HPLC analysis of a bioactive chalcone and triterpene in the buds of *Cleistocalyx operculatus*

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*Cleistocalyx operculatus* is a well-known medicinal plant, the buds of which are commonly used as an ingredient of tonic drinks in southern China. To evaluate the quality of *C. operculatus* material, a simple, rapid and accurate HPLC method was developed for the quantitative/qualitative assessment of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone and ursolic acid, the principal bioactive compounds in the buds of *C. operculatus*. The HPLC system was equipped with a C<sub>18</sub> reversed phase column, and methanol and aqueous H<sub>3</sub>PO<sub>4</sub> were used as the mobile phase. Peaks were detected at 220nm. The recovery of the compounds was 97.5% and 99.3%, respectively, for compounds 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone and ursolic acid, and the two compounds showed good linearity (r<sup>2</sup> ≥ 0.9997) in a relatively wide concentration range. The concentration of these two phytochemicals in the buds of *C. operculatus*, growing at three different locations in China, was determined to establish the effectiveness of the method as well as the variability of the plant material.

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Introduction

*Cleistocalyx operculatus* (Roxb.) Merr. et Perry (Myrtaceae) is a well-known medicinal plant, the buds of which are commonly used as an ingredient of tonic drinks in southern China. It was reported that a water extract of the buds increases the contractility and decreases the frequency of contraction of an isolated rat heart in a perfusion system (Woo *et al*. 2002). It was also found that the water extract of the buds showed strong protective effects on lipid peroxidation in rat liver microsomes, and exhibited potent protective effects on the trauma of PC12 cells (Lu *et al*. 2003). In continuation of our investigation of new and/or bioactive compounds from the buds, the two main bioactive constituents were characterised spectroscopically as 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (1) and ursolic acid (2) (Figure 1) (Ye *et al*. 2004b). We have shown that 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone significantly inhibited the growth of human cancer cells and could induce apoptosis in cancer cells (Ye *et al*. 2004a). It has also been reported that ursolic acid has various bioactivities, such as inhibition of aflatoxin B<sub>1</sub>-induced mutagenicity (Young *et al*. 1994), cytotoxicity in some tumorous cells (Lee *et al*. 1988) and an inhibitory effect on B16 proliferation (Es-saady *et al*. 1996).

*C. operculatus* is increasingly becoming popular in southern China, so it is essential to establish an analytical quality control method by monitoring the two main active constituents. Although analysis of the leaf oil of the plant by GC and GC/MS has been reported (Dung *et al*. 1994), there is as yet no published analytical method for the non-volatile compounds of the buds. We established a simple and effective HPLC method for the quality assessment of the buds of *C. operculatus*. The levels of the two main constituents in the leaves of plants collected from three geographically different areas of China have been determined to demonstrate the robustness of the developed method as well as the relative variability of the chemical compounds.

Materials and Methods

Plant materials

Three sets of buds of *C. operculatus*, harvested in June 2002 from the Guangdong, Guangxi and Fujian provinces of China, were purchased from the Materia Medica Company in Shenzhen. All materials were identified by ZN Gong with voucher specimens (Nos 20025–20027), deposited at the East China University of Science and Technology, Shanghai 200237, China.

Reagents and standards

2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone (1) and ursolic acid (2) (Figure 1) were isolated by the author from the buds of *C. operculatus*, and their structures characterised by chemical and spectroscopical methods (UV, IR, NMR, MS). These spectroscopic data were compared with the data reported in the literature (Seo *et al*. 1975, Malterud *et al*. 1977, Pant and Rastogi 1977). HPLC grade methanol was purchased from Caledon Laboratories Ltd, Canada. Double-distilled water was made...
in our laboratory. Phosphoric acid (>85%) was purchased from the Shanghai Chemical Company.

**Sample preparation**

Powdered samples (60 mesh, 2g) of the buds of *C. operculatus* were extracted in a Soxhlet with CH$_3$OH-H$_2$O (7:3, v/v) for 8h. The extract was concentrated to dryness and the residue dissolved in a few millilitres of methanol, transferred to a 10ml volumetric flask and diluted to volume with methanol. One millilitre of the solution was diluted in a 5ml volumetric flask with methanol. The solution was filtered through a 0.45µm filter before injection.

**Chromatography**

The HPLC system consisted of an SCL-10AVP System Controller, a LC-10ATVP Pump A and Pump B, a DGU-12A Degasser and a SPD-10AVP Detector (Shimadzu, Tokyo, Japan). Separations were achieved with a reversed phase column (ZORBAX, Eclipse XDB-C$_{18}$, 5µm, 4.6mm x 250mm, Agilent, USA) equipped with a C$_{18}$ guard column. The chromatographic data were recorded and processed by a Class-vp5.0 data processor.

The flow rate of the mobile phase (A, methanol; B, 0.2% H$_2$PO$_4$ aqueous; A:B = 93:7, v/v) was kept constant at 1.0ml min$^{-1}$, and the peaks were detected at 220nm. Injection volume was 10µl.

**Linearity studies**

A mixed stock solution consisting of ursolic acid (4.32mg ml$^{-1}$) and 2',4'-dihydroxy-6'-methoxy-3',5'-dimethyl-chalcone (0.5mg ml$^{-1}$) was prepared; 0.1ml, 0.2ml, 0.5ml, 1.0ml, 2.0ml and 3.0ml of the stock solution each was put into 25ml volumetric flasks and diluted to volume with methanol. 10µl of the above solutions were accurately taken for analysis.

**System suitability test**

The recovery assays of the two compounds were carried out by adding a known amount of standard to the crude drug powder, which was then extracted according to the procedure described in the section 'Sample preparation'. The precision test was carried out by injecting the same sample solution six times. For the stability test, the same sample solution was analysed every 12h over a period of two days.

**Application**

A volume of 10µl of the solution of each sample obtained from plants growing in different locations was used for analysis. The contents of the two compounds in the samples were calculated from the corresponding calibration curve.

**Results and Discussion**

The detection wavelength was chosen at 220nm, because the two compounds have better absorption and sensitivity at this wavelength. Typical chromatograms are shown in Figure 2, which illustrates the separation of the two constituents in this system.

The precision of this method, as well as the stability of the solution, was satisfactory. A study of repeatability showed relative standard deviations (RSDs) of 0.7% and 0.8%, respectively, for compounds 1 and 2 ($n$ = 6), and the solution was found to be rather stable within 48h (RSD < 3.0%).

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**Figure 1:** Structure of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethyl-chalcone (1), and ursolic acid (2)
Good response linearity was obtained for these two compounds studied with $r^2$ values being 0.9999 and 0.9997 (peak area vs concentration), for compounds 1 and 2. The limits of detection (LOD) were 200 ng ml$^{-1}$ for compound 1 (RSD = 2.5%, n = 5) and 400 ng ml$^{-1}$ for compound 2 (RSD = 2.8%, n = 5) (Table 1).

The recoveries of the two compounds were 97.5% and 99.3%, respectively, for compounds 1 (RSD = 4.8%, n = 5) and 2 (RSD = 1.4%, n = 5) (Table 2).

The levels of the two constituents in buds of C. operculatus growing in different locations was also analysed, demonstrating that the levels of these phytochemicals in the plant are dependent on the locality where the plants were collected (Table 3).

In conclusion, the method described herein is effective and suitable to allow direct, rapid and accurate determination of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone and ursolic acid in C. operculatus plant material, but it also fulfills all criteria of a validated method. Thus, it should be useful for both scientific and commercial applications.

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**Table 1:** Retention time ($t_H$), regression equation correlation, coefficient ($r^2$), limit of detection (LOD) and linear range for compounds 1 and 2

| Standard | $t_H$ (min) | Regression equation* | $r^2$ | LOD (ng ml$^{-1}$) | Linear range (µg ml$^{-1}$) |
|----------|-------------|-----------------------|-------|------------------|-----------------------------|
| 2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone | 4.598 | $Y = 4 \times 10^4 X + 6\ 818.2$ | 0.9999 | 200 | 2.00–60.00 |
| Ursolic acid | 9.301 | $Y = 1\ 815.21 X – 26\ 335$ | 0.9997 | 400 | 17.28–518.40 |

* $X =$ concentration (µg ml$^{-1}$); $Y =$ peak areas

**Table 2:** Recovery of compounds 1 and 2 (n = 5)

| Compound | Added (mg) | Initial (mg) | Detected (mg) | Recovery* (%) | RSD (%) |
|----------|------------|--------------|---------------|---------------|---------|
| 2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone | 2.00 | 4.08 | 6.03 | 97.5 | 4.8 |
| Ursolic acid | 17.00 | 35.12 | 52.00 | 99.3 | 1.4 |

* Recovery = ((Detected – Initial) / Added) x 100%

**Table 3:** The mean contents of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone and ursolic acid in C. operculatus from three different localities in China (n = 5)

| Collection place | 2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone | | Ursolic acid |
|------------------|---------------------------------------------|----|-----------------------|
| Guangdong | Content (mg g$^{-1}$) | RSD (%) | Content (mg g$^{-1}$) | RSD (%) |
| Guangxi | 1.005 | 1.7 | 3.549 | 1.3 |
| Fujian | 1.201 | 1.2 | 2.857 | 0.9 |
| | 0.973 | 1.9 | 3.016 | 1.8 |
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