

Original Article

Anti-hepatoma effect of safrole from *Cinnamomum longepaniculatum* leaf essential oil *in vitro*

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Abstract: The aim of this study was to study the anti-hepatoma effect of safrole and elucidate its molecular mechanism, the human hepatoma BEL-7402 cells were incubated with various concentrations (40, 80, 160, 320 and 640 µg/ml) of safrole and the cell proliferation and apoptosis were evaluated. The results showed that both the cell proliferation determined by 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) assay and cell colony determined by soft agar assay were significantly suppressed by safrole in a dose-time-dependent manner. Characteristic morphological and biochemical changes associated with apoptosis, including cells shrinkage, deformation and vacuolization of mitochondria, nuclear chromatin condensation and fragmentation, formation of apoptotic bodies were observed when treated with safrole for 24 h and 48 h. Cell cycle changes evaluated by flow cytometry analysis showed that the safrole could induce accumulation of cells arrested at G₁ and S phases of the cell cycle. These results demonstrated that safrole is potent anti-hepatoma agent and the underlying mechanism may be attributed to suppress tumor cell growth by inducing cell apoptosis.

Keywords: Safrole, human hepatoma, *Cinnamomum longepaniculatum*, cell proliferation, apoptosis

Introduction

Natural products are always been a research hotspot for development of new anticancer agents, which might replace chemotherapeutic agents. Essential oils recently received much attention due to their broad-spectrum of anti-cancer activity [1-3]. There are a variety of essential oils and their major active components are potential candidates for control of various cancers [4]. Hepatocellular carcinoma (HCC) is the most common primary malignant tumor that occurred in men and women, which causes 0.5-1 million new infections per year [5]. To date, a number of essential oils and their components were demonstrated to be potent inhibitors of HCC [6-8].

Cinnamomum longepaniculatum (Gamble) N. Chao is an endemic tree that grows widely in China. The safrole is an important component of the *C. longepaniculatum* leaf essential oil and also is a critical raw material for synthesis of many useful compounds, like piperonyl

butoxide, heliotropine, vanillin and so on [9]. A few studies have demonstrated that the safrole and its oxide can inhibit the growth of human oral cancer HSC-3 cells and human lung cancer cells *in vitro* [10, 11]. However, there was yet no report about the anti-hepatoma effects of safrole on human hepatoma cell line BEL-7402. Therefore, the purpose of this study was to assess the processes by which safrole inhibits cancer cell growth and induces apoptosis of BEL-7402 cells, and to elucidate the molecular mechanism of these actions.

Materials and methods

Preparation of the safrole and essential oil

The safrole (safrole content 90%) from the *C. longepaniculatum* leaf essential oil were provided by the Yibin Chuanhui Company (Sichuan, China). A stock solution of 50 mg/mL of the safrole and the essential oil were made in 100% dimethyl sulfoxide (DMSO) and filtered via a 0.22 µm minipore membrane. The two stock

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solutions were stored at -20°C and diluted with the culture medium before use. The final concentration of DMSO for all treatments was less than 0.25%.

Cell culture and treatment

Human hepatoma BEL-7402 cells were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). BEL-7402 cells were grown in RPMI 1640 (Gibco®) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mmol/L HEPES (pH 7.4). In this study, cells were either incubated in the absence or presence of different concentrations of the test compounds for 48 h.

Measurement of cell growth inhibition

Growth inhibition of BEL-7402 cells by the safrole was measured by 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cancer cells were seeded into 96-well microtiter plates at appropriate densities to maintain the cells in an exponential phase of growth during the experiment. BEL-7402 cells were exposed to the safrole either at 40, 80, 160, 320 or 640 µg/mL for 48 h. Each concentration was tested in quadruplicate. At the end of the treatment, 20 µL of 5 mg/mL MTT (Sigma, USA) was added to each well and the plates were incubated for 4 h at 37°C. DMSO (150 µL) was then added to each well and the plates were rotated for 10-20 min at 200 rpm/min by a shaker (THZ-C, Shenhua BioTech Co., Shenzhen, China). The optical absorbance was read on a plate reader (Bio-Rad, USA) at a wavelength of 570 nm. The inhibitory rate of cell proliferation was calculated using the following formula: Growth inhibition (%) = $(A_{\text{control}} - A_{\text{treated}}) / A_{\text{control}} \times 100$.

Measurement of cell colony inhibition

The soft agar colony formation assay is a common method to monitor anchorage-independent growth, which measures proliferation in a semisolid culture media after 3-4 weeks by manual counting of colonies. Base agar consisted of 0.5% Agar, 1 × RPMI 1640 medium and 10% FBS was prepared and added 1.5 mL to each 35 mm petri dish. After 5 min for allowing agar to solidify, the 1.5 mL top agarose was added, which contained 0.35% Agar, 1 × RPMI

1640 medium, 5% FBS, single cell suspension and safrole. Then, the plates were incubated at 37°C in humidified incubator for 10 to 30 days. At last, the plates were stained with 0.5 mL of 0.005% crystal violet for more than 1 h. The colonies were counted using a dissecting microscope. The colony inhibitory rate of cell colony was calculated using the following formula: Colony inhibition (%) = $(N_{\text{control}} - N_{\text{treated}}) / N_{\text{control}} \times 100$.

Light microscopy assay

BEL-7402 cells were seeded in sterile culture dishes contained cover slips at a density of 1×10^5 cells/mL. Cells were incubated in RPMI 1640 medium containing 10% FBS. For treatment group, the safrole (320 µg/mL) was added and incubated for 24 h and 48 h. Then, the cover slips were washed with PBS, and stained by hematoxylin-eosin staining (HE). Finally, the cells fixed on the cover slips were analyzed, and photographed under light microscope (BH-2, Olympus, Japan).

Transmission electron microscopy assay

BEL-7402 cells were incubated with or without 320 µg/mL the safrole for 24 h. After treatment with 0.25% trypsin, 5×10^6 cells were collected, centrifuged at 10000 g for 5 min and then washed twice with PBS for 5 min. Cell pellets were fixed in 2.5% glutaraldehyde in PBS at 4°C overnight and then washed three times, each time for 15 min, with cacodylate-buffer. Specimens were then post-fixed for 2 h in 1% osmium tetroxide (OsO₄) dissolved in cacodylate-buffer at room temperature and washed with cacodylate-buffer (three times, 15 min each). Samples were dehydrated in ethanol and embedded in Epon-Araldite resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a PHILIPS CM 10 transmission electron microscope (TEM) (Philips Scientifics, Eindhoven, The Netherlands).

Flow cytometric analysis

The suspended single cell solutions subjected to treatment with safrole at 320 µg/mL for different times (12, 24, 36, and 48 h) were harvested. Each group had three culture bottles. Cells were washed with PBS, fixed with 70% ethanol at -20°C for 30 min and stored at 4°C

Table 1. Inhibitory effect of the safrole and *C. longepaniculatum* leaf essential oil on hepatoma cell line BEL-7402

Concentrations ($\mu\text{g}/\text{mL}$)	Safrole		Essential oil	
	$A_{570\text{nm}}$	Inhibition rate (%)	$A_{570\text{nm}}$	Inhibition rate (%)
0	0.4167 ± 0.0213		0.3360 ± 0.01259	
40	0.3835 ± 0.0169	8.13%	0.3350 ± 0.01418	0.41%
80	0.3740 ± 0.0208	10.31%	0.3280 ± 0.02529	2.03%
160	0.3227 ± 0.0389	22.78%	0.3102 ± 0.01436	7.63%
320	0.2227 ± 0.0198	51.56%	0.2690 ± 0.01906	19.82%
640	0.1030 ± 0.0121	75.36%	0.2370 ± 0.01517	29.51%

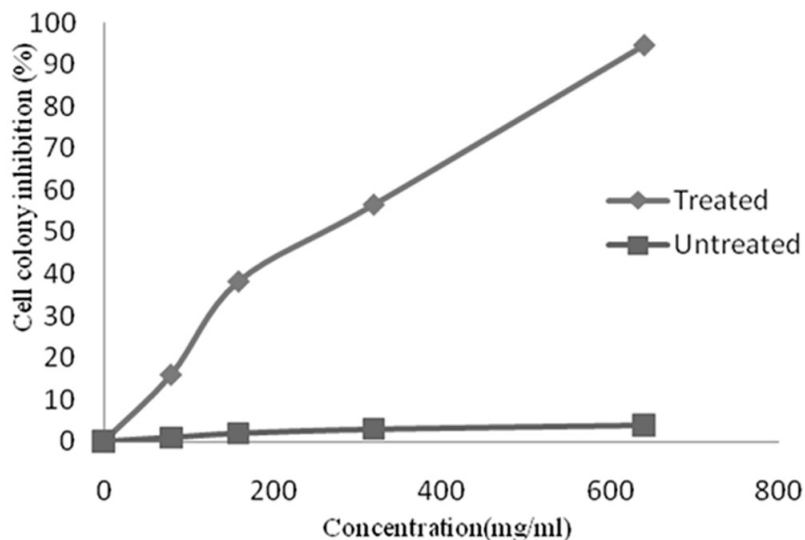


Figure 1. Effects of the safrole on inhibition of BEL-7402 cell colony.

overnight, then washed with PBS again and treated with 100 mg/L PI 100 μL at 4°C for 30 min in darkness. Apoptotic cells were assayed using FACSsort Becton Dickinson Flow Cytometer at 488 nm and data were analyzed with CELLQuest Software. For each sample, 20 000 cells were measured.

Statistical analysis

All data were expressed as mean \pm standard deviation. Statistical analysis was performed by *t* test using software SPSS11.0. $P < 0.05$ was considered significant.

Results

Measurement of cell growth inhibition

Human hepatoma BEL-7402 cells were incubated with the safrole and *C. longepaniculatum*

leaf essential oil for 48 h at concentrations ranging 40-640 $\mu\text{g}/\text{mL}$ and analyzed by MTT assay. The safrole inhibited the BEL-7402 cells proliferation in a dose-dependent manner. The cell inhibition rates were 8.13%, 10.31%, 22.78%, 51.56%, and 75.36% after the treating with 40, 80, 160, 320, or 640 $\mu\text{g}/\text{mL}$ of the safrole for 48 h, respectively (Table 1). In contrast, the essential oil exhibited relatively lower anti-hepatoma activity and the inhibition rates were 0.41%, 2.03%, 7.63%, 19.82% and 29.51%, respectively.

Measurement of cell colony inhibition

The BEL-7402 cells were cultured in the presence or absence of the safrole and cell number was determined over 10 days. The safrole produced a dose-dependent inhibition of cell colony (Figure 1). After exposure of BEL-7402 cells to 80, 160, 320, and 640 $\mu\text{g}/\text{mL}$ of the safrole for 10 days, the inhibition rate of cell colony was $15.97 \pm 1.18\%$, $38.22 \pm 1.80\%$, $56.53 \pm 2.56\%$, and $94.56 \pm 1.81\%$, respectively.

Light microscopy assay

Under light microscope, untreated BEL-7402 cells displayed extended, flat cell bodies with uniform chromatin in the nuclei, normal cells appeared obvious mitosis (Figure 2A and 2B). The BEL-7402 cells that treated with the safrole (320 $\mu\text{g}/\text{mL}$) for 24 h showed that part of cells morphologic alteration, including size reduction, nuclear condensation and boundary aggregation (Figure 2C). After treated with the safrole (320 $\mu\text{g}/\text{mL}$) for 48 h, cell amount decreases, including cells size reduction, vacuoles appeared and cell nucleus pyknosis (shrunken and dark) (Figure 2D).

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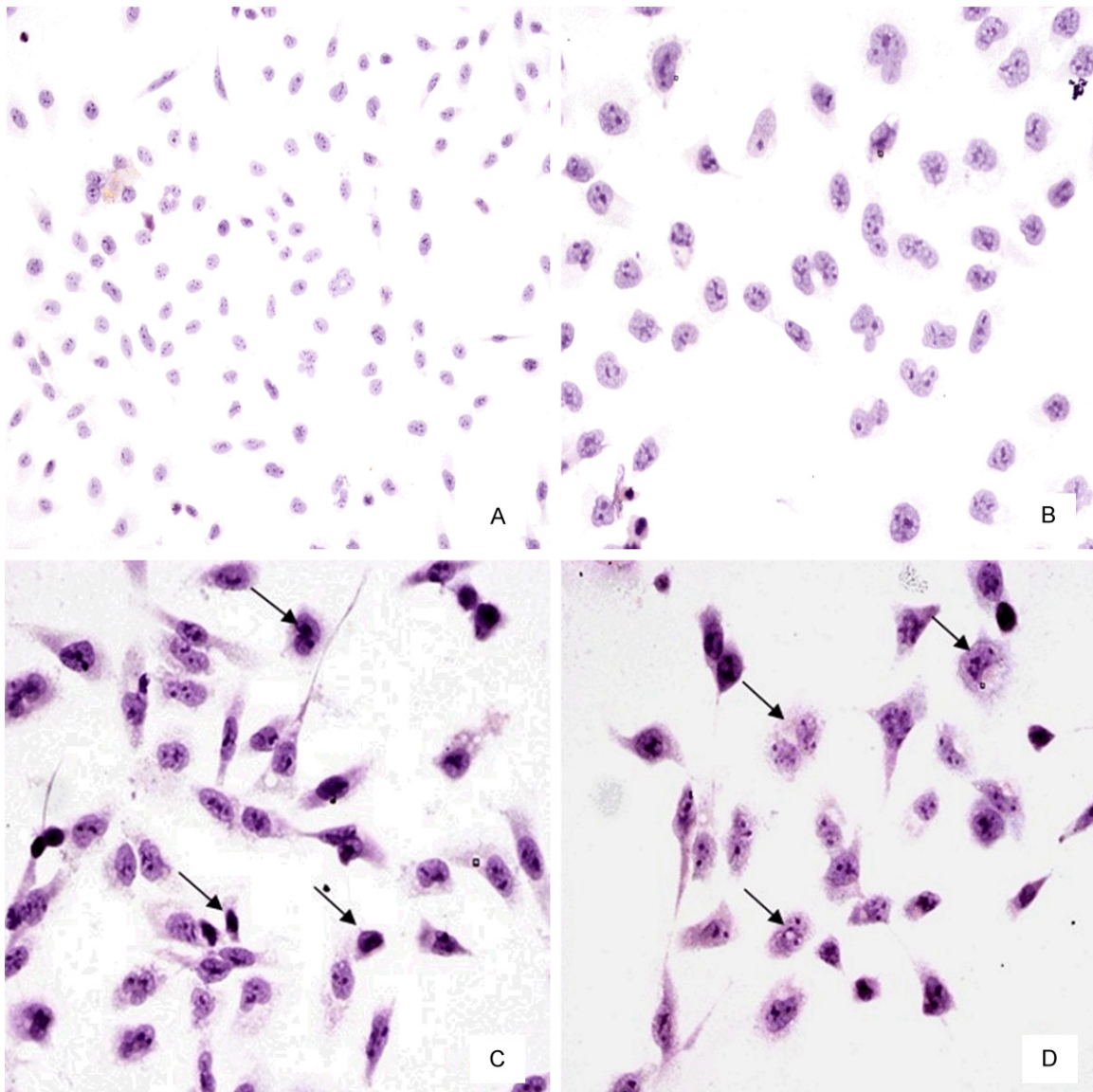


Figure 2. Light micrographs of BEL-7402 cells. Normal cells (A \times 200, B \times 400). BEL-7402 cells treated with 320 μ g/mL for 24 h (C \times 400) showed part of cells morphologic alteration, including size reduction, nuclear condensation, and boundary aggregation (denoted by arrowheads). After treated with the safrole (320 μ g/mL) for 48 h (D \times 400), cell amount decreases, including cells size reduction, vacuolues appeared and cell nucleus pyknosis were observed (denoted by arrowheads).

Transmission electron microscopy assay

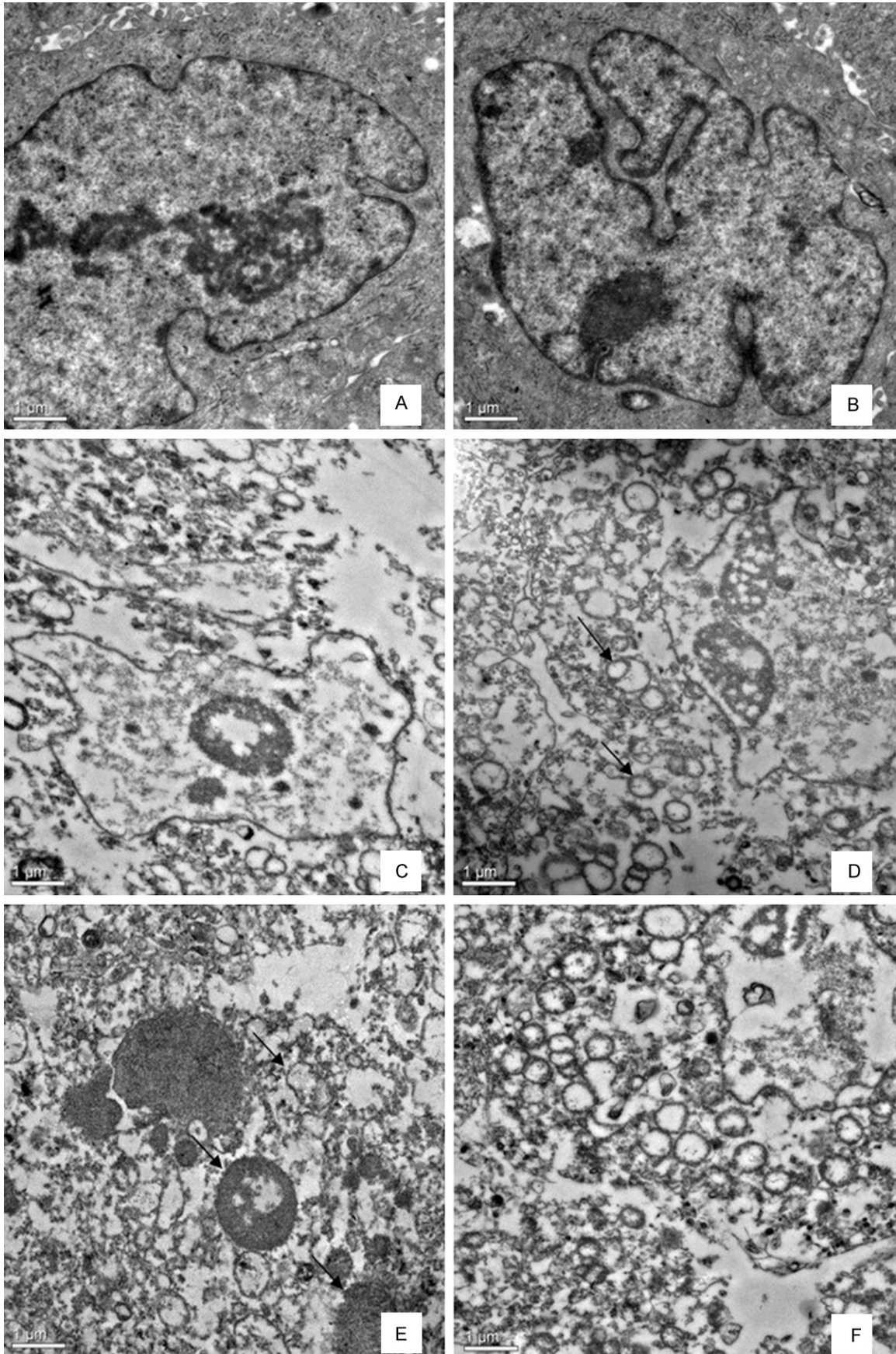
Under transmission electron microscopy (TEM), control cells were big and round, with intact nuclear membrane, electron dense granules in nuclear chromatin (**Figure 3A**) and normal cell mitosis (**Figure 3B**). However, the cells that treated with the safrole (320 μ g/mL) for 24 h exhibited characteristics of apoptosis including cell membrane shrinkage, low density in nuclear chromatin (**Figure 3C**), deformation and vacuolization of mitochondria (**Figure 3D**). After 48

h, cell membrane occurred rupture; the deformation and vacuolization of mitochondria became serious (**Figure 3E**); nuclear chromatin became condensation and fragmentation; apoptotic bodies appeared (**Figure 3F**).

Flow cytometry analysis

The results showed that incubation with safrole of 320 μ g/mL for different time periods resulted in accumulation of cells at G₁ or S phase and a blockade of cell proliferation compared with

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Figure 3. Transmission electron micrographs of the BEL-7402 cell. Without treatment, cells were normal (A, B). After treatment with safrole (320 µg/mL) for 24 h (C, D) and 48 h (E, F), characteristics of apoptosis was observed.

control group (**Figure 4A**). When the treated time was prolonged, the number of cells in different cell cycle has changed (**Figure 4B**). Most of cells were arrested at G₁ or S phase eventually led to cell death as the prolonged incubation response that resulted in the rapid appearance of cells with DNA content less than that at G₁ phase, which is characteristic of apoptotic cells. The percentage of apoptotic cells that untreated with safrole was just 0.45%. The rates of apoptotic cells increased from 4.71% to 14.80%, 42.00%, and 67.34% after 12, 24, 36, and 48 h of exposure to safrole (320 µg/ml), respectively (**Figure 4C**). Therefore, safrole induced apoptosis of BEL-7402 cells in a time-dependent manner.

Discussion

Hepatoma is one of the most common malignant tumors in China and Asia [12]. Therefore, the human hepatoma cell line BEL-7402 was chosen as an in vitro model to explore the effects of the safrole on cancer cell growth and apoptosis. The effects of safrole at various concentrations on the growth (**Table 1**) and cell colony (**Figure 1**) of BEL-7402 cells showed that safrole significantly suppressed cell proliferation in a dose-dependent manner. After human hepatoma BEL-7402 cells were treated with various concentrations of safrole for 48 h, MTT colorimetric analysis showed that safrole could significantly inhibit the proliferation of BEL-7402 cells with IC₅₀ values of 0.28 mg/mL and exhibited obvious anticancer activity at concentration of 0.64 mg/mL. Moreover, *C. longepaniculatum* leaf essential oil exhibited relatively lower anti-hepatoma activity than safrole (**Table 1**), indicating that safrole was main active component of anticancer activity. Manosro et al., 2006 reported that essential oil had the IC₅₀ values between 0.125 and 5 mg/ml as moderate possibility to be developed to cancer therapeutic agent [13]. Thus, safrole exhibited possibility to be developed to cancer therapeutic agent. Similar results that the essential oil from *Schizonepeta tenuifolia* Briq exhibited inhibitory effect of human lung cancer at the concentration of 2 mg/mL [14].

Under electron microscopy, apoptosis cells were seen to have integrated membrane and

nuclear morphological changes at early stage, and then followed by the appearance of apoptotic bodies [15]. In the present study, characteristic morphological and biochemical changes associated with typical apoptosis were observed after BEL-7402 cells were treated by safrole for 24 h and 48 h (**Figures 2 and 3**). In addition, Mitochondria have a pivotal role in apoptosis. Our study also found that the exposure of hepatoma cells to safrole could impair mitochondrial function, leading to a marked deformation and vacuolization of mitochondria.

Flow cytometry can elucidate the cell cycle changes and apoptosis rates [16]. The apoptosis cells would arrested at G₁ and S phases of the cell cycle and the number of cells at G₂ has reduced, inducing less and less cell turn into cell division, even cell death [17]. As shown in our study, quantified by FCM, safrole induced apoptosis of BEL-7402 cells in a time-dependent manner; the longer the time of treatment with safrole, the more apoptotic cells there were (**Figure 4**). Cell cycle changes revealed that the safrole could induce accumulation of cells arrested at G₁ and S phases of the cell cycle.

The concept of apoptosis described the death of cells by fragmentation of DNA, cell shrinkage, followed by cell fragmentation and formation of apoptosis bodies [18]. Many studies have revealed that the uncontrolled growth of neoplasm is not only due to the over proliferation but also due to the loss of natural apoptosis [19, 20]. Therefore, exploring new medicine that could induce cancer cell apoptosis would benefit for neoplasm treatment [21, 22]. This study provide a novel candidate that safrole could induce human hepatoma cell apoptosis in vitro. Given the potent anti-hepatoma activity, safrole exhibits potential for the control of HCC.

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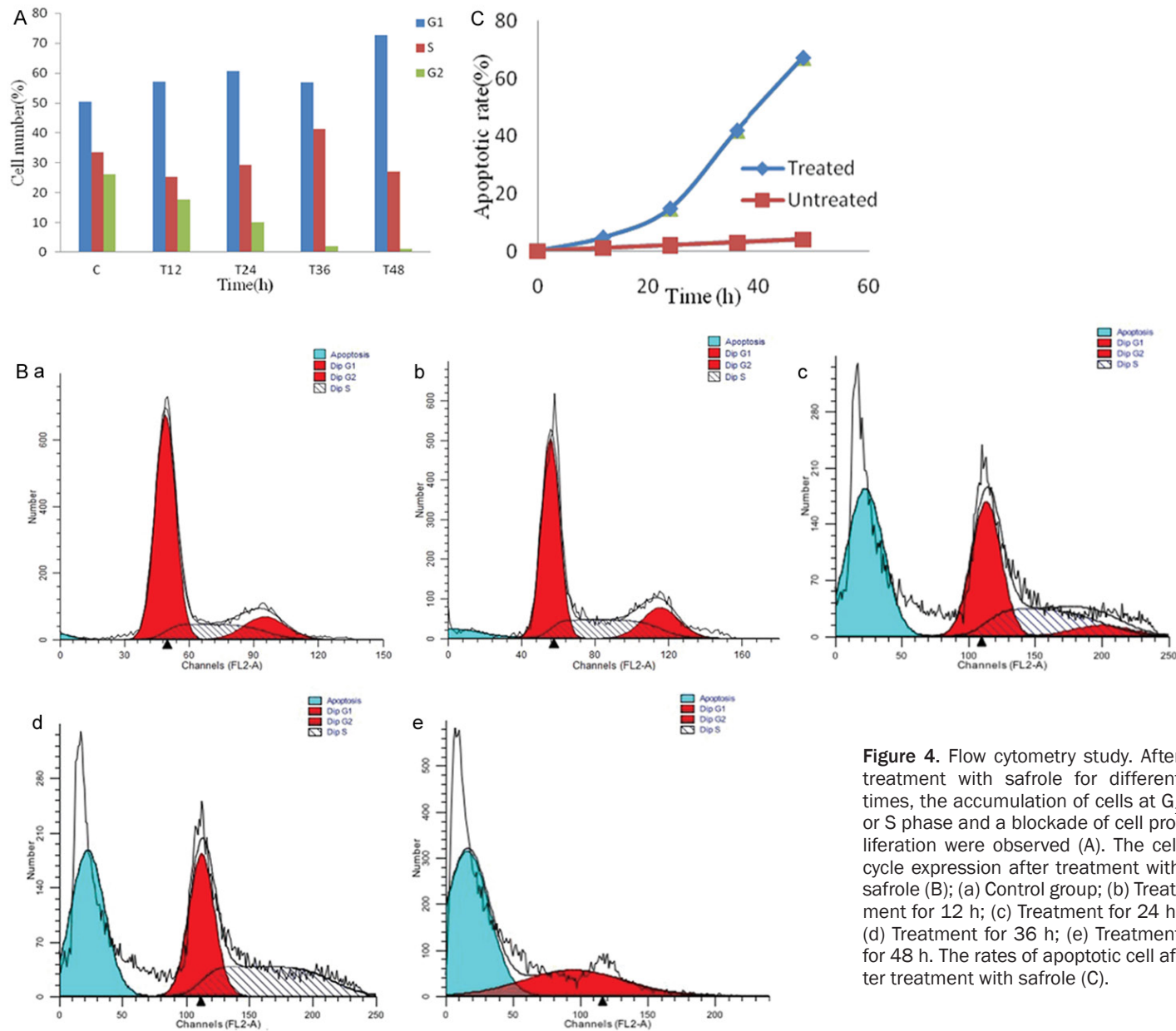


Figure 4. Flow cytometry study. After treatment with safrole for different times, the accumulation of cells at G₁ or S phase and a blockade of cell proliferation were observed (A). The cell cycle expression after treatment with safrole (B); (a) Control group; (b) Treatment for 12 h; (c) Treatment for 24 h; (d) Treatment for 36 h; (e) Treatment for 48 h. The rates of apoptotic cell after treatment with safrole (C).

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Disclosure of conflict of interest

None.

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