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Research Article

Ethanol extract of Ginger *Zingiber officinale* Roscoe by Soxhlet method induces apoptosis in human hepatocellular carcinoma cell line

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ABSTRACT

Introduction: Ginger has had a long history of use in healthcare in Asian countries, including Vietnam. Internationally, there have been a few studies done to assess the anticancer activity and potency of ginger. In this study, we investigated the activity (cytotoxicity) of various ginger root extracts that were produced by different preparation methods on human hepatocellular carcinoma cell line (HepG2 cell line). **Methods**: Ginger (*Zingiber officinale* Roscoe) root extract were extracted from the fresh ginger roots by three methods, which included maceration (G1), Soxhlet (G3), and ultrasonic (G5) methods. The extracts were then evaluated for cytotoxic activity on HepG2 hepatocellular carcinoma cell line based on IC_{50} calculation. The most potent extract was then evaluated for its cytotoxicity by Propidium iodide (PI) staining method and microscopic examination by fluorescence microscopy for both 2D and 3D culture conditions. After treatment, cells were also stained with Annexin-V to assess the apoptosis-inducing ability of the extract. **Results**: The results showed that the ginger root extract prepared by the Soxhlet method (G3) had the strongest activity, and showed significant differences compared to extracts from methods G1 and G5, on the hepatocellular carcinoma cell lines cultured in both 2D and 3D conditions. Indeed, the IC_{50} value of ginger root extract of the G3 was 83.3 ± 0.9189 μg/ml, compared to 159 ± 7.6 μg/ml of G1, and 284 ± 5.116 μg/ml of G5 in 2D culture latform; the IC_{50} value was 228 ± 33.52μg/ml for G3 compared to 341 ± 3.93 μg/ml for G1, and 603.7 ± 56.33 μg/ml for G5 in 3D culture platform. The results of PI staining of cells in 2D and 3D culture conditions showed that ginger root extract killed HepG2 cells in a concentration-dependent manner. The nuclei of HepG2 cells in 2D culture showed nuclear fragmentation when treated with ginger root extract; this is one of the apoptosis indicators. Moreover, Annexin-V staining results showed that HepG2 cells underwent apoptosis when treated with ginger root extract. **Conclusion**: The results of the study show that ginger root extract prepared by the Soxhlet method induced strong cytotoxicity of hepatocellular carcinoma cells cultured in both 2D and 3D conditions, through induction of apoptosis. The evidence suggests that ginger is a potential agent for anticancer therapy. It is necessary to perform further research to isolate compounds from fresh ginger root extract by the Soxhlet method to evaluate the mechanism of anti-tumor activity.

Key words: Apoptosis, fresh Ginger root extract, HepG2, liver cancer, mitotic resistance

INTRODUCTION

Traditional medicine has played a necessary role in improving patient health for thousands of years. Traditional herbal treatments are beneficial in that they are a complementary remedy to reduce complications during treatment and can reduce tumor growth [1,2]. In particular, herbal medicines and their derivatives have been documented to cure diseases via their antioxidant, anti-inflammatory, anti-diabetic, and anticancer properties [3].

Ginger root (*Zingiber officinale*) is a member of the family Zingiberaceae. Since ancient times, ginger has been widely used as a spice and condiment in food and beverages in many Eastern countries [4]. In oriental medicine, ginger has also been regarded primarily as a remedy for digestive disorders, such as colic, gastritis, vomiting, dyspepsia, nausea, and diarrhea [5]. Furthermore, ginger root extract and its pungent components (such as gingerol, paradol, and shogaol [3,6]) have potential anti-inflammatory [7], antioxidant [8], and anticancer [9–11] activities.

Inflammatory disorders are caused not only by infectious agents, such as parasites, bacteria, and viruses, but also by physical and chemical factors, including heat, acid, smoke and physical damage [12,13]. Recently,

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many non-steroidal drugs are commonly used for anti-inflammatory treatment, but side effects and gastric ulcers are some drawbacks[3]. Gingerol, shogaol, and structurally-related substances in ginger were evidenced to inhibit inflammation by down-regulating the synthesis of pro-inflammatory cytokines, including NFκB-regulated genes[14], TNF-α, IL-1, and IL-8[15].

Antioxidant properties play a significant role in balancing the production and neutralization of free radicals and oxidative stress[16]. Ginger extract and its constituents show antioxidant effects and protect macromolecules from damages induced by free radicals and oxidative stress[3]. Gingerol and gingerol-related compounds[16,17] paradol[18], zingerone[19,20], and ginger flavonoids[21] are the main ingredients of ginger extract and have been proven to have high antioxidant effects.

Tumor development and formation progress through various stages, including genetic and metabolic changes[22]. Ann M. Bode *et al.* provided evidence that [6]-gingerol can inhibit VEGF, and [6]-paradol can induce apoptosis in transformed cells[23].Other investigations have demonstrated that [6]-gingerol and constituents of ginger play a significant role in suppression of transformation and hyper-proliferation that relate to stages of carcinogenesis, metastasis, and tumorigenesis[24,25]. Furthermore, Nidhi Nigam *et al.* pointed out that [6]-gingerol increased benzo[a]pyrene (B[a]P)-suppressed p53 and Bax levels while decreasing Bcl-2 expression[25].

This research study was performed to optimize the method for ginger root extraction and investigate its anticancer activity. Alamar Blue staining was used to analyze cell viability and IC_{50} value. Furthermore, flow cytometry was performed with Annexin-V/ Propidium Iodide (PI) staining, and DNA fragmentation was observed with Hoechst 33342 staining to detect apoptosis induction.

MATERIALS- METHODS

Ginger roots

Ginger root (*Zingiber officinale*) within the age of 7-8 months was collected from Daklak, a province in the highlands of Vietnam. The whole root of fresh ginger was used in this study.

Extracts from ginger roots by Soxhlet, Sonication, and Maceration

Extracting the ginger root by Soxhlet

Twenty gram of the fresh ginger root was grinded. Then, 100ml of absolute ethanol solvent (Merck, Germany) was added into the flask together with the ginger root. The process was conducted at 78.4^{o}C for 12 hours, with 5-6 heat cycles in a heating mantle (MTOP, Republic of Korea) for 1 hour.

Extracting the ginger root by Sonication

100 g of the fresh ginger root was grinded and placed in a flask. Then, 500 ml of absolute ethanol was placed into the Ultrasonic Cleaner machine (JPS, CA, USA). The ultrasound process was conducted at a temperature of 15^{o}C for 30 minutes. The mixture after the ultrasound was filtered. The process was continued by refining 2-3 times with Whatman filter paper (Sigma Aldrich, MO, USA) to remove residue completely.

Extracting the ginger root by Maceration

100 g of the fresh ginger root was soaked in 500 ml of absolute ethanol in Becher for 72 hours. After 72 hours, the whole mixture was filtered to remove residue.

Cell culture (2D)

Human hepatocarcinoma cell line HepG2 was purchased from ATCC (Manassas, VA, USA). The cell line was grown in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO, USA) and 5% antibiotic (Thermo Fisher Scientific). HepG2 cells were put into a 5% CO_2 humidified atmosphere at 37°C. All the experiments were performed in triplicate.

Cell culture (3D)

The HepG2 cell line was seeded in a Hanging drop 96-well plate (3D Biomatrix, Ann Arbor, MI, USA) with DMEM/F12 medium supplemented with 10% FBS and 5% antibiotic. The cell density was 5000 cells per well (in 50 μl volume). The cells tended to cluster together and form spheroids after three days.

Apoptosis assay

Cells were labeled with 3 μl of Annexin V-FITC (Miltenyi Biotec, Germany) and 3 μl of Propidium Iodide (PI) (Miltenyi Biotec) in 500 μl of binding buffer for 15 min to detect apoptotic and necrotic cell death using a FACSCalibur Flow Cytometer (BD, Franklin Lake, NJ, USA). Data were analyzed by Cellquest software (BD).

Cytotoxicity assay on 2D and 3D cultures

After 2 days for 2D culture and 3 days for 3D culture, the cells in 96-well plates were treated with the ginger

root extract by Soxhlet, Sonication, and Maceration. The concentration of these extracts was diluted 2-fold serially from 2000 μg/ml to 15 μg/ml. Following 48h of incubation, the cells were evaluated for viability by staining with Alamar Blue and reading the measurements through a DTX 880 machine (Beckman Coulter, CA, USA).

Nuclei staining assay

To detect cell death after treatment with the extracts, cells were stained with 10 μl of PI. After that, the cells were incubated in a 5% CO_2 humidified atmosphere at 37°C. Cell images were captured after 1 h by a Zeiss Axio Imager fluorescence microscope (Zeiss, Germany).

Statistical analysis

The raw data from the cytotoxicity assay on the 2D and 3D culture cells were calculated for IC_{50} values. Accordingly, based on the untreated group (negative control) with 100% live cells, the calculation would detect the concentration of 50% of cells. The experiments were repeated three times and processed statistically with 95% confidence intervals. Statistical analysis was done using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Ginger root extracts inhibited HepG2 pro liferation in 2D and 3D models

HepG2 cells were treated with 3 kinds of ginger root extracts (from Maceration (G1), Soxhlet (G3), and Sonication (G5) methods) in 2D cell culture conditions to select for the extract with the highest cytotoxicity on HepG2 cells. The results showed that Soxhlet extract had the strongest cytotoxic activity on HepG2 with an IC_{50} of 83.3 $\pm$ 0.9189 μg/ml, while the IC_{50} values of the extract from methods of Maceration and Sonication were 159 $\pm$ 7.6 μg/ml and 284 $\pm$ 5.116 μg/ml, respectively. These results showed that the ginger root extract from Soxhlet showed the stronger antitumor cytotoxicity against HepG2.

After the treatment of HepG2 with ginger root extract in 2D conditions (**Figure 1**), we continuously used ginger root extract to treat HepG2 in 3D conditions (**Figure 2**). The results showed no significant differences in IC_{50} between the ginger root extracts from the different preparation methods; the IC_{50} of G3 extract was 228 $\pm$ 33.52 μg/ml, while IC_{50} of G1 was 341 $\pm$ 3.93 μg/ml and of G5 was 603.7 $\pm$ 56.33 μg/ml. The data show that G3 has more potent cytotoxicity compared with G1 and G5.

Ginger root extracts induced apoptosis of HepG2 cells in 2D culture

As aforementioned, from the results of IC_{50} of ginger root extract on 2D and 3D culture conditions, it turned out that G3 (the ginger root extract by Soxhlet method) had the strongest anti-toxicity on HepG2 in both 2D and 3D culture conditions. Therefore, we selected G3 to evaluate its ability to induce apoptosis of HepG2 cells. We treated HepG2 cells with G3 extract at the concentrations of 200 μg/ml, 100 μg/ml, 50 μg/ml, 25 μg/ml, or 0 μg/ml (control). The results demonstrated G3 extract induced apoptosis in a dose-dependent manner. At the concentration of 200 μg/ml, almost all cells underwent apoptosis, with 78.77% in late apoptosis.

To perform Annexin-V staining assay, cells were stained with PI and Hoechst 33342 and observed under microscopy. The results showed that at the concentration of 200 μg/ml (**Figure 3D**), 400 μg/ml (**Figure 3E**), and 800 μg/ml (**Figure 3 F**), almost all cells were stained with PI (red color) (**Figure 4**). At the high microscope magnification, the nuclei of cells treated with G3 extract at 200 μg/ml were condensed and disintegrated (**Figure 5**). These kinds of features indicate that the cells underwent apoptosis.

Death cells of HepG2 spheroids after treatment with ginger root extract

We also evaluated how the G3 extract impacted HepG2 spheroids in 3D culture conditions. After culturing HepG2 cells in 3D and following spheroid development, the spheroids were treated with G3 extract and compared with G1 and G5 extracts (**Figure 6**). The HepG2 spheroids showed to break apart and to stain with PI at the concentration 250 μg/ml of Ginger extract, while untreated spheroids still remained the initial shape. At the concentrations of 500 and 1000 μg/ml, the G1, G2, and G3 extracts all had an impact on the HepG2 spheroids. These results demonstrate that G3 has strong activity on HepG2 spheroids.

Ginger extract triggers apoptosis of HepG2 spheroids

We then selected the extract G3 to evaluate its ability to induce apoptosis by flow cytometry. We observed that even in untreated spheroids, there was about 30% of cells which underwent apoptosis (**Figure 7**). This was likely due to the core of HepG2 spheroids, where there is a lack of oxygen and nutrients. However, after spheroids were treated with G3 extract, the apoptotic

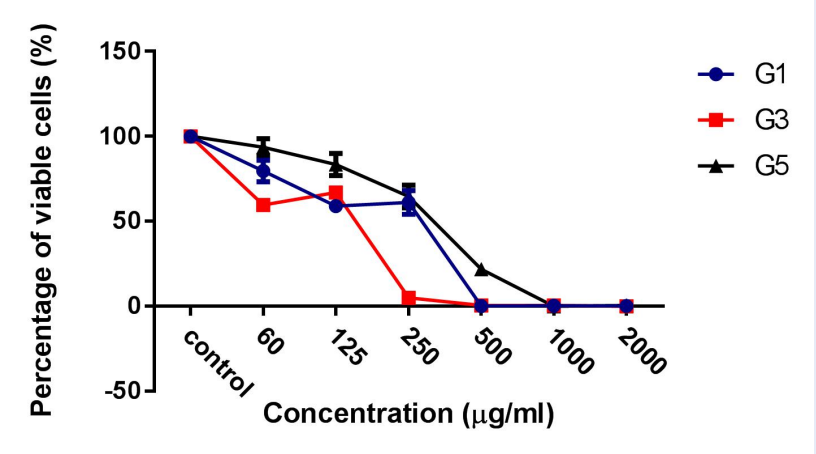

Figure 1: **Inhibition of proliferation of HepG2 cells after treatment with ginger root extract**. HepG2 cells were treated with the ginger root extracts from methods of Maceration (G1), Soxhlet (G3), or Sonication (G5); the range of concentrations were 2000, 1000, 500, 250, 125, and 60 μg/ml, along with untreated (control). After 48 hours of treatment, cells were stained with Alamar Blue, and cell viability was measured by DTX 880 multimode reader. The data was analyzed by Graphpad Prism. The experiment was replicated.

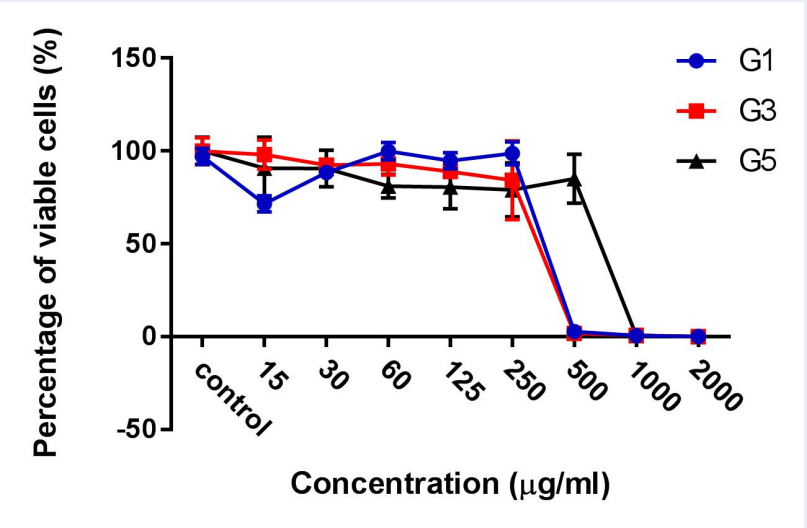

Figure 2: **Inhibition of proliferation of HepG2 cells after treatment with ginger root extract .** HepG2 spheroids were treated with ginger root extract from Maceration (G1), Soxhlet (G3), or Sonication (G5); the range of concentrations were 2000, 1000, 500, 250, 125, 60, 30, and 15 μg/ml, along with untreated (control). After 48 hours of treatment, Alamar Blue was added. Cell viability was measured by DTX 880 machine. Data were analyzed by Graphpad Prism. The experiment was replicated.

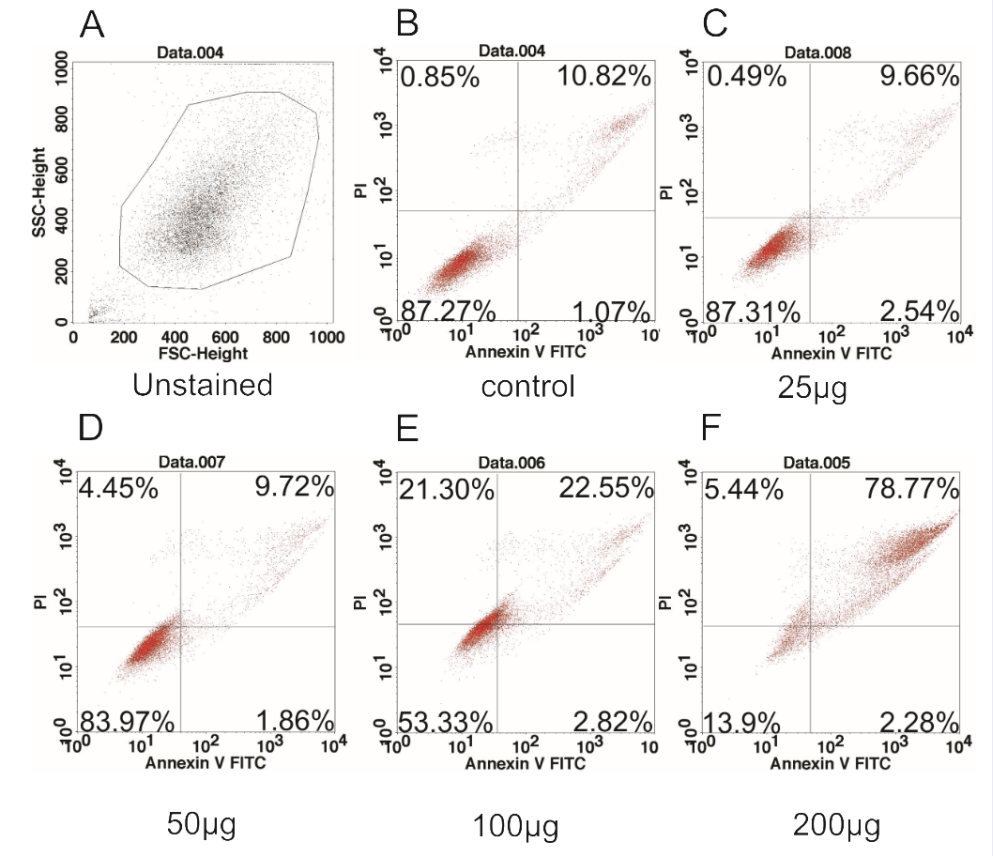

Figure 3: **Apoptosis of HepG2 cells after treatment with G3 extract in 2D cell culture condition.** HepG2 cells were treated with G3 extract at the concentrations of 25 μg/ml, 50 μg/ml, 100 μg/ml, or 200 μg/ml, and compared to control (no treatment). After 48 hours of treatment, cells were stained with Annexin-V to detect the apoptotic cells. At the concentration of 200 μg/ml, almost all the treated cells underwent apoptosis, with 78.77% in late apoptosis and 5.44% in early apoptosis.

populations increased to 40% at the $IC_{50}/2$; 77.04% at IC_{50}; almost all cells were in late apoptosis at IC_{50} x 2 and IC_{50} x 4. These results demonstrated the impact of G3 extract on HepG2 spheroids. Together with the ability to break the HepG2 spheroids apart, the G3 extract induced the HepG2 cells in spheroids to undergo apoptosis.

DISCUSSION

Phytochemical extracts from fruits and vegetables are increasingly being shown to exert potent antioxidant and anti-proliferative effects. It is widely becoming appreciated that chemo-preventative agents offer superior potential in the long term over chemotherapeutic agents. Life style and dietary habits have been identified as major risk factors, particularly in cancer growth and progression.

Ginger root is extensively used in the form of a fresh paste or dried powder to flavor food and beverages. Recently, a study showed that treatment with 2 mg/ml of ginger with 31 mg/ml of gelam honey inhibited the growth of most HT29 cells and induced apoptosis in a dose-dependent manner, with the combined treatment yielding the highest apoptosis rate. This was caused by the downregulation of gene expression of *Akt*, *mTOR*, and *Raptor*, while *cytochrome C* and *caspase 3* genes where shown to be upregulated[26] Recently, ginger root extract also showed anti-leukemia and anti-drug resistant effects[27].

In the present study, we show that ginger extracted by Soxhlet has stronger cytotoxic activity on HepG2 cells than extracts from Maceration and Sonication. The ginger extract could induce apoptosis of HepG2 cell lines cultured in both 2D and 3D conditions. Sonication and Maceration methods were not strong enough to break down the cell wall of the ginger plant to release certain active agents, but the Soxhlet method was capable of that. Recently, some studies have shown

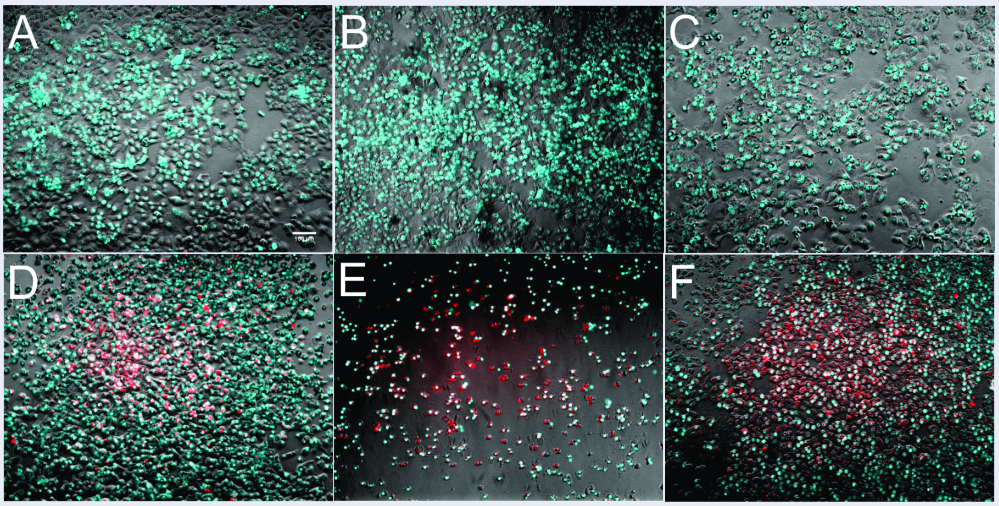

Figure 4: **PI-staining of dead HepG2 cells in 2D culture condition**. HepG2 cells were treated with G3 extract at various different concentrations. **A**. Control; **B**. 50 μg/ml; **C**. 100 μg/ml; **D**. 200 μg/ml; **E**. 400 μg/ml; **F**. 800 μg/ml. After 48 hours of treatment, cells were stained with PI (red dead cells) and Hoescht 33342 (blue nucleus). Cells were then observed under fluorescent microscopy. All Images taken at same magnification, at 10X.

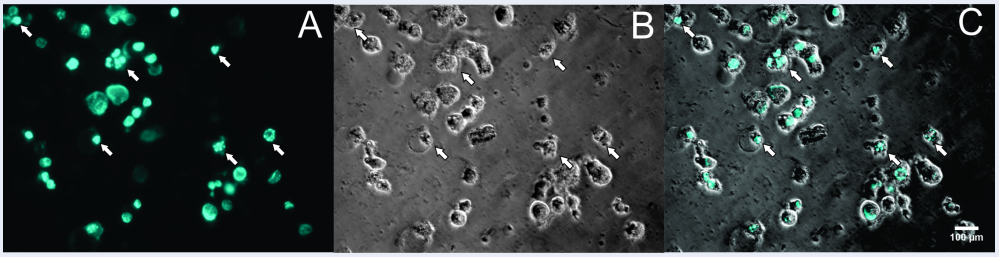

Figure 5: **Disintegrated nuclei of ginger-treated HepG2 cells.** HepG2 cells were treated with the extract at the concentration of 200 μg/ml in 48 hours. Cells were then stained with Hoechst 33342 and observed under a fluorescent microscope. Apoptotic cells exhibited the characteristic condensed and debris-full nucleus. The white arrow indicates apoptotic cells. All Images taken at same magnification, at 20X.

that ginger polysaccharides induced cell cycle arrest and apoptosis of HepG2 through up-regulation of *Bax*, *caspase-3*, and *p53*, and down-regulation of *Bcl-2*[28]. We observed the typical apoptotic morphological changes in HepG2 cells, including cell shrinkage and detachment, and nuclear condensation and fragmentation in 2D culture conditions of HepG2. This was in accordance with a study by Elkady *et al.*[29] which demonstrated that ginger extract induced the upregulation of *p53* and *p21*, which inducing down-regulation of *cyclin D1* and *cyclin-dependent kinanse-4* expression, resulting in G2/M2 arrest.

We firstly demonstrated the anti-tumor effect of ginger extract in 3D conditions. The extract induced HepG2 spheroids to break part from the original round shape. We also found that ginger extract induced cells in the HepG2 spheroids to undergo apoptosis. This might have caused the cells to lose their cell-to-cell contact, resulting in the disassembly of the HepG2 spheroids.

6-shogaol is a major active constituent of dietary ginger that has been demonstrated to inhibit cell proliferation in osteosarcoma[30], lung cancer[31], and colon cancer[32], through increasing apoptosis pathways which lead to a decrease of expression levels of anti-apoptotic proteins (*e.g.* survivin and Bcl-2) and increasing pro-apoptotic proteins (*e.g.* Bax). In HepG2 cell lines, 6-shogaol and 6-gingerol (two active components in ginger) showed anti-invasion and anti-metastasis properties *in vitro*[33].

Taken together, ginger extract possesses constituents that are able to block proliferation and induce the

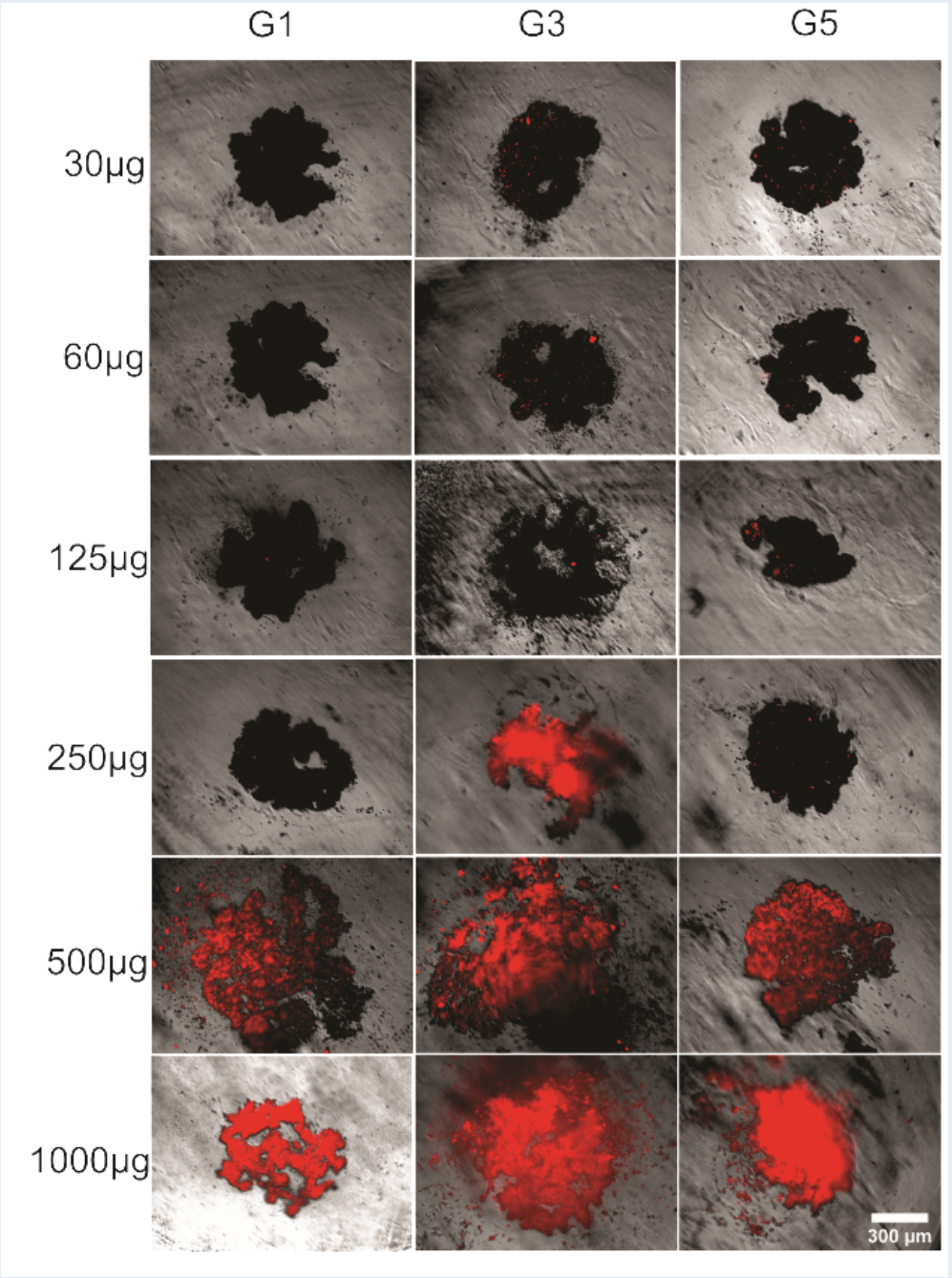

Figure 6: **Degradation of HepG2 spheroids after treatment with ginger extract.** HepG2 cells were cultured in 3D conditions for 3 days, then treated with ginger extracts from Maceration (G1), Soxhlet (G3), or Sonication (G5)- at the concentrations of 30, 60, 125, 250, 500, or 1000 μg/ml. After 2 days of treatment, spheroids were stained with PI and observed under a fluorescent microscope. The red dot in the figure shows the death of cells in the spheroids. All Images taken at same magnification, at 5X.

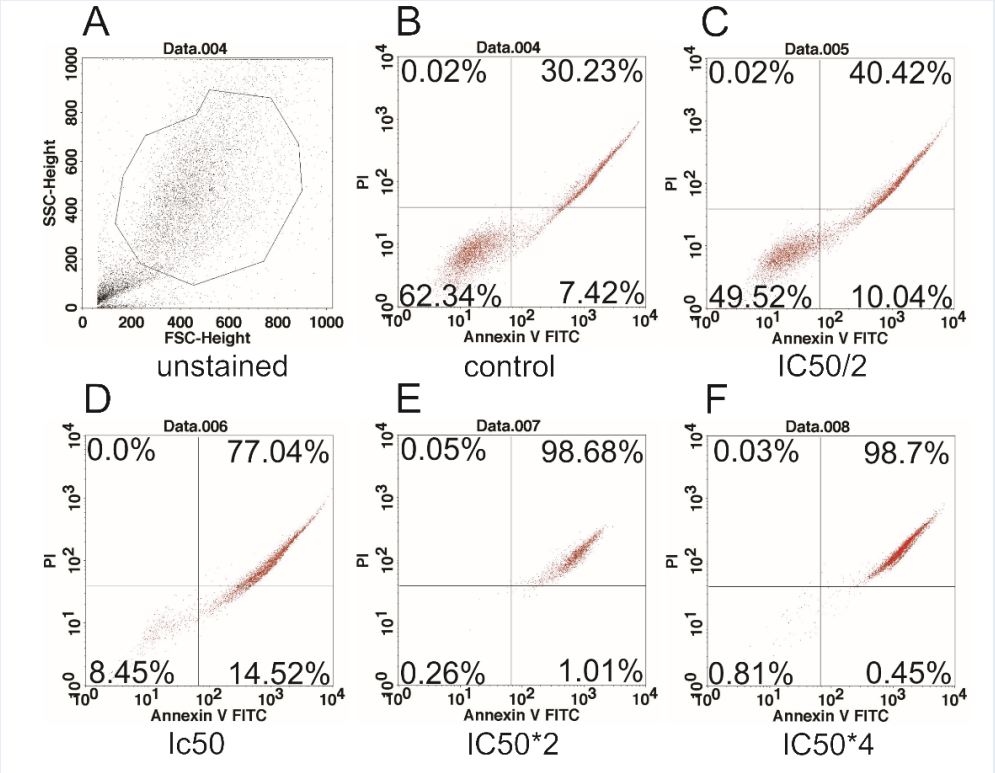

Figure 7: **Apoptosis of cells in the HepG2 spheroids after treatment with ginger root extract**. HepG2 spheroids were treated with ginger root extract at the concentration of IC_{50}, IC_{50}/2, IC_{50} x 2, or IC_{50} x 4. After 2 days of treatment, cells in the HepG2 spheroids were detached from the spheroids and suspended into single cells. The cells were then stained with Annexin V and PI, and analyzed by BD FACSCalibur machine. Data were analyzed by Cellquest Pro software.

death of HepG2 cells in both 2D and 3D culture conditions.

CONCLUSION

Ginger has a long history of use in the diet and medicine of humans. This study contributes further evidence of the potency of ginger root extract. We show that ginger possesses potent cytotoxicity on HepG2 cells grown in 2D or 3D, suggesting its potential benefit as an anticancer agent in disease treatment. Some studies on the constituents of ginger have further documented its ability to induce apoptosis *in vitro*. Further studies of ginger root extract, and its active constituents, *in vivo* using animal models, are needed to ascertain its anticancer potency prior to its application for human disease.

ABBREVIATIONS

G1: ginger root extract by Maceration method
G3: ginger root extract by Soxhlet method
G5: ginger root extract by Ultrasonic method
NFκB: nuclear factor kB
TNF- α: tumor necrosis factor alpha
IL: interleukin
VEGF: vascular endothelial growth factor

CONFLICT OF INTETEST

We have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Sinh Nguyen, Phuc Van Pham conceived and planned the experiments. Phuc Hong Vo, Thao Duy Nguyen, Nghia Minh Do, Bao Huu Le, Duong Thuy Dinh performed the experiments. Kiet Dinh Truong, Sinh Nguyen analysed the data. Sinh Nguyen wrote the manuscript. Phuc Pham and Kiet Dinh Truong revised the manuscript.

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