

Communication

Anti-Proliferative Effect of Camellianin A in *Adinandra nitida* Leaves and Its Apoptotic Induction in Human Hep G2 and MCF-7 Cells

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Abstract: Leaves of *Adinandra nitida* constitute a kind of flavonoid-rich plant food. In this study, camellianin A, the main flavonoid in the leaves of *Adinandra nitida*, was prepared and identified by high performance liquid chromatography-photodiode array detector-electrospray ionization mass spectrometry (HPLC-PDA-ESI/MS). In the anticancer assay, it was found camellianin A could inhibit the proliferation of the human hepatocellular liver carcinoma Hep G2 and human breast adenocarcinoma MCF-7 cell lines in a dose-dependent manner and induce the significant increase of the G0/G1 cell population. After treated by camellianin A, phosphatidylserine of Hep G2 and MCF-7 cells could translocate significantly to the surface of the membrane. The increase of an early apoptotic population of Hep G2 and MCF-7 cells was observed. It was concluded that camellianin A not only affected the progress of the cell cycle, but also induced cells to enter into apoptosis.

Keywords: *Adinandra nitida*; camellianin A; flavonoid; apoptosis; cell cycle

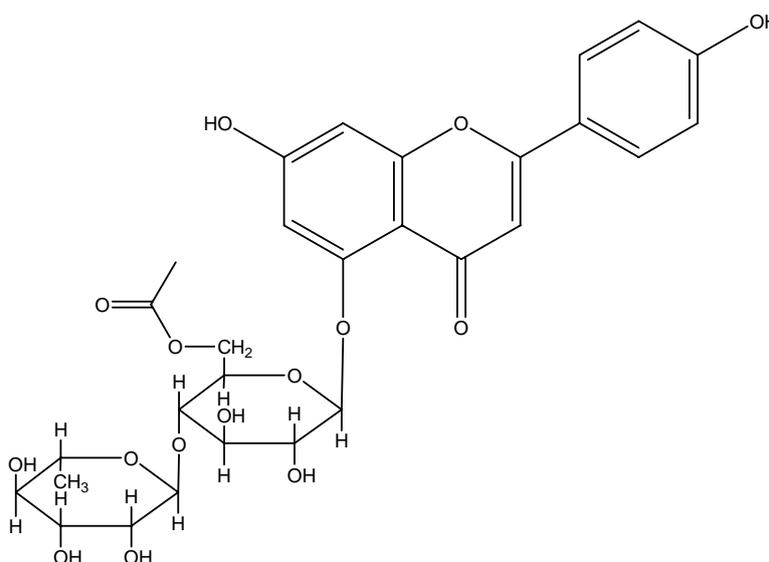
1. Introduction

Flavonoids have received great attention and have been studied extensively for their antioxidant, antibacterial, analgesic antitumor properties. It is believed that a higher intake of flavonoid-rich foods

is associated with decreased risk of many degenerative diseases, particularly cardiovascular diseases and cancer [1,2].

Adinandra nitida (Theaceae), a wild plant from South China, is a flavonoid-rich plant source. Its leaves have been consumed as a health tea (Shiyacha) and herbal medicine for hundreds of years [3,4]. It is reported to have many curative effects, such as reducing blood pressure, as well as antibacterial, antioxidant, and analgesic properties [5]. According to our previous studies [6–8], the total flavonoid content in *A. nitida* leaves can be more than 20%. Nowadays, *A. nitida* can be artificially cultivated in China, so there is the possibility for large-scale use of camellianin A in the health food and phytopharmaceutical industry. However, so far *A. nitida* leaves have not been thoroughly studied. For example, it was always thought that the anticancer activity of *A. nitida* leaves was due to its flavonoids, but this was not supported by any experimental evidence. In this study, camellianin A (Figure 1), the main flavonoid in *A. nitida* leaves, was separated and its apoptotic induction in human Hep G2 and MCF-7 cells were investigated for the first time.

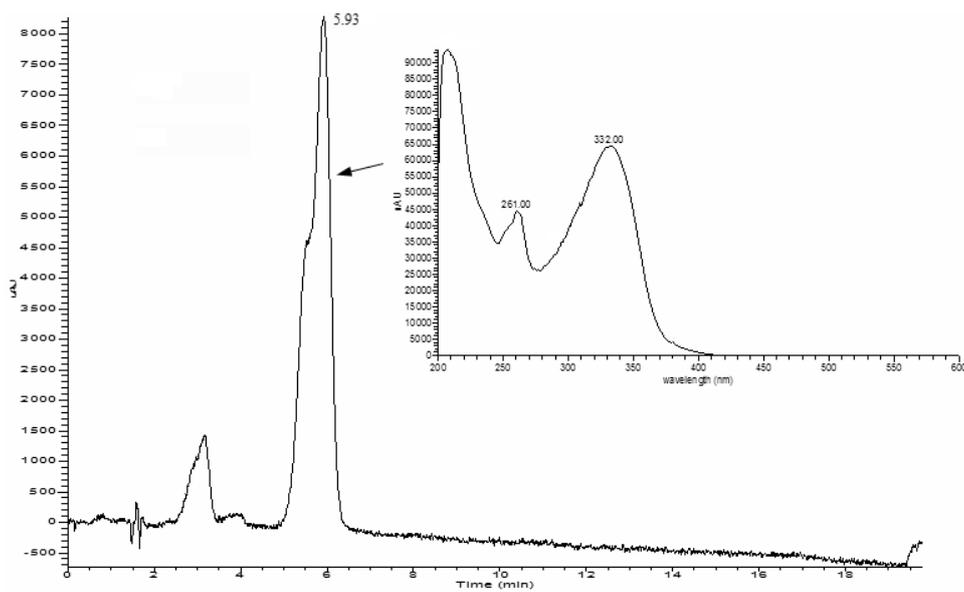
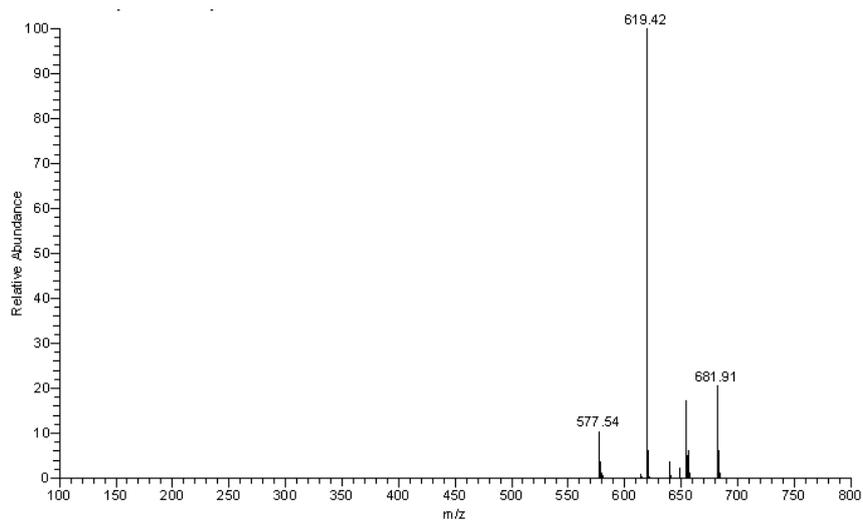
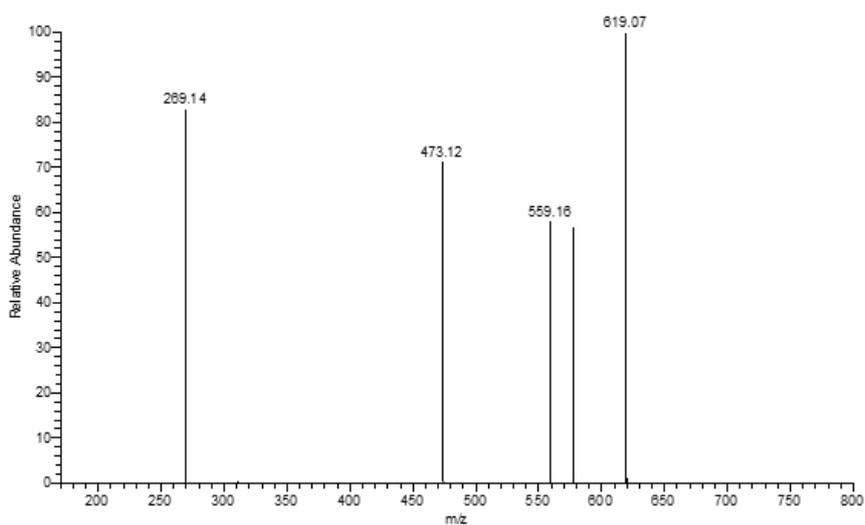
Figure 1. Chemical structure of camellianin A.



2. Results and Discussion

2.1. Identification and determination of Camellianin A

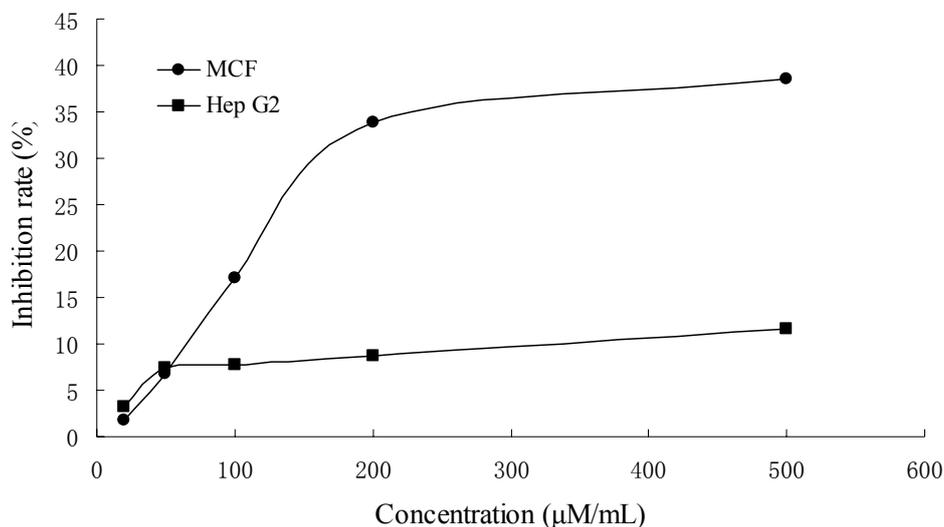
By using HPLC-PDA-ESI/MS, the compound eluted at 5.93 min in the HPLC (Figure 2) was identified as camellianin A. The UV and ESI-MSⁿ data (Figures 3 and 4) were as follows: UV, λ_{\max} (nm) 261, 332; ESI-MS² negative ion m/z : 681.91 ([M+NO₃]⁻), 654.87 ([M+Cl]⁻), 619.42 ([M-H]⁻), 577.54 ([M-Acetyl-H]⁻), 473.12 ([M-Rham-H]⁻), 269.14 ([M-Acetyl-Rham-Glu-H]⁻), which coincided with our previous study [6]. The content of camellianin A in *A. nitida* leaves was determined as $19.25 \pm 0.24\%$ by HPLC.

Figure 2. HPLC-PDA profile of camellianin A.**Figure 3.** ESI-MS of camellianin A.**Figure 4.** ESI-MS² of camellianin A.

2.2. Anti-proliferative effect of camellianin A on human Hep G2 and MCF-7 cells

The inhibitory effects of camellianin A on the proliferation of Hep G2 and MCF-7 cells were tested at different concentrations for 48 h and the inhibition rates were determined (Figure 5).

Figure 5. Effect of camellianin A on Hep G2 and MCF-7 cell proliferation.



Camellianin A induced a dose-dependent inhibitory effect. Nearly 33.8%, 8.7% of MCF-7 and Hep G2 cells were inhibited by 200 μM camellianin A. Compared with Hep G2 cells, inhibition of MCF-7 cell proliferation by camellianin A was more effective. The differential activity of camellianin A on both cell lines represented the selectivity of its antitumour action. Though camellianin A could inhibit the proliferation of Hep G2 and MCF-7 cells, there were other compounds present in *A. nitida* leaves which could also contribute to its antiproliferative activity.

2.3. Cell cycle analysis

To determine whether the Hep G2 and MCF-7 cells treated with camellianin A undergo the apoptosis pathway, the cell distribution in the cell cycle was examined by PI staining.

Figure 6. Effect of camellianin A on cell cycle distribution in MCF-7 cells.

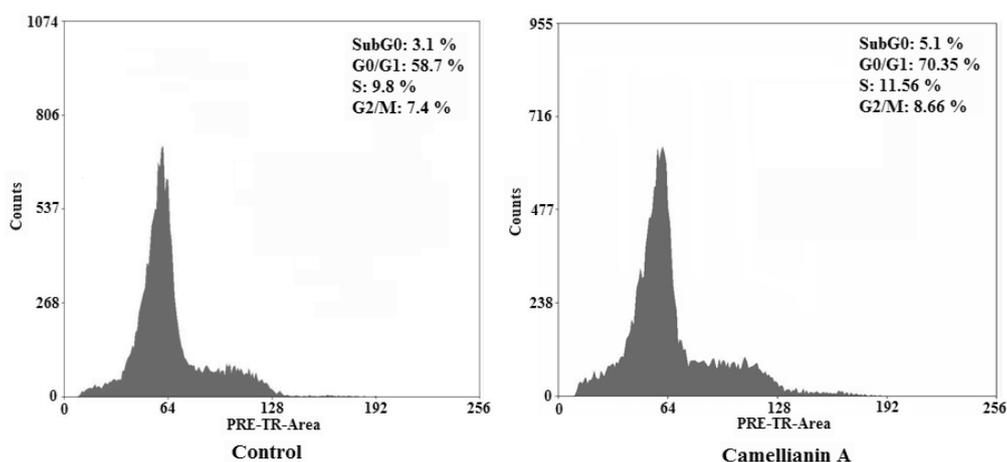
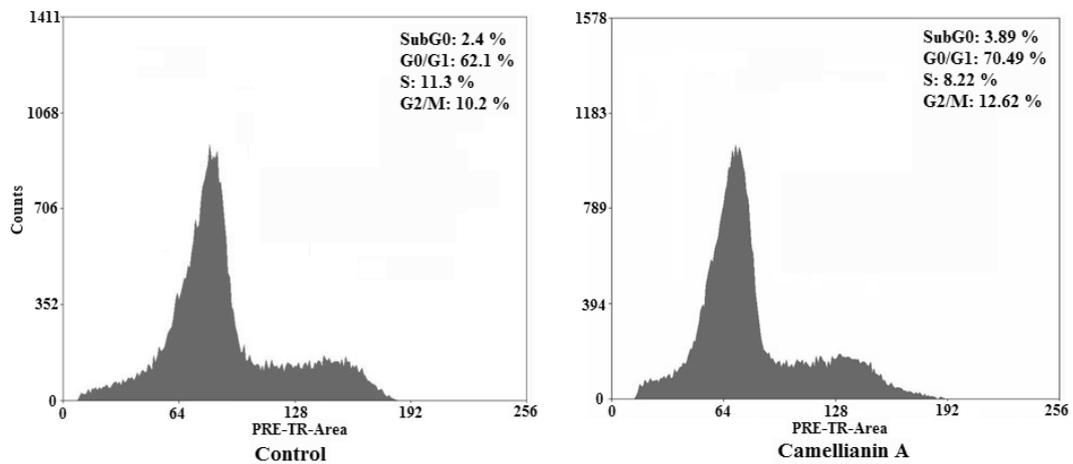


Figure 7. Effect of camellianin A on cell cycle distribution in Hep G2 cells.

Camellianin A induced the increase of the cell population in the cell population in the G0/G1 phase in MCF-7 and Hep G2 cells (Figures 6 and 7). It was found that the G0/G1 population of treated tumor cells increased dramatically which was suggested to be the apoptotic DNA.

2.4. Flow cytometry analysis of cell apoptosis

Apoptosis is a highly regulated cell death process with characteristic biochemical features [9,10] and membrane-bound apoptotic bodies [11]. The hallmark of early apoptotic cells is the transverse redistribution of plasma membrane phosphatidylserine (PS); thus, the annexin V binding assay was performed to detect the surface exposure of PS. Figures 8 and 9 showed the FACS histograms with dual parameters including annexin V-FITC and PI.

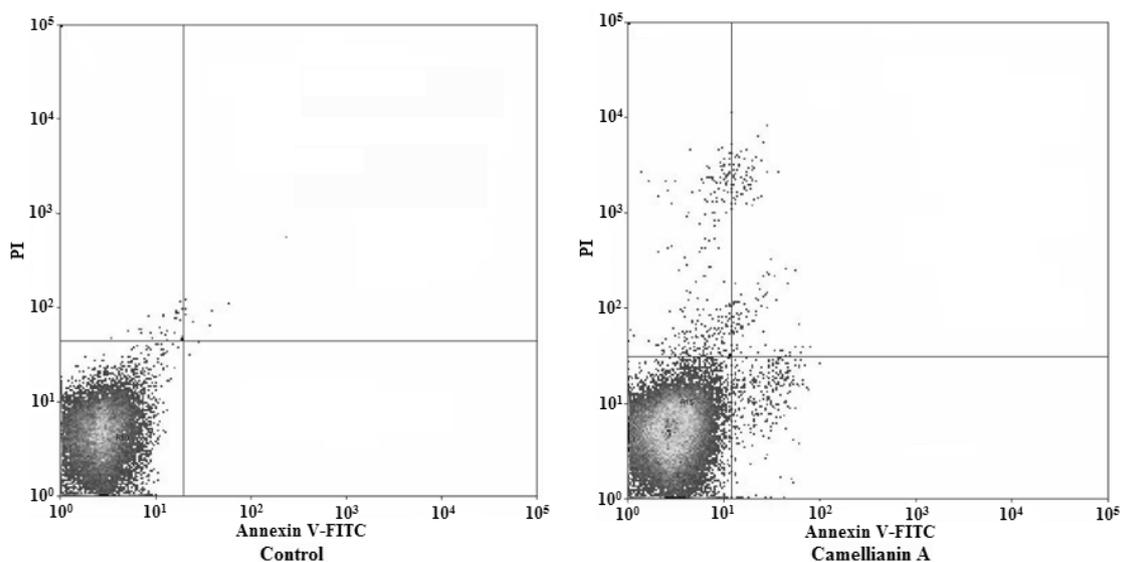
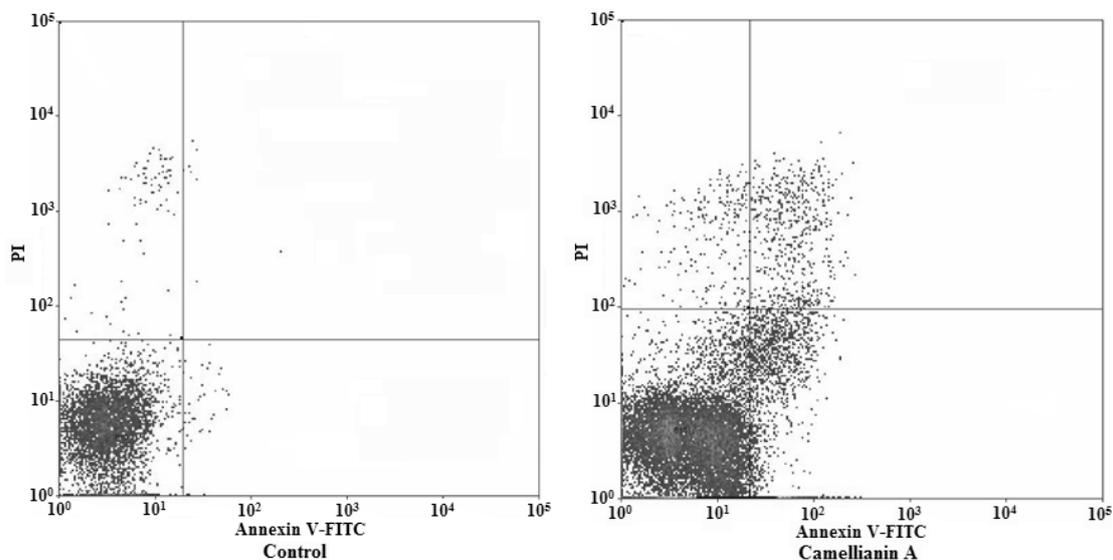
Figure 8. Flow cytometric analysis of phosphatidylserine externalization and cell membrane integrity in MCF-7 cells undergoing apoptosis.

Figure 9. Flow cytometric analysis of phosphatidylserine externalization and cell membrane integrity in Hep G2 cells undergoing apoptosis.



The dual parametric dot plots combining annexin V-FITC and PI fluorescence showed the viable cell population in the lower left quadrant (annexin V-negative/PI-negative), the early apoptotic cells in the lower right quadrant (annexin V-positive/PI-negative), and the late apoptotic cells in the upper right quadrant (annexin V-positive/PI-positive). In untreated MCF-7 cells, 0.01% of cells were the early apoptotic cells, 0.1% of cells were the late apoptotic cells. The early and late apoptotic cells increased to 1% and 0.46%, respectively, after being treated with camellianin A at a concentration of 0.5 mM for 48 h. For Hep G2 cells, the control cells, only 0.7% cells were the early apoptotic cells and 2.7% cells were the late apoptotic cells. The early and late apoptotic cells were 15.08% and 2.7%, respectively after the cells were treated with camellianin A at the concentration of 0.8 mM for 48 h. Our work demonstrated that PS could significantly translocate to the surface of the membrane in the Hep G2 and MCF-7 cells. Early apoptotic cells were observed by both annexin V-FITC and PI staining. These result suggested that camellianin A in *A. nitida* leaves could inhibit the proliferation of Hep G2 and MCF-7 cells in a dose-dependent manner and induced the cells to enter into apoptosis.

3. Experimental

3.1. Materials and chemicals

Leaves of *A. nitida* (2009 production, moisture content 9.3%) for this study were purchased in Pingle, China, and identified by Professor Yousheng Zhang in Guangdong Academy of Agricultural Sciences. The voucher specimen was deposited in the Laboratory of Food Chemistry of Henan Institute of Science and Technology. The camellianin A standard was obtained from our previous study. The human hepatocellular liver carcinoma Hep G2 and human breast adenocarcinoma MCF-7 cell lines were obtained from the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences. Powdered Dulbecco modified eagle medium and trypsin solution were purchased from GIBCO (Grand Island, NY, USA). Fetal bovine serum (FBS) and antibiotics (penicillin and

streptomycin mixture) were purchased from Hyclone Laboratories, Inc. Propidium iodide (PI) and 3-[(4, 5)-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. An annexin V-FITC apoptosis detection kit was purchased from Clontech Laboratories Inc. Other chemicals were of analytical grade.

3.2. Preparation of Camellianin A

About 24.38 g of powdered *A. nitida* leaves was extracted with 500 mL of 60% ethanol (v/v) in a water bath at 70 °C for 2 h and then filtered. The filtrate was concentrated under vacuum at 55 °C. The obtained solution was kept for 24 h at room temperature. The filtered precipitate (6.72 g) was collected and purified to obtain the white crystals by recrystallizing three times from 30% ethanol (v/v). After drying, about 3.50 g of the product was gained for identification and anticancer tests.

3.3. HPLC-PDA-ESI/MS analysis

A sample of the obtained above powder (10 mg) was dissolved and diluted to 100 mL with methanol. After filtration through 0.45 µm Millipore filter, a 10 µL volume of the solution was injected for HPLC-PDA-ESI/MS analysis, which was performed using an LCQ Deca XP MAX system (Finnigan, USA). Separation was performed on an Eclipse×DB-C18 column (150 × 4.6 mm i.d.; 3.5 µm particle size). The mobile phase consisted of methanol and water (6:4). The flow rate was 0.8 mL/min. The first detection was by Photodiode Array Detector (PDA), and the second detection employed an Electrospray Ionization Mass Spectrometry (ESI-MS). Due to the acidic nature of flavonoids, the mass analyzer was set to record the negative spectra. The scan range was set from 100 to 800 m/z.

3.4. Determination of the content of Camellianin A in *A. nitida* leaves by HPLC

Dried powdered leaves (1.029 g) were placed in a Soxhlet extractor and refluxed at 80 °C for 10 h with methanol (150 mL) and then the extract was collected and diluted to 500 mL with methanol. After filtered through a 0.45 µm Millipore filter, a 10 µL volume of the solution was injected for HPLC analysis. For the calibration curve, the stock solution of camellianin A standard was diluted with methanol in the concentration range from 28.65 to 573 µg/mL for camellianin A, All the solutions were stored at 4 °C. The sample was separated on a reversed phase column, SunFire™ C18 column (4.6 × 250 mm; 5 µm particle size) made by Waters. The mobile phase consisted of water and methanol (1:1) with a flow rate of 1.0 mL/min. The column temperature was set at 30 °C. The HPLC analysis was performed on a Waters Alliance HPLC system, which consisted of a Waters 2695 separations module and a Waters 2487 dual wavelength detector. The injection volume was 10 µL and the wavelengths for detection were set at 265 nm and 330 nm. The quantitative analysis of camellianin A in the sample was based on an external standard. The chromatographic data were recorded and processed by Empower 2 software.

3.5. Cell culture and drug treatment

The human MCF-7 and Hep G2 cells were cultured in DMEM medium with 10% FBS, 100 UI/mL penicillin and 100 µg/mL streptomycin in humidified air at 37 °C with 5% CO₂, respectively. The exponentially growing cells were collected and re-suspended in fresh medium for 4 h and then exposed to various concentrations of camellianin A.

3.6. MTT assay

Survival of cells was evaluated by using a system based on MTT, which was reduced by living cells to yield a soluble formazan product that could be detected colorimetrically. Cells were suspended in 96-well plates of 90 µL medium at a density of 2×10^4 cells/well and 10 µL camellianin A at different concentrations. These were then incubated in humidified air at 37 °C with 5% CO₂ for 48 h, exposed to 10 µL MTT (5 mg/mL) and incubated for another 4 h under the conditions mentioned above. The formazan precipitate was dissolved in 100 µL DMSO. IC₅₀ values were tested through the MTT method [12]. The inhibition rate was calculated as follows: Inhibition Rate % = (mean control absorbance - mean experimental absorbance) / mean control absorbance × 100%.

3.7. Flow cytometry analysis

The flow cytometry analysis was performed on a FACS Calibur Flow cytometer (BeckmanCoulter, USA). Cell pellets were fixed in 70% ethanol at -20 °C for at least 12 h or overnight. After being washed twice with ice-cold PBS, they were incubated in RNase A/PBS (1 mg/mL) at 37 °C for 30 min, and stained with PI (0.5 mg/mL) at room temperature for 15 min. The intracellular DNA was then labeled with PI and the PI fluorescence of individual nuclei determined by a FACSCalibur fluorescence-activated cell sorter at 488 nm excitation. Surface exposure of phosphatidylserine in apoptotic cells was measured by the annexin V-FITC apoptosis detection kit according to the manufacturer's instructions. Additional exposure to PI made it possible to differentiate the early apoptotic cells (annexin V-positive/PI-negative) from the late apoptotic cells (annexin V-positive and PI-positive).

3.8. Statistical analysis

The data obtained in this study were expressed as the mean of three replicate determinations and standard deviation (SD). Statistical comparisons were carried out using student t test. P values of <0.05 were considered to be significant.

4. Conclusions

Though flavonoids exist ubiquitously in plants, few plant species contain the levels needed to achieve large-scale production. *Adinandra nitida* leaves are rich in camellianin A. In this study, it was found that camellianin A could inhibit the proliferation of the human hepatocellular liver carcinoma Hep G2 and human breast adenocarcinoma MCF-7 cell lines in a dose-dependent manner by inducing cells to enter into apoptosis. The obtained result could be the basis for its alleged health

promoting potential of *Adinandra nitida* leaves and *A. nitida* leaves could serve as a new source of natural nutraceuticals with potential applications in the food and medicine industries.

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Sample Availability: Samples of the compounds are available from the authors.

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