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Potential anticancer activity of litchi fruit pericarp extract against hepatocellular carcinoma in vitro and in vivo

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Abstract

Litchi fruit pericarp (LFP) extract contains significant amounts of polyphenolic compounds, and exhibits powerful antioxidative activity against fat oxidation in vitro. The purpose of this study is to confirm the anticancer activity of LFP extract against hepatocellular carcinoma in vitro and in vivo, and to elucidate the mechanism of its activity. Human hepatocellular carcinoma cell line was tested in vitro for cytotoxicity, colony formation inhibition, and cell cycle distribution through flow cytometry after treatment with water-soluble crude ethanolic extract (CEE) from LFP. Murine hepatoma bearing-mice were fed doses of 0.15, 0.3, and 0.6 g/kg/day of water-soluble CEE in DH_2O p.o. for 10 days, respectively, to test the anticancer activity and BrdU incorporation of cancer cells in vivo. LFP extract demonstrated a dose- and time-dependent inhibitory effect on cancer cell growth; IC_{50} was 80 μg/ml, and significantly inhibited colony formation in vitro, tumor growth and BrdU incorporation into cancer cells in vivo. The tumor inhibitory rates at doses of 0.15, 0.3, and 0.6 g/kg/day were 17.31% ($P > 0.05$), 30.77% ($P < 0.05$), and 44.23% ($P < 0.01$), respectively. BrdU labeled tumor cells of treated animals were 11.80 ± 2.79%, and were significantly lower than that in untreated controls (23.00 ± 5.42%, $P < 0.05$). Our findings showed that LFP extract exhibited potential anticancer activity against hepatocellular carcinoma in vitro and in vivo through proliferating inhibition and apoptosis induction of cancer cells.

Keywords: Litchi fruit pericarp (LFP) extract; Anticancer activity; Hepatocellular carcinoma; Growth inhibition; Apoptosis

1. Introduction

Malignant cancer is the second leading cause of death worldwide. There is a continuing need for development of new anticancer drugs, drug combinations and chemotherapy strategies, through methodical and scientific exploration of the enormous pool of synthetic, biological and natural products [1]. In light of the continuing need for effective anticancer agents, and the association of fruit and vegetable consumption with reduced cancer risk, edible plants are increasingly being considered as sources of anticancer drugs [2]. In the past several decades, natural products were regarded as important sources that could produce potential chemotherapeutic agents. Over 50% of anticancer drugs approved by United States Food and Drug Administration, since 1960 originated from natural resources, and especially from terrestrial plants [3,4].

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Epidemiological studies revealed that consuming food and beverages rich in polyphenols (e.g. catechins, flavones, and antocyanines) was associated with a lower incidence of cancer [5]. Experimental investigation has demonstrated that many naturally occurring agents and plant extracts have exhibited antioxidant and anticancer potential in a variety of bioassay systems and animal models having relevance to human diseases [6]. Crude methanolic extract (CME) from the pericarp of *Garcinia mangostana* (family *Guttiferae*) has antiproliferative, apoptotic and antioxidative activities against human breast cancer cell line in vitro [7]. The antioxidant and anticancer activity of the extracts from medical plants and herbs are associated with components of phenolic compounds. The major types of phenolic compounds included phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids [8].

Litchi (*Litchi chinensis, Sapindaceae*) is a tree that originates from China and is cultivated for its sweet fruit worldwide in warm climates [9]. Litchi fruit pericarp (LFP) contains significant amounts of polyphenolic compounds. The principal characteristic of the polyphenolic compounds is their *ortho*-diphenolic structure, which gives them high oxidability. The major components of fresh LFP extract are condensed tannins (polymeric proanthocyanidins), epicatechin and procyanidin A2 [10]. The main components of mature and premature LFP extract are phenolic compound and flavonoids, which exhibit powerful antioxidative activity against fat oxidation in vitro [11].

Combinations of polyphenols naturally found in fruits and vegetables have been suggested to be optimal for cancer prevention [12–14]. Anticarcinogenic effects of polyphenolic compounds in fruits and vegetables are well established [12]. Therefore, based on the main components of LFP extract and its antioxidant properties [10,11], we hypothesized that LFP extract might have anticancer activities against some cancer cell lines in vitro and animal models in vivo. Currently, no reports have been published to this effect. The inhibitory effects of LFP extract on growth of human hepatocellular carcinoma cells in vitro and the growth inhibition of murine hepatoma in vivo, along with the mechanism of activity were investigated in this experimental study to confirm the hypothesis.

2. Material and methods

2.1. Reagents

RPMI-1640 was purchased from Gibco/BRL Invitrogen (Gaithersburg, MD). Fetal bovine serum (FBS) was purchased from Huaxi Biology Institute (Chengdu, People's Republic of China). Trypsin, methylthiazdyldiphenyl-tetrazolium bromide (MTT) and DMSO were purchased from the Sino-American Biotechnology Company of Beijing (Beijing, People's Republic of China), 5-Bromo-2-deoxy-uridine (BrdU) was purchased from Roche (Nutley, NJ). S-P immunohistochemical staining kit (SP9001) was purchased from Zhongshan Biological Technology (Beijing, People's Republic of China). All other chemicals and reagents were obtained from Sigma (St. Louis, MO).

2.2. Preparation of extracts

Mature Baila litchi (*Litchi chinensis, Sapindaceae* in Guangdong, China) fruit pericarp was collected, dried naturally at room temperature and powdered. The powdered material (100 g) was extracted with 95% ethanol (800 ml, two times) for 48 h at room temperature, and blended continuously with a magnetic force stirrer. The extract was filtered and concentrated to remove the solvent at 75 °C for 4 h, and then frozen dry. More than 22.0 g of crude ethanolic extract (CEE) was eventually yielded, and the percentage of yield was exceeded than 22.0%. 60% of the extract was water-soluble, and 40% was soluble in ethanol and acetone.

2.3. Cell line and culture

Human SMMC-7721 hepatocellular carcinoma cell line was obtained from Shanghai Cell Biology Institute of Chinese Academy of Sciences (Shanghai, People's Republic of China). The cells were maintained in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 50 μg/ml CO_2 at 37 °C.

2.4. In vitro assay for cytotoxic activity (MTT assay)

The cytotoxicity of water-soluble CEE from LFP was determined by a tetrazolium (MTT) assay [15]. Cells (1×10^3/well) were plated in 100 μl of medium/well in 96-well plates (Costar Corning, Rochester, NY). After overnight incubation, water-soluble CEE was added in various concentrations (20, 40, 80, 160, and 320 μg/ml). Five wells were included in each concentration. After treatment with water-soluble CEE for 1, 2, 3, 4, and 5 days, 20 μl of 5 mg/ml MTT (pH 4.7) was added to each well and cultivated for another 4 h. The supernatant fluid was then removed, 100 μl/well DMSO was added and samples were shaken for 15 min. Absorbance at 570 nm was measured with a microplate reader (Bio-Rad,

Richmond, CA) using wells without cells as blanks. Three independent experiments were performed. The effect of water-soluble CEE from LFP on proliferation of human hepatocellular carcinoma cells was expressed as the %cytoviability using the following formula: %cytoviability = A_{570} of treated cells/A_{570} of control cells × 100%.

2.5. Clonogenic survival determination

The cells were assayed for colony-forming ability by replating them in specified numbers (300/well) in six-well plates treated with 60, 120, and 210 μg/ml of water-soluble CEE, respectively. After 12 days of incubation, the cells were stained with 0.5% crystal violet in absolute ethanol, and colonies with >50 cells were counted under a dissection microscope. Three independent experiments were conducted in triplicate.

2.6. Cell cycle analysis (FCM)

Cell cycle was analyzed by flow cytometry analysis [16]. Briefly, cells were treated with 60 μg/ml of water-soluble CEE for 72 h. After 72 h incubation, a total of 1×10^8 cells were harvested from the treated and control cultures. The cells were washed twice with PBS and fixed in 70% ice-cold ethanol for 1 h. The sample was concentrated by removing ethanol and treated with 1% (v/v) Triton X-100 and 0.01% RNase for 10 min at 37 °C. Staining of cellular DNA was performed with 0.05% propidium iodide for 20 min at 4 °C in darkness. The cell cycle distribution and apoptotic cells were detected with FACScan (Fluorescence Activated Cell Sorting, Becton-Dickinson, San Jose, CA). More than 1×10^4 cells were analyzed with the CellFit software package (Becton-Dickinson, San Jose, CA).

2.7. In vivo tumor growth inhibition study

2.7.1. Cancer cell line and animals

A murine hepatoma H_{22} cell line was kept in liquid nitrogen for regular use in our laboratory. Inbred ICR mice were purchased from the Experimental Animal Center at Sichuan University. Procedures involving the care and use of animals were conducted in accordance with institutional guidelines for Laboratory Animal Care of Experimental Animal Center at Sichuan University.

2.7.2. Tumor growth inhibition experiment in vivo

six-week-old inbred female ICR mice were inoculated with murine hepatoma H_{22} cells [17] s.c. 24 h after tumor cell inoculation, tumor-bearing mice were randomized into four experimental groups, each having 13 mice. Three treatment groups were fed doses of 0.15, 0.3, and 0.6 g/kg/day of water-soluble CEE from LFP in DH_2O p.o. for 10 days, respectively. The control mice were given 0.2 ml DH_2O each. At the end of experiment all animals were sacrificed, and tumors were dissected and weighed. The tumor inhibitory rates were calculated using the following formula: tumor inhibitory rate (%) = (mean tumor weight of the control mice—mean tumor weight of the treated mice) ÷ mean tumor weight of the control mice × 100%.

2.8. BrdU incorporation in vivo

Six h before animal sacrifice, the high dose treated (0.6 g/kg/day) mice and the control mice were injected with 500 mg/kg of BrdU [18]. The animals were then sacrificed, the tumors were excised and the tumor specimens were fixed in 4% paraformaldehyde for 24 h and processed conventionally. Paraffin sections were made for immunohistochemical staining to detect BrdU labeled cells and hematoxylin and cosin (HE) staining for histopathological examination.

Briefly, the sections were de-paraffinized and re-hydrated through graded alcohols, washed with PBS for 5 min, then immersed in 2 N HCl for 1 h at room temperature. The sections were incubated in 0.1 M borate buffer (pH 8.5, 0.1 M boric acid, 25 mM $Na_2B_4O_7$, and 75 mM NaCl) twice for 5 min each, followed by three washes (10 min) in PBS. Next, the sections were incubated with BrdU mouse monoclonal antibody (11B5, Zymed, USA) at a dilution of 1:100 overnight at 4 °C, with biotylated second antibody for 20 min, and with streptavidin/peroxidase for 30 min at room temperature. Subsequently, the sections were subjected to color reaction with 0.02% 3,3 diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 in PBS (pH 7.4) and counterstained lightly with hematoxylin. A percentage of BrdU labeled cells was determined by counting several fields of 200 cells (in areas of the slide containing the most labeled cells) [19].

2.9. Statistical analysis

All experimental data were expressed as mean ± SD. The statistical significance of the difference between control and LFP extract treated groups was determined by one-way ANOVA followed by Tukey's test for multiple comparisons. Dunnett's *t*-tests (2-sided) were employed, as needed, and the result was considered significant at $P<0.05$.

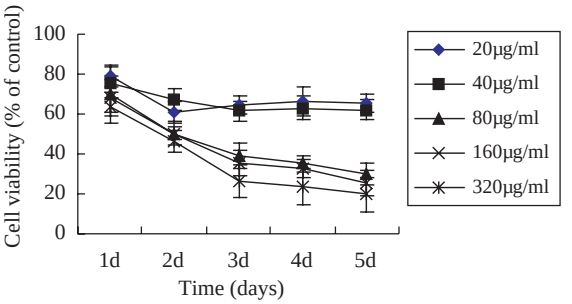

Fig. 1. Inhibition of human SMMC-7721 hepatocellular carcinoma cells growth by water-soluble CEE from LFP. Cells were seeded onto 96-well plate at 3×10^3/well and were treated with water-soluble CEE from LFP at different concentrations; and percentage of cell viability was determined by MTT assay after 1, 2, 3, 4 and 5 days of treatment, respectively. Dose- and time-dependent growth inhibition was observed at concentrations ranging from 80 to 320 μg/ml. Results are mean values ± SD of three independent experiments, each in five wells.

3. Results

3.1. Cytotoxic activity of water-soluble CEE against human hepatocellular carcinoma cells

The result of cytotoxic activity of water-soluble CEE from LFP against human hepatocellular carcinoma cells is shown in Fig. 1. The percentage of growth inhibition of water-soluble CEE at various concentrations on human hepatocellular carcinoma cells was determined as the percentage of viable-treated cells in comparison with viable cells of untreated controls. It showed a dose- and time-dependent inhibitory effect on cell growth. IC_{50} was 80 μg/ml, and maximal inhibition of cell growth (>80%) was obtained at 320 μg/ml.

3.2. Inhibition of colony formation

The result of colony formation inhibition of water-soluble CEE from LFP is presented in Fig. 2. Untreated hepatocellular carcinoma cells produced 115 ± 16 colonies, and the colony numbers were suppressed to 98 ± 11 ($P>0.05$), 56 ± 9 ($P<0.05$) and 12 ± 5($P<0.05$) with 60, 120, and 240 μg/ml of the water-soluble extract treatment, respectively. A dose-dependent colony-forming inhibition effect was observed.

3.3. Apoptosis induction and G_1 phase arrest

The results of apoptotic induction and cell cycle analysis of cells treated with water-soluble CEE from LFP are presented in Fig. 3. After treatment with 60 μg/ml of water-soluble CEE for 72 h, the apoptotic cells increased significantly ($P<0.05$) from 4.5% (Fig. 3(A)) to 11.3% (Fig. 3(B)). The cells in G_0/G_1 phase increased from 64.5% (Fig. 3(C)) to 79.2% (Fig. 3(D), $P<0.01$). The cells in G_2/M phase decreased from 20.4% (Fig. 3(C)) to 0.3% (Fig. 3(D), $P<0.01$). This suggested that a portion of cells were arrested in G_0/G_1 phase after treatment with water-soluble CEE from LFP.

3.4. Tumor growth inhibition in vivo

The result of tumor growth inhibition of water-soluble CEE from LFP is shown in Fig. 4. To investigate its anticancer activity in vivo, murine hepatoma bearing-mice were fed water-soluble CEE from LFP p.o. for 10 days. At the end of experiment, the mean tumor weight of the control mice was 1.04 ± 0.20 g. The mean tumor weights of the mice treated with 0.15, 0.3, and 0.6 g/kg/day of water-soluble CEE were 0.86 ± 0.15, 0.72 ± 0.13, and 0.58 ± 0.11 g, respectively. The tumor inhibitory rates were 17.31% ($P>0.05$), 30.77% ($P<0.05$), and 44.23% ($P<0.01$), respectively. The experimental finding shows that there is a significant dose-dependent inhibition of tumor growth in the treated mice compared to the control mice. No evidence of toxicity was identified in the treated animals through comparison of body weight increase, histopathological changes of major organs, and blood biochemistry analysis of both group animals (data not shown).

3.5. Decrease of BrdU incorporation in tumor cells of the treated mice

The result of inhibition of BrdU incorporation into tumor cells of water-soluble CEE from LFP is

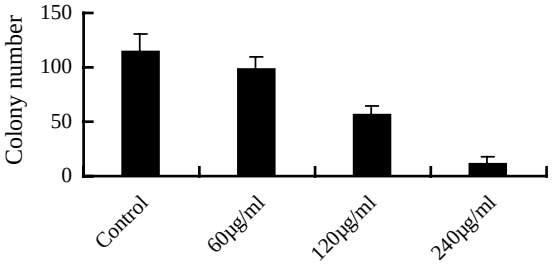

Fig. 2. Inhibition of human SMMC-7721 hepatocellular carcinoma cell colony formation by the water-soluble CEE from LFP. Cells were seeded onto six-well plate (300/well) and treated with the water-soluble CEE at different concentrations; the colony number was counted under dissection microscope. Dose-dependent colony formation inhibition was found. Results are mean values ± SD of three independent experiments, each in triplicate.

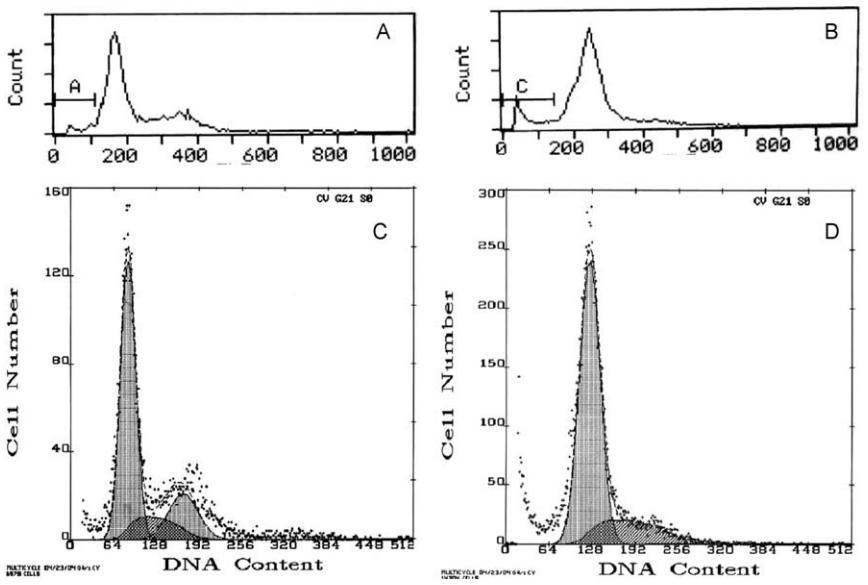

Fig. 3. Cell cycle analysis of human SMMC-7721 hepatocellular carcinoma cells treated with the water-soluble CEE from LFP. Cells were treated with 60 μg/ml of the water-soluble CEE from LFP for 72 h, both the control and treated cells were harvested and subjected to flow cytometric analysis: (A) apoptotic cell percentage of the control cells; (B) apoptotic cell percentage of the treated cells increased significantly; (C) cell cycle distribution of the control cells; (D) cell cycle distribution of the treated cells, cells in G_0/G_1 phase increased from 64.5% to 79.2% ($P<0.01$), and the cells in G_2/M phase decreased from 20.4% to 0.3% ($P<0.01$).

presented in Fig. 5. The inhibitory effect of water-soluble CEE from LFP on tumor growth in vivo was further confirmed using BrdU incorporation in tumor cells of control mice and high dose treated (0.6 g/kg/day) mice in vivo. BrdU labeled cells in tumor cells of the treated animal were 11.80±2.79% (Fig. 5(B)), and lower than that in untreated controls (23.00±5.42%, Fig. 5(A)). This was statistically significant ($P<0.05$).

4. Discussion

Litchi fruit pericarp (LFP) contains significant amounts of polyphenolic compounds, including condensed tannins (polymeric proanthocyanidins), epicatechin, procyanidin A2, and flavonoids [10,11]. Previous study has shown that LFP extract possesses antioxidant properties against fat oxidation in vitro [11]. No other bioactivity of LFP extract has been reported. During in vitro cytotoxicity assay, some of the plant extract exhibited potential antioxidant and anticancer properties [7,20] and inhibited proliferation of multiple human cancer cells [21,22]. Animal studies have demonstrated that a dietary polyphenol known as tannic acid (TA) exhibits anticarcinogenic activity in chemically induced cancers [23]. Based on the potent antioxidant activity of LFP extract in vitro and its components of polyphenolic compounds and flavonoids [10,11], it might be assumed that LFP extract may have anticancer activity against some cancer cells and/or animal models. Our study confirms that water-soluble CEE from LFP has strong dose- and time-dependent anticancer activity against human hepatocellular carcinoma cells ($IC_{50}=80$μg/ml) and inhibited colony growth potential of cancer cells in a dose-dependent manner in vitro.

Although MTT assay correlated well to data derived from trypan blue exclusion [24], the antioxidant activity of resveratrol was recently shown to interfere with results from the MTT reduction assay, resulting in a higher concentration of converted formazan [25]. In this experimental study, the colony formation assay shows an effect of LFP extract at 120 μg/ml whereas the cell viability data from MTT assay shows an effect at a

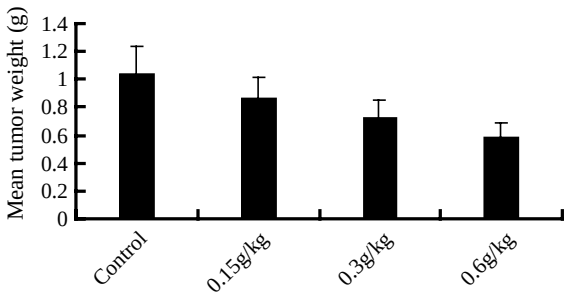

Fig. 4. Inhibition of tumor growth in murine hepatoma bearing-mice. The histogram shows that there was a significant difference of mean tumor weight between the untreated and treated mice, the tumor inhibitory rates were in dose-dependent manner.

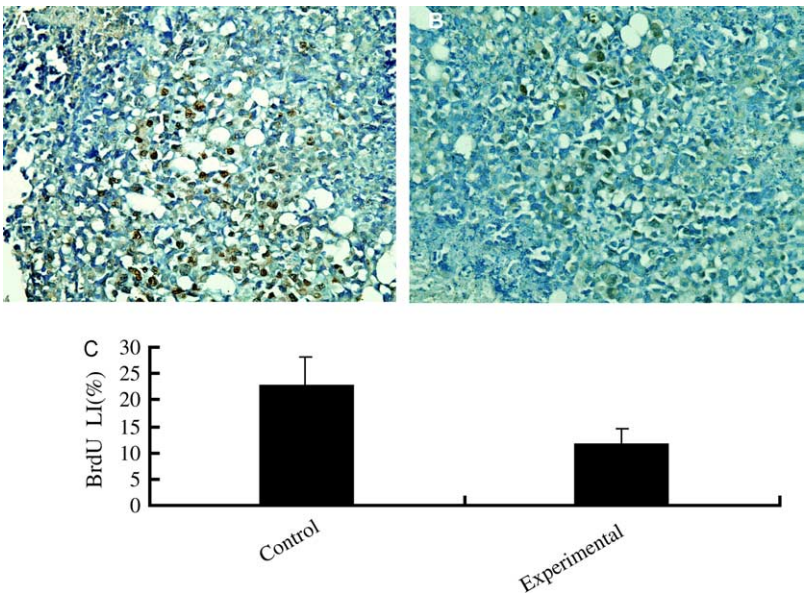

Fig. 5. BrdU incorporation in vivo. Before 6 h of animal sacrifice, the high dose of the water-soluble CEE from LFP treated (0.8 g/kg/day) mice and the control mice were injected with 500 mg/kg of BrdU, BrdU labeled cells were detected by immunohistochemical staining: (A) BrdU labeled cells in the tumor of control mouse; (B) BrdU labeled cells in the tumor of high dose of the water-soluble CEE from LFP treated mouse; (C) the histogram shows that there was a significant decrease of BrdU labeled cells in the tumor of the mice treated with water-soluble CEE from LFP.

lower concentration ($IC_{50}=80$ μg/ml). As the main components of LFP extract are polyphenolic compounds (antioxidants), the effects of LFP extract observed in the MTT reduction assay in this study are likely based on the same phenomenon caused by resveratrol [12].

Cultured cancer cells are valuable reagents for rapid screening of potential anticancer agents as well as for elucidating the mechanism of their activity. Prior to clinical trials, however, it is essential that the in vivo efficacy of potential anticancer agents is determined in a suitable animal model [26]. Hepatocellular carcinoma (HCC) is one of the most common malignancies, responsible for an estimated one million deaths annually [27]. Therefore, it is important to discover the new agents that are effective on growth inhibition of hepatocellular carcinoma.

As no reports exist about the clinical usage of LFP extract, we designed the in vivo experiment according to the results of in vitro cytotoxicity and in vivo toxicity (data not shown). Murine hepatoma bearing-mice were fed doses of 0.15, 0.3, and 0.6 g/kg/day of water-soluble CEE from LFP, respectively. The high concentration of LFP extract (0.6 g/kg/day) used in the tumor growth inhibition experiment in vivo equaled to approximately 10 times relevant to what would be a human situation. A dose-dependent tumor growth inhibitory effect was demonstrated (Fig. 4) without any untoward toxicity. Our findings suggest that LFP extract may have potential anticancer activity on liver cancer.

The anticancer activity of LFP extract was demonstrated further by FCM analysis and BrdU incorporation in vivo. After treatment with LFP extract for 72 h, apoptotic cell death increased significantly ($P<0.01$). Cells in G_0/G_1 phase increased from 64.5% to 79.2% ($P<0.01$), and cells in G_2/M phase decreased from 20.4% to 0.3% ($P<0.01$), which suggested that treatment from LFP extract resulted in apoptotic cell death and G_0/G_1 phase arrest of cancer cells (Fig. 3). The same findings were observed in cell proliferation inhibitory effect of epigallocatechin-3-gallate [28]. Inhibition of DNA synthesis and proliferation of cancer cells was verified by the ability to reduce BrdU incorporation into cancer cells, which correlates with decreased cell proliferation [29]. BrdU labeled cells in tumor cells of the treated animal were significantly lower than those in untreated controls ($P<0.05$), which indicated that LFP extract inhibited proliferation of cancer cells through DNA synthesis inhibition of cells. Therefore, it could be assumed that the anticancer activity of LFP extract might result, at least in part, from inhibition of DNA synthesis, cell proliferation and apoptosis induction of cancer cells.

In conclusion, for the first time, the potential anticancer activities of LFP extract against human

hepatocellular carcinoma cells in vitro and murine hepatoma in vivo were investigated in this experimental study. The results demonstrated that CEE from LFP has a powerful antiproliferation effect by inducing apoptotic cell death, inhibiting DNA synthesis of cancer cells, and causing G_0/G_1 phase arrest of cancer cells. Some constituents from LFP extract might serve as a powerful novel antitumour agent, and need to be investigated further.

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