzu, Japan). A UV detector operated at 365 nm was applied. The column used was Lichrospher C18 (4.6 mm × 250 mm, 5 μm) (GL Sciences, Japan). The temperature was 35°C. The mobile phase was a mixture of acetonitrile and ultrapure water (φr = 3 : 1). The flow rate was set to 1.0 mL min⁻¹. Pachypodol separated in our laboratory was used as an external standard. The content of pachypodol was 1.983 mg g⁻¹.