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Analytical Methods

Preparative separation of flavonoids in *Adinandra nitida* leaves by high-speed counter-current chromatography and their effects on human epidermal carcinoma cancer cells

Erdong Yuan [a], Benguo Liu [b,*], Zhengxiang Ning [a], Chungang Chen [b]

[a] College of Light Industry and Food Science, South China University of Technology, Guangzhou 510640, China
[b] School of Food Science, Henan Institute of Science and Technology, Xinxiang, Henan 453003, China

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ABSTRACT

Preparative separation of camellianins A and B in *Adinandra nitida* leaves, which is a flavonoid-rich plant source with great practical prospects, was conducted by high-speed counter-current chromatography (HSCCC) with a solvent system composed of ethyl acetate–ethanol–water (5:1:5, v/v). Apigenin (their aglycone) was prepared by hydrolysing a water extract and recrystallising. The purity and identity of these flavonoids was confirmed by HPLC-ESI/MS, and ^{1}H and ^{13}C NMR. These flavones were used in cultures of the A431 cell line. Their inhibitory effect was evaluated by using the MTT assay. The results showed that three flavones could significantly inhibit the growth of human epidermal carcinoma cancer cells in a concentration-dependent manner. After 48 h of treatment, IC_{50} values of camellianin A, camellianin B and apigenin against A431 tumour cells were 9.09 μM, 12.5 μM, 21.0 μM, respectively.

1. Introduction

Flavonoids, widespread in fruits, vegetables, teas and medicinal plants, have received great attention and have been studied extensively, because they are a group of biologically active plant compounds. They capture pro-oxidants and free radicals as potent antioxidants (Burda & Oleszek, 2001). Recent studies report that they are also potent inhibitors of cellular growth (Bestwick & Milne, 2006; Kirszberg, Esquenazi, Alviano, & Rumjanek, 2003), having anticancer (Kobayashi, Nakata, & Kuzumaki, 2002), antibacterial (Kuroyanagi, Arakawa, Hirayama, & Hayashi, 1999) and anti-viral (Wu, Wang, Yi, & Lee, 2003) activities.

Today, more than 4000 kinds of flavonoids have been identified or synthesized. However, only rutin can be widely used in the fields of food and medicine. One reason is that few kinds of plants can contain enough flavonoids to achieve large-scale production, although flavonoids exist ubiquitously in plants. *Adinandra nitida*, a particular wild plant in south China, is a flavonoid-rich plant source. Its leaves have been consumed as health tea (Shiyacha) and herbal medicine for hundreds of years. It is reported to have many curative effects, such as reducing blood pressure, as well as antibacterial, antioxidant and analgesic properties (Liu, Ning, Gao, & Xu, 2008a; Wang et al., 2003). Our previous studies (Liu, Ning, Zhan, Xu, & Gao, 2008b; Liu, Zhan, Ning, Gao, & Xu, 2008c) showed that the content of flavonoids in *A. nitida* leaves can be more than 20%, including camellianin A, camellianin B and apigenin, and camellianin A is the main flavonoid. Today, *A. nitida* may be artificially planted in Guangxi province, China. So, there is possibility of large-scale production of camellianin A for use in functional food and in the phytopharmaceutical industry. Because of the heat-instability of the ester bond of camellianin A, it was difficult to separate camellianins A and B with high purity, as is required for quality control and screening of different biological activities. Due to the gentle operation conditions, high-speed counter-current chromatography (HSCCC) was used to prepare camellianins A and B in this study. The cytotoxic activities of the two flavonoids and apigenin (their aglycone) against human epidermal carcinoma cancer A431 cells were also evaluated by using the MTT method. To the best of our knowledge, the HSCCC separation and cytotoxic activities of camellainin A and camellianin B are now reported for the first time.

2. Materials and methods

2.1. Plant materials

Leaves of *A. nitida* (2007 production, moisture content 9.3%) for this study were purchased in Pingle, China, and identified by

* Corresponding author. Tel.: +86 0373 3040674; fax: +86 0373 3040709.
E-mail address: zzgclbg@126.com (B. Liu).

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Professor Yousheng ZHANG in Guangdong Academy of Agricultural Sciences.

2.2. Chemicals and reagents

A431 cell line was obtained from ATCC (Rockville, MD, USA). DMEM medium supplemented with 25 mM Hepes buffer, 60 mg/l of Penicillin, 2 g/l of sodium bicarbonate and 100 mg/l of streptomycin, DMSO (dimethylsulfoxide) and MTT (3-[4,5)-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were from Sigma Chemical Corporation. Foetal Calf Serum (FCS) was the product of Gibco (Invitrogen Corporation). Ethanol and ethyl acetate of analytical grade were used as received, and were purchased from Jingke Chemical Co., Guangzhou, China.

2.3. HSCCC apparatus

A model TBE-300A high-speed counter-current chromatograph was manufactured by Tauto Biotech Co. Ltd, Shanghai, China. It was equipped with a 260 ml multilayer coil column made of a 1.6 mm i.d. PTFE (polytetrafluoroethylene) tubing and a 20 ml sample loop. The HSCCC system was equipped with a model AKTA Prime plus (GE Co., Ltd.-Healthcare) as constant-flow pump, UV detector and fraction collector. The data were recorded and processed by a model N2000 workstation (Zhejiang University, Huangzhou, China).

2.4. Preparation of sample

One hundred grams of *A. nitida* leaves was extracted with 1500 ml of boiling water for 60 min and then filtered. The residue was extracted with 1200 ml of boiling water for 60 min and filtered. The filtrate was mixed and stored for 24 h at 2 °C and then filtered. The resultant precipitate was then collected and twice recrystallized from water. After drying at 60 °C for 3 h, 8.35 g of yellow product was obtained.

2.5. Separation of camellianins A and B

The HSCCC experiments were performed with a two-phase solvent system composed of ethyl acetate–ethanol–water (5:1:5, v/v). After thoroughly equilibrating the mixtures in a separatory funnel at room temperature, two phases were separated shortly before use. The upper organic phase was used as the stationary phase and the lower aqueous phase as the mobile phase. The multi player coiled column was first entirely filled with the upper phase as the stationary phase. Then the apparatus was rotated at 900 rpm, whilst the lower phase was pumped into the column at a flow rate of 2.0 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, 5 ml (for analysis) or 10 ml (for preparation) of sample solution containing 30 mg or 200 mg of the crude sample obtained in 2.4. were injected through the injection valve. The effluent of the column was continuously monitored with a UV detector at 280 nm, and the column temperature was set at 25 °C. The effluent was collected with a fraction collector in order to analyse and collect needed fractions.

2.6. Preparation of apigenin

Apigenin was prepared according to our previous study (Liu, Ning, Zhan, Gao, & Xu, 2008 c), slightly modified as follows: the leaves of *A. nitida* (200 g) were extracted twice with 2000 ml of boiling water for 1 h and then filtered. After cooling, sulphuric acid was added to the extract in the ratio of 1:50 (by volume). The mixture was heated for 20 min and then filtered to collect the yellow precipitate. The precipitate was washed on the filter until neutral pH was obtained and then dried. About 21.5 g of raw product were obtained from the yellow powder. By recrystallizing, four times, from ethanol, about 4.2 g of pure product were obtained.

2.7. HPLC-ESI/MS analysis

HPLC-ESI/MS analysis was performed on a Waters ZQ 2000 mass spectrometer (Milford, MA, USA) equipped with a Waters

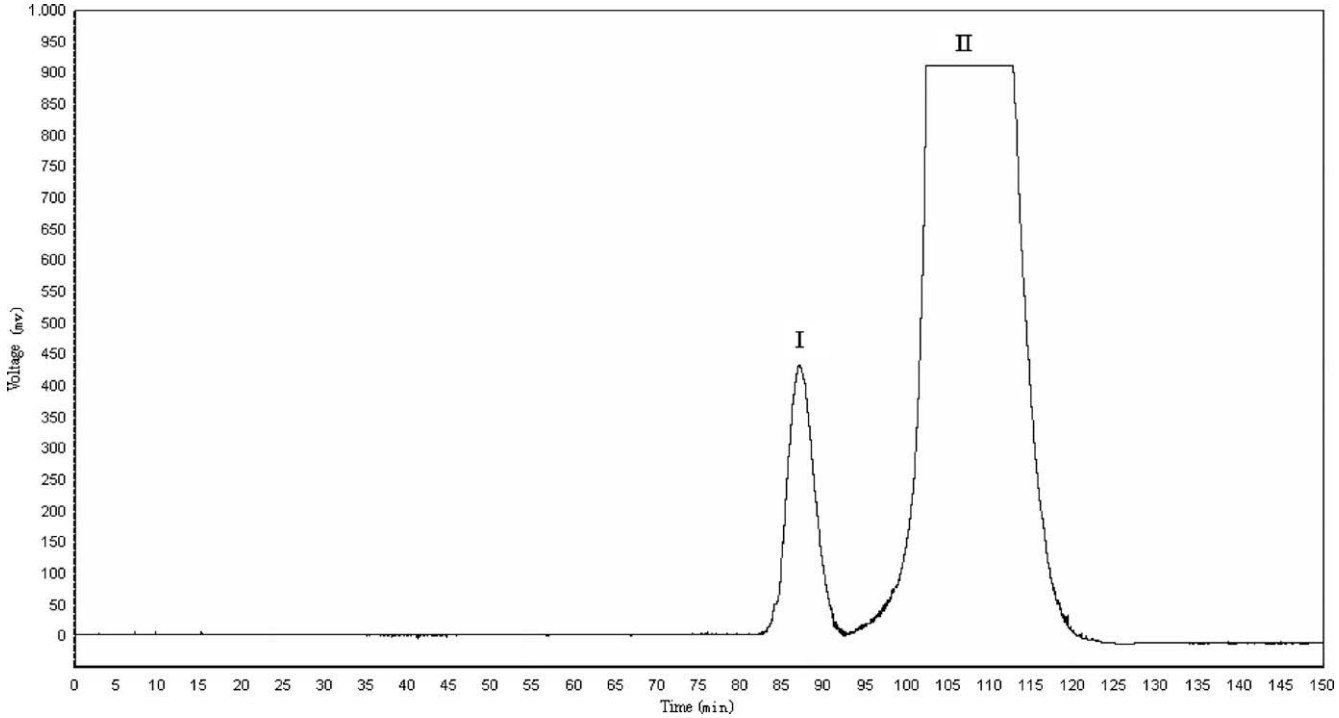

Fig. 1. HSCCC separation chromatogram of the crude sample.

1525 binary HPLC pump (Milford, MA, USA) and a Waters 2487 dual λ absorbance detector (Milford, MA, USA). Separation was performed on a reversed phase, Diamonsil C18 column (4.6 × 250 mm; 5 μm particle size, DIKMA, Beijing, China). The mobile phase consisted of methanol and water (1:1) with a flow rate of 1.0 ml/min. The wavelength for detection was set at 265 nm. The mass spectrometer was operated in a negative electrospray mode to obtain the best result with a source voltage of 3 kV. Deprotonated molecular ions were observed at cone voltages of 40 and 80 V. Nitrogen was used as a nebulizing gas with a cone gas flow at 50 l/h and a desolvation gas flow at 300 l/h. Source and desolvation temperatures were set at 110 and 300 °C, respectively. The scan range was set from 100 to 1500 m/z. The data were recorded and processed by Masslynx software ver. 4.1 (Milford, MA, USA).

2.8. NMR analysis

^{13}C and ^{1}H NMR spectra were recorded in DMSO-d_6 using an AVANCE Digital 400 MHz NMR spectrometer (Bruker, Germany) at 400 MHz.

2.9. Cellular viability with MTT

The A431 cell lines were kept by passages twice a week in DMEM medium (Hyclone) with 10% foetal calf serum (FCS) at 37 °C in 5% CO_2 atmosphere. The MTT method was used to measure the cellular viability (Mosmann, 1983). The cells at 2×10^4 cells/ml, were incubated with the samples for 48 h or 72 h in 96-well plates. After this period, 100 μl of MTT (1 μg/ml) per well were dispensed into the wells. The plate was incubated for 4 h in the dark, at 37 °C in 5% CO_2. After this, the plate was centrifuged again for 5 min at 1100 rpm, and the pellet was resuspended in 200 μl of DMSO. Optical density was measured on a Tecan multiwell scanning spectrophotometer reader Elisa at 570 nm and 630 nm (reference). IC_{50} values of the samples were calculated by using the programme of probit analysis for continued variables in a DPS 9.5 program package (Zhejiang University, Hangzhou, China) (Tang & Feng, 2007).

3. Results and discussion

3.1. HSCCC separation of camellianins A and B

HSCCC is a support-free all-liquid chromatographic technique that is widely used in natural product analysis due to the gentle operation conditions. HSCCC offers several advantages compared to preparative HPLC. As no solid stationary phase is used, no irreversible adsorption on active surfaces can occur. Sample loads in HSCCC are high; that is, isolation of up to several hundred milligrams of pure compounds can be achieved in a single HSCCC run (Degenhardt, Hofmann, Knapp, & Winterhalter, 2000).

Fig. 1 shows the HSCCC separation result of a 30 mg of crude sample, which gave two peaks I and II (Fig. 1) corresponding to peaks 1 and 2 in HPLC-ESI/MS analysis of the crude sample, respectively (Fig. 2). The two sections of the fractions' corresponding peaks, I–II, were combined and freeze-dried to yield 11.3 mg of

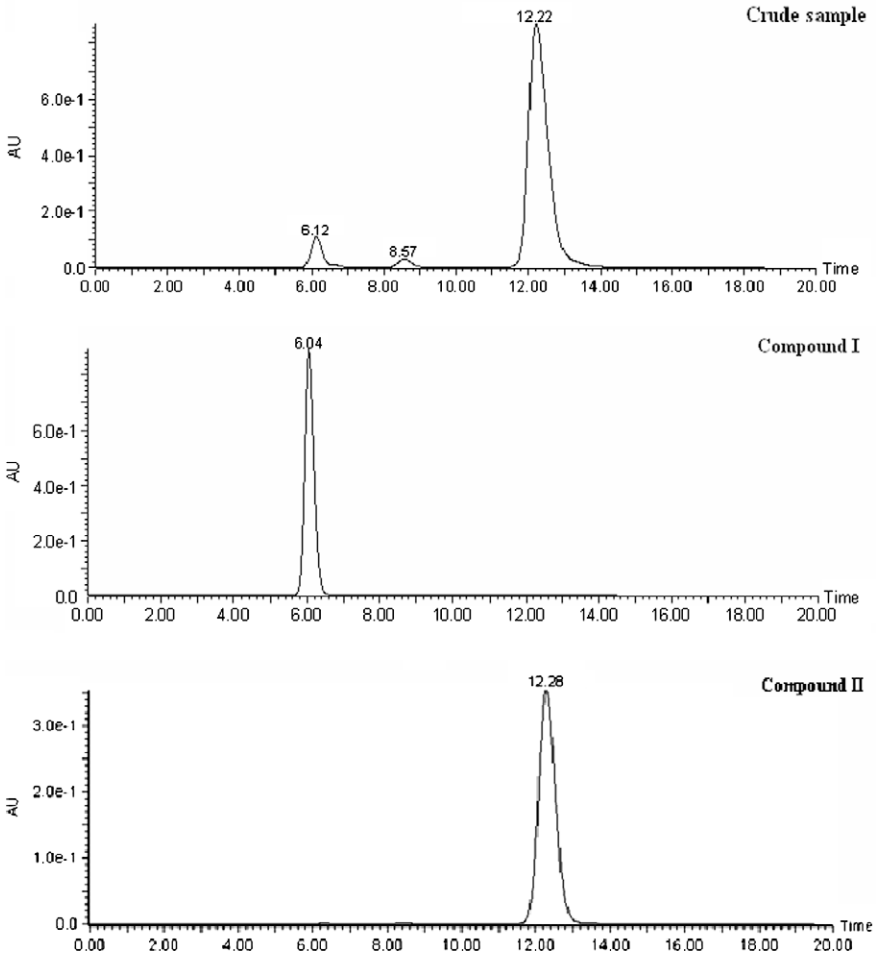

Fig. 2. HPLC-UV chromatogram recorded at 265 nm.

component I and 145.8 mg of component II from 200 mg of crude sample. To check the purity and identity of isolated flavonoids, HPLC-ESI/MS, ^{1}H and ^{13}C NMR were used.

Compound I was identified as camellianin B (Fig. 4). Negative ESI-MS m/z: 577.32 ([M-H]$^{-}$) (Fig. 3); ^{13}C NMR: 175.95 (C-4), 164.07 (C-2), 161.15 (C-4′), 160.35 (C-7), 159.35 (C-9), 158.01 (C-5), 128.23 (C-2′ and C-6′), 121.79 (C-1′), 116.38 (C-3′ and C-5′), 107.23 (C-10), 106.21 (C-3), 100.00 (C-1″), 99.78 (C-6), 98.10 (C-1‴), 96.79 (C-8), 77.49 (C-4″), 77.23 (C-3″), 76.91 (C-5″), 72.75 (C-2″), 71.00 (C-3‴), 70.84 (C-4‴), 70.35 (C-2‴), 68.95 (C-5‴), 61.15 (C-6″), 18.41(C-6‴); ^{1}H NMR: 7.82 (2H, d, J = 8.6 Hz, 2′- and 6′-H), 6.88 (2H, d, J = 8.6 Hz, 3′- and 5′-H), 6.49 (1H, s, 8-H), 6.44 (1H, s, 3-H), 5.26 (1H, d, J = 6.6 Hz, 1″-H), 5.20 (1H, s, 1‴-H), 3.0–5.0 (10H, m, Hs in sugar), 1.04 (3H, d = 6.0 Hz, 6‴-H).

Compound II was identified as camellianin A (Fig. 4). Negative ESI-MS m/z: 619.29 [M-H]$^{-}$ (Fig. 3); ^{13}C NMR: 176.31 (C-4), 170.70 (C-7″), 162.82 (C-2), 161.37 (C-4′), 160.90 (C-7), 159.36 (C-9), 157.68 (C-5), 128.46 (C-2′ and C-6′), 121.98 (C-1′), 116.51 (C-3′ and C-5′), 107.95 (C-10), 106.43 (C-3), 100.28 (C-1″), 99.75 (C-6), 97.79 (C-1‴), 96.94 (C-8), 77.47 (C-4″), 77.11 (C-3″), 74.03 (C-5″), 72.83 (C-2″), 71.11 (C-3‴), 71.02 (C-4‴), 70.55 (C-2‴), 69.25 (C-5‴), 63.50 (C-6″), 20.90 (C-8″), 18.52 (C-6‴); ^{1}H NMR: 10.73 (1H, s, 7-OH), 10.20 (1H, s, 4′-OH), 7.86 (2H, d, J = 8.6 Hz, 2′- and 6′-H), 6.92 (2H, d, J = 8.6 Hz, 3′- and 5′-H), 6.62 (1H, s, H-8), 6.53 (1H, s, H-3), 6.50 (1H, s, H-6), 5.51 (1H, d, J = 6.6 Hz, 1″-H), 5.41 (1H, d, J = 6.0 Hz, 1′-H), 5.20 (1H, s, 1‴-H), 3.16–4.64 (10H, m, Hs in sugar), 1.87 (3H, s, 8″-H), 1.09 (3H, d, J = 6 Hz, 6‴-H);

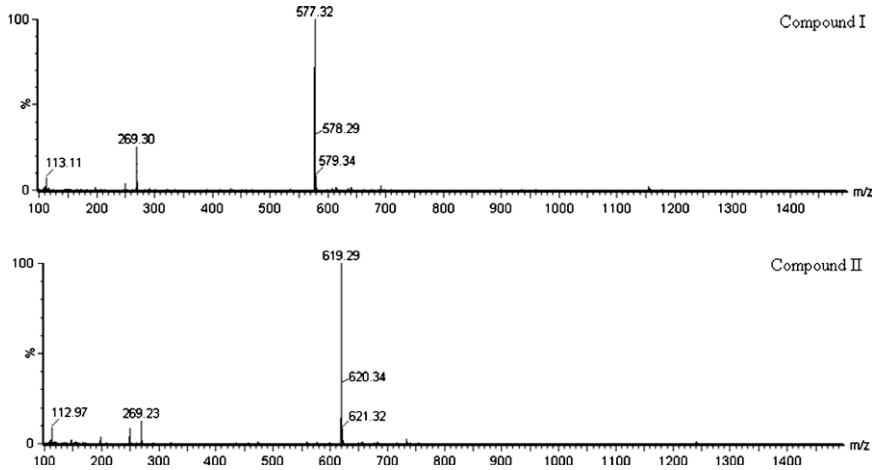

Fig. 3. ESI-MS spectra of compounds I and II.

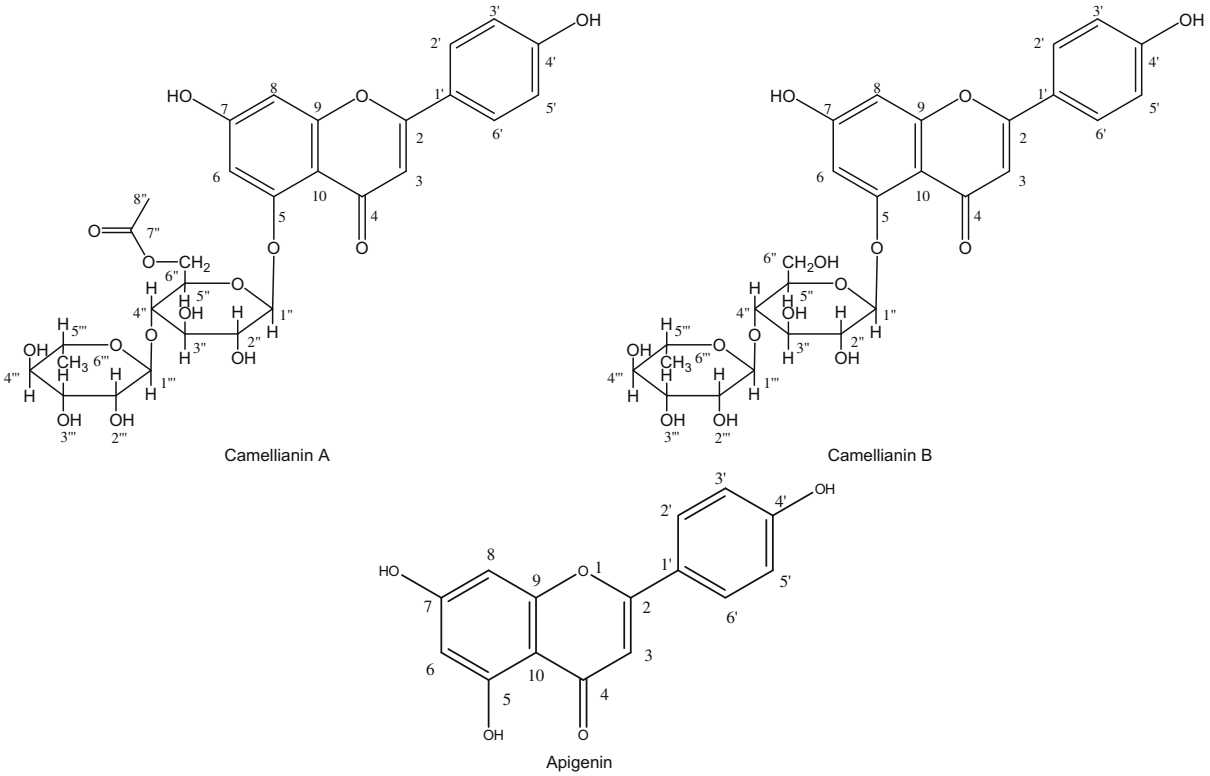

Fig. 4. Chemical structures of apigenin, and camellianins A and B.

In our previous study (Liu et al., 2008b; Liu et al., 2008c), the crystal of camellianin A was obtained by recrystallizing 10 times from water. But in the following HPLC-ESI/MS analysis, existence of camellianin B in the crystal of camellianin A could still be found, which could attribute to the heat-instability of the ester bond of camellianin A. The results in this study demonstrate that HSCCC is an excellent technology: it could separate two flavonoid glycosides with little difference in chemical structure in leaves of *A. nitida*, which could not be separated by the traditional method of recrystallizing. This is the first report of the isolation of flavonoids from *A. nitida* leaves by HSCCC.

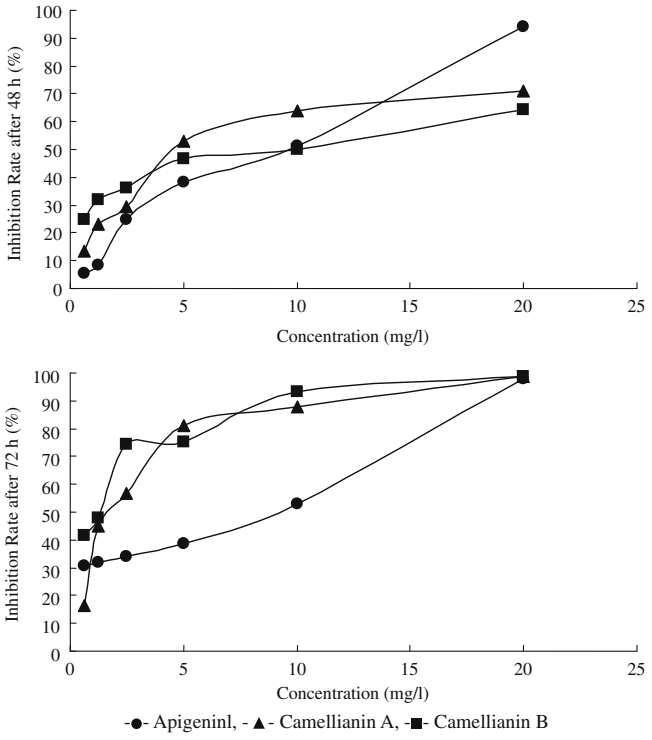

Fig. 5. Inhibitory effects of camellianin A, camellianin B and apigenin on A431 cells.

3.2. Preparation of apigenin

In order to investigate the anticancer activity-structure relationship of camellianins A and B, the anticancer activity of their aglycone–apigenin was also investigated in this study. The existence of apigenin in *A. nitida* leaves had been proven by our previous study (Liu et al., 2008b) but due to the low content of apigenin in leaves of *A. nitida*, it was difficult to obtain it by using HSCCC. So in this study, apigenin (Fig. 4) was prepared by hydrolysing a water extract and recrystallising. Its purity and identification were ensured by the following spectral data: Negative ESI-MS m/z: 539 $[2M-H]^-$, 269 $[M-H]^-$; ^{13}C NMR (400 MHz,DMSO-d_6) δ, 182.189 (C-4), 164.576 (C-7), 164.166 (C-2), 161.902 (C-5), 161.610 (C-4'), 157.748 (C-9), 128.906 (C-2' and C-6'), 121.622 (C-1'), 116.395 (C-3' and C-5'), 104.147 (C-10), 103.278 (C-3), 99.278 (C-6), 94.404 (C-8); ^1H NMR (400 MHz, DMSO-d_6) δ, 12.970 (1H, s, 5-OH), 10.584 (2H, s, 4' and 7-OH), 7.918 (2H, d, J = 8.8 Hz, 2'–6'-H), 6.923 (2H, d, J = 8.8 Hz, 3'–5'-H), 6.773 (1H, s, 3-H), 6.477 (1H, br, 8-H), 6.193 (1H, br, 6-H). By the simple process and high yield, the method for preparing apigenin proposed in this study is very interesting from the point of view of industrialisation.

3.3. Effect of three flavonoids on tumour cell viability

Cancer is the second leading cause of death in the world. The prognosis for a patient with metastatic carcinoma (lung, colon, hepatic, or prostate) remains a concern and accounts for more than one half of all cancer deaths. Almost all artificial agents currently being used in cancer therapy are known to be toxic and they cause severe damage to normal cells. Therefore, chemoprevention or chemotherapy via nontoxic agents could be one approach for decreasing the incidence of these cancers. Naturally occurring dietary antioxidants found in food and medicinal plants could, in theory, serve as alternatives to chemically designed anticancer agents (Rao, Geethangili, Fang, & Tzeng, 2007).

Leaves of *A. nitida* have been consumed over hundreds of years in south China; their safety has been accepted. However, no scientifically proven information is available on the anticancer effects of the flavonoids obtained from this plant with food and medicinal functions. In this study, the cytotoxic activities of camellianin A, camellianin B and apigenin against human epidermal carcinoma cancer A431 cells were evaluated by the MTT assay, and the results

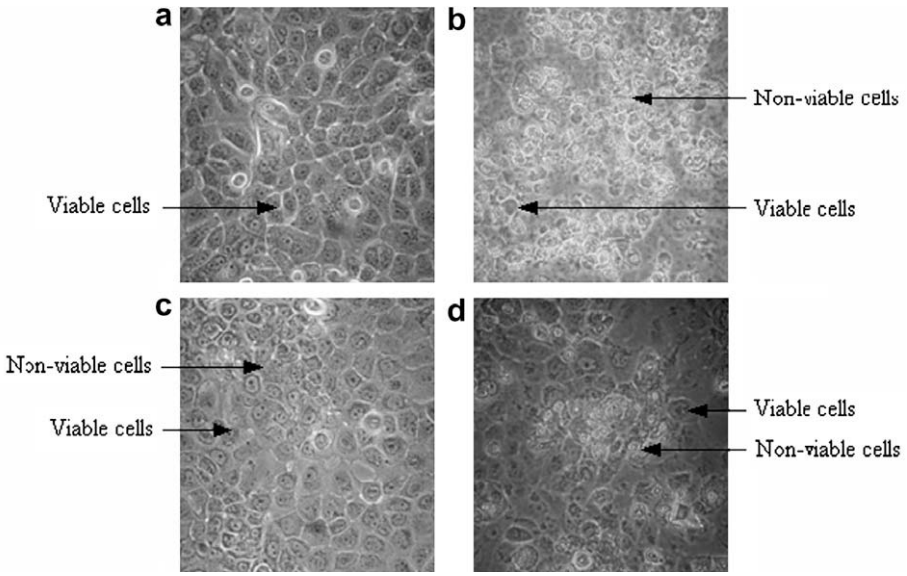

Fig. 6. Morphology of human epidermal carcinoma cancer A431 cells treated by camellianin A, camellianin B and apigenin.

are shown in Fig. 5. The three flavones showed significant anticancer effects against A431 tumour cells by affecting cell proliferation and changing the cell morphology (Fig. 6). The viable cells could retain their common shape, but the non-viable cells lost their shape and broken into pieces. The inhibitory effects of three flavones were also observed in a concentration-dependent manner. After 48 h of treatment, IC_{50} values of camellianin A, camellianin B and apigenin against A431 tumour cells were 9.09 μM, 12.5 μM and 21.0 μM, respectively. The inhibitory effect of camellianin A was superior to that of apigenin. After 72 h of treatment, the performance of camellianin A, with an IC_{50} value of 2.98 μM, was still better than that of apigenin, with an IC_{50} value of 11.65 μM.

The antitumour effect of flavonoids has been extensively studied on several tumour cell lines. Different mechanisms have been linked to flavonoid-mediated cytotoxicity, including proteasome inhibition, inhibition of fatty acid synthesis, topoisomerase inhibition, inhibition of phosphatidyl-inositol 3-kinase, induction of cell cycle arrests, accumulation of p53 or enhanced expression of c-fos and c-myc (Plochmann et al., 2007).

The 4-carbonyl group of flavonoids has been reported to correlate with inhibition of fatty acid synthesis (Brusslmans, Vrolix, Verhoeven, & Swinnen, 2005). The presence of the 2–3 double bond has been linked to efficient binding and inhibition of the P-glycoprotein (P-gp) (Plochmann et al., 2007). The three flavones in leaves of *A. nitida* possess the above functional structures. As a result, they show inhibitory effects on A431 tumour cells to different degrees. Although it is reported that high lipophilicity seems to be associated with enhanced cytotoxicity, the cytotoxicity of apigenin with high lipophilicity was significantly less than that of camellianins A and B with low lipophilicity, which may be attributed to the poor water-solubility of apigenin. In conclusion, we examined the cytotoxic activities of 3 pure flavones, prepared from A. nitida leaves, against human epidermal carcinoma cancer A431 cells. The results could explain the anticancer activity of this plant.

Acknowledgements

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References

Bestwick, C., & Milne, L. (2006). Infuence of galangin on HL-60 cell proliferation and survival. *Cancer Letters, 243*, 80–89.

Brusslmans, K., Vrolix, R., Verhoeven, G., & Swinnen, J. V. (2005). Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. *The Journal of Biological Chemistry, 280*, 5636–5645.

Burda, S., & Oleszek, W. (2001). Antioxidant and antiradical activities of flavonoids. *Journal of Agricultural and Food Chemistry, 49*(6), 2774–2779.

Degenhardt, A., Hofmann, S., Knapp, H., & Winterhalter, P. (2000). Preparative isolation of anthocyanins by high-speed countercurrent chromatography and application of the color activity concept to red wine. *Journal of Agricultural and Food Chemistry, 48*(12), 5812–5818.

Kirszberg, C., Esquenazi, D., Alviano, C. S., & Rumjanek, V. M. (2003). The effect of a catechin-rich extract of *Cocos nucifera* on lymphocytes proliferation. *Phytotherapy Research, 17*, 1054–1058.

Kobayashi, T., Nakata, T., & Kuzumaki, T. (2002). Effect of flavonoids on cell progression in prostate cancer cells. *Cancer Letters, 176*, 17–23.

Kuroyanagi, M., Arakawa, T., Hirayama, Y., & Hayashi, T. (1999). Antibacterial and antiandrogen flavonoids from *Sophora flavescens*. *Journal of Natural Products, 62*(12), 1595–1599.

Liu, B. G., Ning, Z. X., Gao, J. H., & Xu, K. Y. (2008a). Preparing apigenin from leaves of *Adinandra nitida*. *Food Technology and Biotechnology, 46*(1), 111–115.

Liu, B. G., Ning, Z. X., Zhan, Y., Xu, K. Y., & Gao, J. H. (2008b). Characterization and 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of methanol and supercritical carbon dioxide extracts from leaves of *Adinandra nitida*. *Journal of Food Biochemistry, 32*(4), 431–442.

Liu, B. G., Zhan, Y., Ning, Z. X., Gao, J. H., & Xu, K. Y. (2008c). Characterization and antioxidant activity of flavonoid extract from leaves of *Adinandra nitida* Merr. ex Li. *Chemistry and Industry of Forest Products, 28*(1), 6–10.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assays. *Journal of Immunological Methods, 65*(1–2), 53–63.

Plochmann, K., Korte, G., Koutsilieri, E., Richling, E., Riederer, P., Rethwilm, A., et al. (2007). Structure-activity relationships of flavonoids-induced cytotoxicity on human leukemia cells. *Archives of Biochemistry and Biophysics, 460*, 1–9.

Rao, Y. K., Geethangili, M., Fang, S. H., & Tzeng, Y. M. (2007). Antioxidant and cytotoxic activities of naturally occurring phenolic and related compound: A comparative study. *Food and Chemical Toxicology, 45*(9), 1770–1776.

Tang, Q. Y., & Feng, M. G. (2007). *DPS data processing system: Experimental design, statistical analysis, and data mining*. Beijing: Science Press.

Wang, Y., Chen, S. B., Ni, J., Yao, X., Ye, W. C., & Zhao, S. X. (2003). Chemical Studies on the *Adinandra nitida*. *Journal of China Pharmaceutical University, 34*(5), 407–409.

Wu, J. H., Wang, X. H., Yi, Y. H., & Lee, K. H. (2003). Anti-AIDS agents 54. A potent anti-HIV chalcone and flavonoids from genus *Desmos*. *Bioorganic and Medicinal Chemistry Letters, 13*(10), 1813–1815.