SUPPLEMENTARY MATERIAL

Antitumor activity on human bladder cancer T-24 cells and composition analysis of the core of *Camellia osmantha* fruit

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In this study, antitumor activity and composition analysis of the core of *Camellia osmantha* fruit were investigated. The cores were extracted by 80% ethanol to obtain the crude extract (TF), which was successively distributed by different concentrations of methanol with MCI resin to obtain three different parts (Fr.s 1 to 3). Cytotoxicity activities showed that TF, Fr.2, and Fr.3 exhibited good inhibition against T-24 with IC$_{50}$ values of 6.7 ± 1.3, 6.9 ± 0.9, and 6.7 ± 1.74 μg/mL, respectively. Fr.3 has a strong inhibitory activity on T-24 cells, mainly due to effectively increasing the release of intracellular calcium ions and reactive oxygen species. In addition, Fr.3 can inhibit T-24 cells migration and invasion. Further composition analysis on Fr.3 was detected by LC-Q-TOFMS implying the main components to be ellagic acid and its derivatives.

Keywords: *Camellia osmantha*; Composition analysis; T-24; LC-Q-TOFMS; Cell migration

Abbreviations: liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-Q-TOFMS); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); reactive oxygen species (ROS); High-performance liquid chromatography (HPLC); Fetal bovine serum (FBS); Dulbecco's Modified Eagle Media (DMEM); Roswell Park Memorial Institute (RPMI-1640); Dimethyl sulfoxide (DMSO); and trifluoroacetate (TFA); phosphate buffer solution (PBS); 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA); Flow Cytometry/financial capacity model (FCM); mean fluorescence intensity (MFI).

1 Experimental

1.1 Instrument and reagents

HPLC was performed on a Dionex UltiMate3000 HPLC system (Dionex, UltiMate 3000, USA). LC-HR/MS was measured on an Agilent 6545B Q-TOF LC/MS (Agilent technologies, Quebec, Canada). FBS was obtained from Gibco, USA. DMEM medium and RPMI-1640 medium were from Hyclome. MTT, trypsin, PI, RNase, and Hoechst 33342 were purchased from Sigma. Reactive oxygen detection kit and calcium ion
detection kit were purchased from Biyuntian Institute of Biotechnology, China. DMSO and TFA were purchased from Xilong Chemical Industrial Co., Ltd. Guangdong, China. HPLC-grade acetonitrile and methanol were from Fisher Scientific, UK.

1.2 Preparation of sample

The cores of *C. osmantha* fruit (200g) were collected in Nanning, Guangxi. It was dried, smashed, and passed through an 80 mesh sieve. The samples were extracted three times with 80% ethanol and filtered. The solvent was removed under reduced pressure to give 2.02 g of crude product (TF) as a brown solid. The crude extract was dissolved in water and separated into three parts (*Fr.s 1 to 3*) by gradient elution with 0%, 50%, and 100% methanol using a column of MCI-gel CHP 20P. All fractions are dried and stored at -4°C.

1.3 Cell cultures

Five cell strains used for proliferation inhibitory activity in this experiment *in vitro*, that is, WI-38 human normal liver cell line, MGC-803 human gastric cancer cell line, four human bladder cancer cell lines (T-24, 5637, and EJ), Hela human cervical cancer cell line, and 7402 human liver cancer cell line were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The above cells were placed in DMEM medium containing 5% fetal bovine serum, 100 U/mL penicillin or RPMI-1640 medium, and cultured in an incubator containing 5% CO₂ at 37 °C. The cells were observed under a microscope at intervals, and after the cells were grown to 80% to 90%, they were washed twice with a buffer solution (pH=7.43) of 0.05 mol/L PBS. Passage was digested with 0.25% trypsin and logarithmic growth cells were used for the experiment.
1.4 Cell viability tests

Cell viability tests were evaluated by MTT assay reported from the literature methods (Elbaz et al. 2012). Seven cells in the logarithmic growth phase were seeded at a density of $1.0 \times 10^4$ cells/mL in a 96-well culture plate. In the experimental group, 200 μL of sample with HAM / F12 medium were added to each well to a final concentration of 1, 2, 5, 10, and 40 μg/mL, and DMSO with the same concentration as the control group. After 24 and 48 hours of incubation, 5 g / L of MTT solution was added to each well. The supernatant was aspirated and shaken by adding 150 μL of DMSO four hours later. After mixing and dissolving the crystals, the absorbance (OD value) was measured at 490 nm on an enzyme-linked immunosorbent detector, and each dose was repeated to 4 wells in parallel. The inhibition rate was calculated according to the following formula:

$$\text{the inhibition rate} = \left(1 - \frac{A_{\text{test}}}{A_{\text{control}}} \right) \times 100\%.$$

The semi-inhibitory concentration ($IC_{50}$) of each fraction against various cell lines can be calculated by the Bliss method. All experiments were repeated three times and averaged.

1.5 Morphology analysis of apoptosis stained by Hoechst 33342

Morphology analysis of T-24 was observed by stained with Hoechst 33342 according to the literature (Tan et al. 2015). T-24 cells in logarithmic growth phase were inoculated into a 24-well plate with a density of $2.5 \times 10^5$ cells/mL. After adding 24 μg/mL Fr-3 to the plate for 24 h, the culture solution was aspirated and 4% paraformaldehyde was added to fix for 2 h, and then rinse twice with PBS. Finally, 200 μL of Hoechst 33342 staining solution were added with a final concentration of 20 μmol/L, staining for 30 min in the dark and rinse twice with PBS again. Photos were observed with the inverted fluorescence microscope.
1.6 Determination of release of intracellular calcium ions

Release of intracellular calcium ions (Driesbaugh et al. 2014). T-24 cell lines were collected at different times and digested after Fr-3 was added with the concentration of 12 and 24 μg/mL. Then T-24 cells were washed twice with RPMI1640 with a mass fraction of 0.1%, stained with trypan blue with a mass fraction of 0.25%, and the viable cells were counted above 95%. The cell suspension was pre-warmed at 37 °C for 5 min, then washed with PBS and calcium-free Tyrode for 2 to 3 times. 100 μL of Fluo-3/AM dye solution was added and incubated at 37 °C for 45 min. The fluorescence intensity of the cells was recorded using a laser scanning confocal microscope with the excitation wavelength at 488 nm under a 40-fold objective lens. The fluorescence intensity of the cells reflected the concentration of Ca$^{2+}$.

1.7 Determination of Reactive Oxygen Production Status in T-24 Cells by FCM

24 μL of T-24 cell suspension (1×10^6 /mL) was treated with different concentrations of Fr-3 (12, 24 μg/mL) for 24 h, then washed twice with PBS. 5 μmol/L of DCFH-DA 2 mL was added to each tube after the supernatant was removed by centrifuged at 1500 rpm for 5 min. 2 mL of PBS was added as the control group. After shaking at 37 °C for 20 min in a constant temperature water bath, centrifugation for 5 min at 1500 r / min, 600 μL of PBS was added to the supernatant, and DCF green fluorescence intensity was measured by fluorescence microscopy (TH4-200 Olympus, Japan) after DCFH-D reaction with FCM. The FCM sub-laser has an excitation wavelength of 488 nm and a power of 10 mW. The results are expressed as MFI.

1.8 Cell wound-healing assay

T-24 cell migration was measured by a scratch test (Jiang et al. 2010). T-24 cells were seeded in a 6-well plate at a cell concentration of 5×10^5 /mL per well. When the cells
were almost full, a 200 μL pipette tip was used to draw a trace in the middle of the cells. Images were captured using fluorescence microscopic (Cytation 5 Cell Imaging Multi-Mode Reader, BioTek Instruments, Inc., USA) at 0 h and 36 h after treatment with 0, 12 and 24 μg/mL of Fr-3. The floating cells were washed with PBS. The supernatant (induced migration) of mouse (Mus musculus) embryonic fibroblast cells (NIH-3T3) treated by Fr-3 with different concentrations (12, 24 μg/mL) was added. Photographs were taken with an inverted fluorescence microscope at 0, 36, and 48 h.

1.9 Components analysis by HRLC-MS

Chemical composition analysis of the samples was performed using a DIONEX U-3000 HPLC. Gradient elution must be used to separate. The gradient elution conditions were optimized after ensuring that the main components of all samples were able to flow out. The column was a YMC-C18 reverse phase column; flow rate: 1.0 mL / min; column temperature 30 °C. Wavelength was detected with a DAD detector. Liquid chromatography condition is mobile phase A: acetonitrile+ 0.04% TFA, mobile phase B: water + 0.04% TFA. Gradient elution procedure: 0 minutes to 20 minutes, A: 10% → 60%, B: 90% → 40%.

Chromatographic system for MS analysis was composed of an 1260 Infinity series HPLC unit using a Supelcosil ABZ+PLUS column (150 mm × 4.6 mm, 3 μm) with 0.1% formic acid of water as mobile phase A and 0.1% formic acid of methanol as mobile phase B. The linear gradient was 0–3 min, 30% B; 3–7 min, 30–100% B; 7–10 min, 100% B, flow rate of 0.3 mL / min, detection wavelength of 210 and 254 nm, injection volume of 0.3 μL. The mass spectrometer uses an electrospray ionization source (ESI), negative ion scan, scan range (m/z): 100 ~ 1 500; dry gas flow rate 8 L/min, nebulizer pressure 20 psi, sheath gas temperature 350 °C, Sheath gas flow rate
11 L/min, capillary voltage 3 500 V.

1.10 Statistical analysis

Experimental data was performed in triplicate. Data were recorded as the mean and standard deviation and analyzed by Microsoft Excel 2003. The one-sample t-test was run on SPSS 19.0. When $P < 0.05$ was considered to be statistically significant, $P < 0.01$ was considered to be statistically significant.

2. Results and discussion

2.1 Morphological changes of Fr-3-induced T-24 cells apoptosis stained with Hoechst 33342 staining

It can be seen from the Figure S1 that the living cells grew adherently and the density was uniform, the nucleus was full and the structure was normal. While treated with Fr-3, the adherent cells decreased significantly, the shrinkage, rounding and exfoliated cells gradually increased. Moreover, the morphology of apoptotic cells appeared gradually: the cell nucleus shrunken and deformed obviously and some cells showed dense and dense staining, showing high cohesion and marginalization. Several cells were judged death according to cleaving into fragments or producing apoptotic bodies. The result indicates that Fr-3 can trigger early apoptosis of T-24 cells.

Figure S1. Assessment of nuclear morphological changes by hoechst 33342 staining in T-24 cells after 24 h.

2.2 Release of intracellular calcium and ROS in T-24 cells treated with Fr-3

Figure S2 shows the release of intracellular calcium ions and ROS in T-24 cells treated
with **Fr-3** for 24 h. As can be seen from the figure, the cells treated with **Fr-3** appeared brighter green fluorescence compared to the control group. Furthermore, the green fluorescence intensity increased significantly according to the increase of **Fr-3** concentration. It indicated that calcium ions were released from the cells increased significantly and influenced by the concentration-effect relationship. The result showed that **Fr-3** can effectively cause the increasing of calcium ions in T-24 bladder cancer cells. In the same vein, the green fluorescence intensity was increased significantly with the increase of drug concentration, indicating that the amount of ROS released significantly increased. The results showed that **Fr-3** can effectively cause to increasing of ROS in T-24 cells.

![Figure S2](image)

*Figure S2. The accumulation of Ca<sup>2+</sup> and ROS detected by cycation with Fluo-3 AM/DCFH-DA in T-24 cells*

**2.3 Inhibition of cell migration**

As shown in Figure S3, **Fr-3** was applied to T-24 cells, the migration ability of T-24 cells was significantly inhibited compared with the control group, and the inhibition effect at high concentration (24 μg/mL) was stronger than that of low concentration (12 μg/mL). The result indicated that the **Fr-3** inhibited T-24 cell migration in a dose-dependent manner.
From the migration area data of Table S1 (calculated by Image J software), it can be seen that the migration area of the control group gradually decreases as the time increased. The migration area was increased gradually along with the time after Fr-3 treatment at 12 μg/mL, and the velocity also decreased gradually. However, when the concentration of Fr-3 was increased to 24 μg/mL, the migration area was increased with the time, which may be due to the gradual death of the cells and suspended. In summary, Fr-3 can effectively inhibit the migration of T-24 cells.

Table S1 Effect of control and Fr-3 on T-24 cell migration in scratch wound model
2.4 HPLC Analysis of the cores of C. osmantha fruit

The chromatograms of **TF** and **Fr-3** under optimized chromatographic conditions are shown in Figure S4. As can be seen from the Figure S4, **Fr-3** existed four main components (Peaks 1-4), and each chromatographic peak is basically separated to meet the needs for qualitative analysis. Peaks 1 and 2 have a maximum absorption wavelength of 260 and 362 nm, and it is inferred that may be two compounds with numerous conjugated system. Peaks 3 and 4 have similar absorption with maximum wavelengths at 210 nm, which deduced to be components with less conjugated double bonds. Therefore, two type compounds may be the main substances inducing T-24 cells apoptosis.

| parameters                  | control | 12 μg/mL | 24 μg/mL |
|-----------------------------|---------|----------|----------|
| Migration area (10^5 μm^2)  | 0 h     | 9.028    | 9.329    | 8.891    |
|                             | 36 h    | 3.759    | 7.980    | 10.98    |
|                             | 48 h    | 2.948    | 7.894    | 12.37    |
Figure S4. Chemical constituents’ analysis of TF and Fr-3 with wavelength at 254 nm.

2.5 Identification of anti-tumor components

The chemical composition of Fr-3 was further analyzed by LC-MS (Table S2, Figures S5 and S6). In total, four main compounds in Fr-3 were identified by LC Q-TOF MS analysis. The extract ion chromatogram at m/z 300.9974 showed a peak at Rt 5.256 min, and was identified as ellagic acid (Chen et al. 2014). At m/z 343.0444, the chromatogram showed a peak at Rt 6.521 min, which was identified as 3,3’4’-tri-O-methylellagic acid (Yasumoto et al. 2006). At m/z 327.2161 the chromatogram showed a peak at Rt 6.621 min and m/z 329.2321 a peak at Rt 6.821 min, are possibly deduced a type of chain hydroxycarboxylic acids, such as trihydroxyoctadecenoic acid with five oxygen atoms (Falcão et al. 2013).

Table S2 HPLC-ESI-MS analyses of the four compounds identified in Fr-3

| Peak | Rt/min | Measured [M–H]⁻ | Molecular formula | Tentative identification |
|------|--------|-----------------|-------------------|-------------------------|
| 1    | 5.272  | 300.9974        | C₁₄H₆O₈           | ellagic acid            |
| 2    | 6.535  | 343.0444        | C₁₇H₁₂O₈          | 3,3’,4’-tri-O-methylellagic acid |
| 3    | 6.621  | 327.2161        | C₁₈H₃₂O₅          | Unknown                 |
| 4    | 6.821  | 329.2316        | C₁₈H₃₄O₅          | Unknown                 |

Figure S5. Total ion chromatogram and HPLC chromatogram (254 nm) of Fr-3
Figure S6. HRMS spectrometric analysis of the four compounds identified in the Fr-3 by LC- Q-TOFMS. A: peak 1, ellagic acid; B peak 2, 3,3’,4’-tri-O-methylellagic acid; C peak 3; D peak 4.

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