Ficus auriculata (fig) Extracts Induced Cell Cycle Profile Changes and Apoptosis Through Caspase-Independent Pathway in Human Lung Adenocarcinoma Cell Line, A549

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Abstract

Background: *Ficus auriculata* (fig) has immense value of benefits with regards to their medicinal and therapeutic properties. It has been long used in traditional folk medicine, and one of the fruits mentioned in Al-Quran. Many scientific researches have proven the usage of this natural medicine in in vitro and in vivo studies, where anti-cancer is among of its recognized properties.

Objective: To acquire further details on how *F. auriculata* (fig) inhibited cancer growth, we investigated the effects of its exposure on cell cycle profile.

Methods: Subsequently we determined the type of cell death and the mechanism it induced using flow cytometer. Human lung adenocarcinoma cell line A549 was selected as the model of this study.

Results: It is indicated significant decreased of A549 cells in G0/G1and concurrent accumulation of cells in G2/M phase. Cell death analysis revealed significant late apoptosis induced by *F. auriculata* (fig) via caspase independent pathway.

Conclusion: Therefore, we concluded that *F. auriculata* (fig) is one of the promising natural medicines that can be used as cancer preventive strategy, as a result from its effects against cell cycle profile and apoptosis via caspase independent pathway.

Keywords: Anti-cancer, Apoptosis, Natural medicine, Lung cancer

Introduction

Lung cancer, one of the most lethal forms of cancer, is the second leading cause of death among cancer patients. Poor prognosis of lung cancer patients is due to late-stage of diagnosis and ineffectiveness of current chemotherapy medicines. Therefore, the identification and exploration on complementary medicine for lung cancer treatment and prevention is highly needed. One of the promising sources of treatment or prevention is among natural product options. *Ficus*, is a fruit used in many cultures and have been used as cancer treatment in history.

Ficus comprises of more than 800 species belongs to 40 genera of the Moraceae family [1]. This genus is widely distributed at several regions, particularly of the tropics and subtropics area, and may extent to the temperate region [2]. It is divided into three groups based on their geographical distribution; mainly of Central and South America, Asia, Australasia and Africa. The bark has a rough texture and the fruits are reddish brown, pear in shape which located at the base of the trunk [3].

Fig has immense value of benefits with regards to their medicinal and therapeutic properties. Each part is used in treating ailments such as cuts and wounds, dysentery and diarrhea, cholera, mumps and jaundice [1, 4] and cancer [3]. Ethyl acetate, chloroform and ethanol extracts of *Ficus pseudopalma* Blanco is known to induced significant cytotoxic on human prostate (PRST2) cancer cells but not against human foreskin surface epithelial (hFSE) cells. Saini et al. (2012) also revealed cytotoxic activity of *F. palmate*,

F. auriculata and *F. pashia* were selective, where they imposed cytotoxicity against cervical cancer cells C33A without attacking normal peripheral blood mononuclear cells. This provides safety evidences on fig consumption as chemoprevention.

The principle behind cancer inhibition may results from various mechanisms and pathways. Cell cycle arrest is one of possible ways to reduce and limit cancer growth. Cell cycle of eukaryotic cells is typically divided into four phases: Gap 1 (G1), Synthesis (S), Gap 2 (G2) and Mitosis (M) [5] and is regulated by three key checkpoints principally at the entry of S phase (G1/S), M phase (G2/M), and anaphase (spindle checkpoint). Functional checkpoints ensure important events such as DNA replication have completed and cells are ready before mitosis can progress [6]. In the case of DNA damage or defects in the mitotic spindle, cells typically activate checkpoints and arrest the cell cycle [7]. However, checkpoints are often compromised in cancer, and cells frequently enter mitosis with extensive damage.

In addition to cell cycle control, cell death also plays major roles in reducing cancer cells development. Apoptosis is a type of cellular suicide program to remove unhealthy or unnecessary cells from the body following cellular stress as well as during developmental process [8]. It can occur via various pathways in response to multiple stimuli [9]. Two common apoptotic pathways have been discovered including the extrinsic and intrinsic pathway [8, 9].

Therefore, in this study, we examined the ability of *F. auriculata*, a common fig species

available in Malaysia [10] to arrest cell cycle. Subsequently we evaluated the type of cell death and the mechanism induced by this fruit using in vitro model, A549.

Materials and Methods
Plant Specimen

F. auriculata (fig) was collected from Malaysia near Kuantan, Pahang Malaysia. The plant was identified and authenticated at herbarium of International Islamic University Malaysia (IIUM), with voucher specimen number was deposited as PIIUM 0240.

Preparation of Plant Specimen

The fruit was cleaned with tap water followed by distilled water to removed dirt and wiped dried. Sample was cut homogenously into small pieces and stored in ultra-low temperature freezer at -80 °C overnight (Haier DW-86L388). On next day, frozen samples were freeze dried with freeze drying machine (Eyela FDU-1200) and kept in -80 °C until analysis [11].

Cell Line

Human lung adenocarcinoma A549 cells were purchased from ATCC® CCL-185 and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin. Cells were grown at density of 1×10^4 cells/cm^2 in T25 cm^2 flask and incubated at 37 °C, 95 % air and 5 % CO$_2$. Cells were passaged after density reach 80 % confluence. For experimental purposes, the cells were seeded at 1.96×10^5 [12].

Cell Cycle Phase Distribution Analysis

The assessment of cell cycle distribution of a whole cell population was done using Propidium Iodide (PI) staining method. This staining identified proportion of cells in each three distinct interphase stages; G0/G1, S and G2/M using the fluorescent nucleic acid binding dye. Cells growing in exponential phase were seeded in 60 mm culture plates at a density of 1.96×10^5. After 48 h, *Ficus auriculata* (fig) with a concentration of IC$_{50}$ obtained from earlier preliminary study was added for 48 h and harvested. Cells were washed with PBS and TrypLE solution was added to allow cells to detach. The cells were centrifuged at 600 g, washed with PBS twice and decanted. 70 % ethanol was drop wise added and kept at least 1 h in 4 °C freezer. Cells were centrifuged at 865 g and washed with PBS twice. The supernatant was discarded and the cell pellet was treated with 50 μl Ribonuclease A (RNase) solution in PBS (100 μg/ml) to remove unwanted RNA. Approximately 400 μl of PI solution in PBS (50 μg/ml) was added directly to cell pellet and incubated for 10 min at room temperature. Cells were analyzed with a flow cytometer (Guava easycyte) at a flow rate of 400 events per second. Data from three independent experiments were analysed using guavaSoft 2.7 software to determine percentages of cells in each phase: sub G1, G0/G1, S and G2/M. The assay was carried out in triplicate [13].

Type of Cell Death Analysis

The translocation of Phosphatidylserine (PS) from inner plasma membrane to the outer

surface of cells is an indicator of early apoptosis. Quantification of PS can determine the number of apoptotic cells in a whole cell population. This was done using Annexin V protein that has high affinity for PS. The protein was labelled with fluorescein isothiocyanate (FITC) to directly measure the apoptotic cells using flow cytometry. Cells were treated and harvested as in the cell cycle analysis. The procedure was referred to the kit's protocol. Cells were centrifuged at 865 g for 1 min and the supernatant was decanted. The cell pellet was resuspended with ice cold PBS and recentrifuged for 1 min and the supernatant was removed. Cells were resuspended in 200 µl of binding buffer and the density was adjusted to 5×10^7 cells/ml. Approximately 10 µl of Annexin V-FITC was added to the cell suspension, gently mixed and incubated for 10 min at room temperature. Cells were centrifuged for 1 min at 865 g and supernatant was decanted. Later, cell pellet was resuspended with 190 µl of binding buffer and 10 µl of 20 µg/ml PI stock solutions. Cells were analyzed by flow cytometry and results were obtained in percentages of cells in four quadrants. The assay was carried out in triplicate.

Mechanism of Cell Death Analysis

Activation of intracellular caspases is an indicator of programmed cell death. It is responsible for biochemical and morphological changes associated with apoptosis. Caspase 8 and Caspase 9 are initiator of apoptosis signal while Caspase 3 is the effector for apoptosis. Caspase activity was quantitated by cleavage of synthetic peptide para-nitroaniline (ρNA) and the light absorbed by ρNA was measured. Cells were seeded in 6 well plates at 9.6×10^4 and allow to grow for 48 h. The medium was discarded following 48 h and wash with PBS. *F. auriculata* (fig) was applied to the culture plates and incubated for several time points (0 h, 6 h, 12 h 24 h and 48 h) and harvested. No treatment was added to the control plate. Harvested cells were resuspended in 50 µl of chilled cell lysis buffer and incubated for 10 min. Later, it was centrifuged at 10 000 g and the supernatant was transferred and put on ice. Approximately 50 µl of 2x reaction buffers was aliquoted together with 10 µl of 1.0 M DTT (per 1 ml of 2x reaction buffer) were mixed together and added to each sample. Then, 5 µl of 200 µM substrate was added and incubated in the dark for 2 h at 37 °C. Samples were read at 405 nm using microplate reader. Results were interpreted based on fold increase of caspase activities compared to control. The assay was carried out in triplicate.

Statistical Analysis

All experiments were done in triplicate at least, independently. The effect of *F. auriculata* (fig) on cell cycle profile, cell death and the mechanism were evaluated using Wilcoxon matched-pairs signed rank test. The results were expressed as mean ± standard error of mean (SEM). Statistical analyses were performed with GraphPad Prism software 6.0. The value of P≤ 0.05 was considered as statistically significant.

Results
Cell Cycle Profile

Percentage of cells in each phase of the cell cycle was evaluated using Propidium Iodide dye. This dye binds to DNA and therefore is

proportional to DNA content of A549 cells. The results were measured following 48 h of incubation period. Etoposide was used as a positive control to validate the experimental result.

Table 1 shows quantitation of A549 fractions in each cell cycle phase following induction with *F. auriculata* (fig). Untreated A549 cells served as the control of the study showed the highest percentage in G1 phase (59.7 ± 1.4 %) compared to other cell cycle phases; S (14.6 ± 1.9 %), G2/M (25.4 ± 0.7 %) and Sub G1 (0.2 ± 0.2 %). Subsequent treatment with IC_{50} of etoposide (positive control) indicated decreased in G0/G1 from 59.7 ± 1.4 % to 29.0 ± 1.2 % and concurrent increased in G2/M phase from 25.4 ± 0.7 % to 54.9 ± 8.8 %. Co-culture with *F. auriculata* (fig) induced significant decreased in G0/G1 phase (P<0.05) and accumulation of A549 cells in G2/M phase when compared to untreated cells.

Discrimination between Apoptosis and Necrosis

In normal viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, apoptosis initiates PS translocation from the inner to the outer leaflet of cellular membrane, exposing PS to the external cellular environment. Phosphatidylserine exposure allows type of cell death (apoptosis or necrosis) and the degree to be determined using fluorescently labelled Annexin V/PI double label. The mode of cell death induced by *F. auriculata* (fig) is shown in Table 2. In the absence of treatment, A549 cells (control) exhibited higher (94.6 ± 1.4 %) number of non-apoptotic viable cells (Annexin V-PI-) than treated cells. Upon treatment with *F. auriculata* (fig), it triggered A549 cells to undergo late apoptosis (Annexin V+PI+) after 48 h of treatment (14.7 ± 3.4 %; P<0.05) .

Table 1- Relative proportion of A549 cells in cell cycle phases

48 h in Culture	G0/G1 Phase	S Phase	G2/M Phase	Sub G1 Phase
Positive Control (Etoposide)	29.0 ± 1.2	15.6 ± 7.2	54.9 ± 8.8	0.5 ± 0.5
Untreated A549 (Control)	59.7 ± 1.4	14.6 ± 1.9	25.4 ± 0.7	0.2 ± 0.2
F. auriculata (fig)	33.3 ± 13.5 *	16.8 ± 4.4	49.4 ± 13.9	0.5 ± 0.5

This table details the cell cycle distribution following treatments with *F. auriculata* (fig). A549 cells were seeded at 1.96×10^5 in 60 mm plates. Cells were grown for 48 h prior to exposure to *Ficus auriculata* (fig) IC_{50} and harvested after 48 h of exposure. Cells were stained with propidium iodide before analyzed by flow cytometry. Distribution of each phase was adjusted to 100 %. Data shown are the mean ± SEM (n=3). Statistical analysis was done using Wilcoxon matched-pairs signed rank test (* where P<0.05 when compared to untreated).

Table 2- Type of cell death induced by *F. auriculata* (fig)

| 48 h in Culture	Live Cells (%)	Early Apoptotic Cells (%)	Late Apoptotic Cells (%)	Necrotic Cells (%)
Untreated A549 (Control)	94.6 ± 1.4	0.8 ± 0.3	3.2 ± 0.4	1.6 ± 0.8
F. auriculata (fig)	65.1 ± 10.5	10.1 ± 4.5	14.7 ± 3.4 *	1.3 ± 0.7

This table details the percentage of cells from total measured by flow cytometry. Distribution of each quadrant was adjusted to 100 %. Data shown are the mean ± SEM (n=3). Statistical analysis was done using Wilcoxon matched-pairs signed rank test (* where P<0.05when compared to untreated).

## Pathway to Apoptosis: Caspase dependent or independent pathway

Apoptosis is a regulated cell death mechanism that controls tissues homeostasis characterized by DNA fragmentation, nuclear condensation, and membrane blebbing and cell shrinkage. A group of cysteine proteases called caspases are responsible for this action by activating these pro-enzymes via proteolytic cleavage hence disintegrate cells into apoptotic bodies. There are two distinct apoptosis pathways available typically the extrinsic and intrinsic pathways. The first initiates disassembly through caspase 8 following response from extracellular apoptosis inducing ligands while the second via caspase 9 in response to agents that cause cytochrome c release from mitochondria. Later these caspase 8 and caspase 9 were cleaved by caspase 3 thereby causing amplification of apoptosis signal and cascade of caspases.

According to Figure 1 and 2, there was no significant difference of caspase 3 and caspase 8 activities among time points between untreated and fig-treated cells. There was significant difference in caspase 9 activity between time points tested (P<0.05) while no difference in caspase 9 activity was observed between untreated and *F. auriculata* (fig) treated cells (P>0.05).
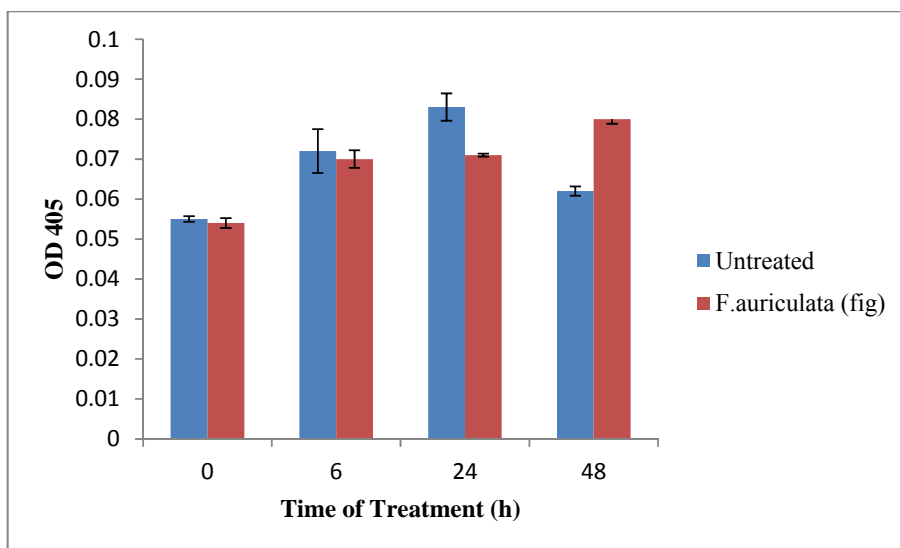
Figure 1- Caspase 3 activities subsequent treatment with *F. auriculata* (fig). A549 cells were seeded at 1.96×10^5 in 60 mm plates. Cells were grown for 48 h prior to exposure to the IC^{50} for 0 h, 6 h, 24 h and 48 h. Following time of exposure, cells were harvested and the caspase 3 activity was measured. Results are the mean ± SEM (n=3). Statistical analysis was done using Wilcoxon matched-pairs signed rank test. There was no significant difference of caspase 3 activity between untreated and *F. auriculata* (fig) treated cells (p>0.05) as well as among time points tested (P>0.05)
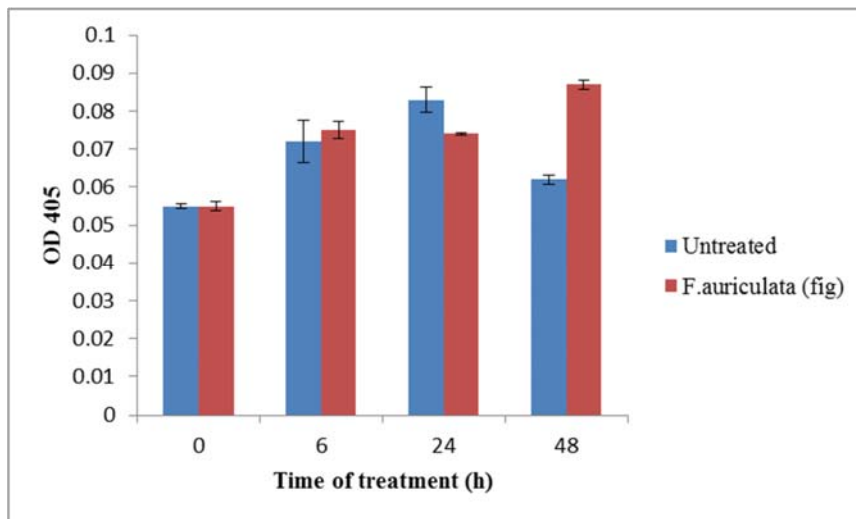
Figure 2- Caspase 8 activities subsequent treatment with *F. auriculata* (fig). A549 cells were seeded at 1.96×10^5 in 60 mm plates. Cells were grown for 48 h prior to exposure to the IC^{50} for 0 h, 6 h, 24 h and 48 h. Following time of exposure, cells were harvested and the caspase 8 activity was measured. Results are the mean ± SEM (n=3). Statistical analysis was done using Wilcoxon matched-pairs signed rank test. Results indicated there was no changes in caspase 8 activity of *F. auriculata* (fig) treated cells when compared to untreated cells
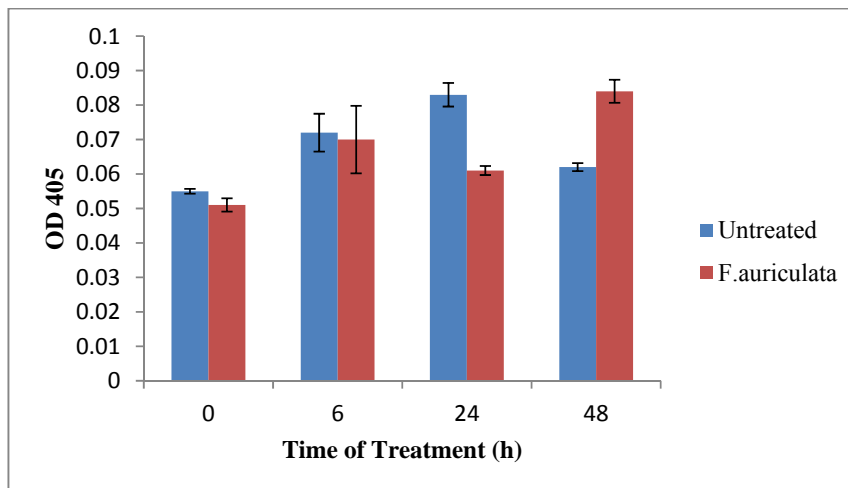
Figure 3- Caspase 9 activities subsequent treatment with *F. auriculata* (fig). A549 cells were seeded at 1.96×10^5 in 60 mm plates. Cells were grown for 48 h prior to exposure to the IC^{50} for 0 h, 6 h, 24 h and 48 h. Following time of exposure, cells were harvested and the caspase 9 activity was measured. Results are the mean ± SEM (n=3). Statistical analysis was done using Wilcoxon matched-pairs signed rank test.

Discussion

Aiming towards the cell cycle is an interesting approach for cancer treatment. Cells transverse through four distinct phases including G1 phase, S phase, G2 phase and M phase with two essentially important regulatory checkpoints; at late G1 and late G2.

These checkpoints are tightly controlled by cumulative receptors to ensure the quality and the rate of cell division is checked. However, cancer cells exhibits malfunctions in the regulatory checkpoints, allowing cells with aberrant DNA damage to transverse through the cell cycle phases. Several drugs have been

developed to target cell cycle checkpoints; most of it modulates the degree of cell cycle arrest. Interestingly, any approaches that can enhance the cell cycle checkpoint arrest may result in enhanced cytotoxicity and higher degree of apoptosis [14].

Previous study done in our laboratory had shown the fig exhibit antiproliferative effect in A549 cells [12]. To further evaluate whether anti-proliferative effect induced by *F. auriculata* (fig) was correlated with compromised cell cycle progression, the cell cycle distribution was assessed by flow cytometry. We assessed cell cycle profile to determine fractions of the cell cycle and to certain extent, cell cycle arrest induced by *F. auriculata* (fig). Our initial findings demonstrated significant decreases in G1 phase and concurrent accumulation of A549 cells in G2/M phase although it was not significant upon treatment with *F. auriculata* (fig) when compared to the untreated cells. These showed that anti-cancer activity induced by *F. auriculata* (fig) was associated with cell cycle distribution. Seiler and Raul (2005) reported an organic compound namely polyamines have the ability to regulate cell cycle by controlling growth-related genes expression, including the one that encode for cytoskeletal proteins [15]. Ghani et al. (2015) proved *F. auriculata* (fig) induced lower intracellular polyamines in A549 cells. It can be suggested that reduction of intracellular polyamines induced by *F. auriculata* (fig) caused significant interruption in regulation and progression of the cell cycle, hence, inhibited cancer growth.

Other than cell cycle distribution and progression, *F. auriculata* (fig) possesses their anti-cancer properties via apoptosis. Apoptosis is the most common form of genetically programmed cell death normally occurs during embryonic development, part of ageing process and maintenance of tissue homeostasis. A significant feature of early apoptosis is the translocation of membrane phospholipid phosphatidylserine (PS) to the outer plasma membrane. The evaluation of type of cell death induced by potential anticancer fruits is crucial to ensure the selected natural product is promising agent. This study has found that *F. auriculata* (fig) induced cell death via apoptosis in A549 cells. Several herbal compounds were identified and proven to activate apoptosis pathway that has been blocked in cancer. Some of the known anti-cancer compounds that induced cell death via apoptosis are curcumin, phenolic compound, ginger, resveratrol and alkaloids [16].

To further investigate the mechanism of cell death underlying the apoptosis, we evaluated caspase activities following 6 h to 48 h of exposure to *F. auriculata* (fig). Here, we determined whether apoptosis activity induced by *F. auriculata* (fig) occur either via caspase dependent or independent pathway. There are two pathways suggested to induce apoptosis in caspase dependent cell death which are intrinsic (mitochondrial pathways) and extrinsic pathway (death receptor-signalled pathways). Intrinsic pathway is usually activated in response to DNA fragmentation, chromatin degradation, protein cross-linking and formation of apoptotic

bodies whereby extrinsic pathway is induce by death receptor-ligand binding (FasL and TNF-α). Caspase 9 is release in intrinsic pathway while caspase 8 in extrinsic pathway. Cleavage of executor caspase 3 initiates either pathway.

Present study evaluated caspase 3, caspase 8 and caspase 9 activities in A549 following exposure to *F. auriculata* (fig). Results showed no caspase activities were observed in A549 suggesting caspase independent cell death has taken place. According to Broker et al. (2005), caspase is not the sole pathway how cell initiates cell death. This is supported by Rieckher and Tavernarakis (2010) who mentioned that apoptosis can be induced in absence of this enzyme. It is believed that caspase were inhibited or compromised when exposed to these treatments [17]. On the other note, absence of caspase does not mean the efficiency to kill the cells are reduced. This is proven by Cauwels et al. (2003) who demonstrated caspase inhibition enhanced the toxicity of TNF-α in mouse [18]. Other caspase independent cell death inducing compounds can be seen in α-Tomatine against mouse colon cancer [19] and flavonoids against osteosarcoma cells [20].

In conclusion, we evaluated the anti-cancer properties of *F. auriculata* (fig) based on cell cycle distribution, cell death analysis and the mechanism of cell death. We concluded *F. auriculata* (fig) induced significant decreased of A549 cells in G0/G1 and stimulated accumulation of cells in G2/M phase. Besides that, *F. auriculata* (fig) also caused cell death through apoptosis via caspase independent pathway.

Abbreviations

TCM, traditional chinese medicine; hFSE, human foreskin surface epithelial; IC^{50}, DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; CO^2, carbon dioxide; PBS, phosphate buffer saline; PS, phosphatidylserine; FITC, fluorescein isothiocyanate; ρNA, para-nitroaniline; half maximal inhibitory concentration; TNF-α, tumor necrosis factor alpha.

Author Contribution

R.A.G designed the experiments. E.F.J executed the experiments, evaluated the data and wrote the manuscript. R.A.G edited the manuscript.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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