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Full Length Research Paper

Ginger extract (*Zingiber officinale*) triggers apoptosis and G_0/G_1 cells arrest in HCT 116 and HT 29 colon cancer cell lines

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Although many studies have shown the antitumor properties of ginger extract (*Zingiber officinale*), little is known regarding the mechanism of its effects. This study was conducted to determine the mechanism of antitumor effects of ginger extract by evaluating apoptosis rate and cell cycle progression status in colon cancer cell lines HCT 116 and p53 defective HT 29. HCT 116 and HT 29 cells were cultured in the presence of ginger extract at various concentrations for 24 h. The percentage of cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-di phenyl tetrazolium bromide (MTT) assay. Our results showed that ginger extract inhibited proliferation of HCT 116 and HT 29 cells with an IC_{50} of 496 ± 34.2 µg/ml and 455 ± 18.6 µg/ml, respectively. We also found that ginger extract at increasing concentrations induced apoptosis dose dependently in both colon cancer cells. Apoptosis rates were 11.15, 35.05 and 57.49% for HCT 116 and 4.39, 19.81 and 28.09% for HT 29 at 200, 500 and 800 µg/ml of ginger extract, respectively. Ginger extract arrested HCT 116 and HT 29 cells at G_0/G_1 and G_2/M phases with corresponding decreased in S-phase. This study suggests that ginger extract may exert its antitumor effects on colon cancer cells by suppressing its growth, arresting the G_0/G_1-phase, reducing DNA synthesis and inducing apoptosis.

Key words: *Zingiber officinale*, HCT 116, HT 29, G_0/G_1 phase, S phase, apoptosis.

INTRODUCTION

Colorectal cancer accounts for 10 to 15% of all cancers and is the second leading cause of cancer-related death in industrialized countries (Virginie et al., 2007). According to National Cancer Registry of Malaysia (Gerard and Halimah, 2003), colon cancer is recognized as the commonest cancer among men and the third most common cancer among women. Colon cancer occurrence is commonly ascribed to the transformation of normal colon epithelium to adematous polyps and ultimately invasive cancer. Since cancer is a disorder of deregulated cell proliferation and cell survival (Evan and Vousden, 2001), inhibiting cell proliferation and increasing apoptosis in tumors are effective strategies for preventing tumor growth (Kim et al., 2005). Recent studies showed that curcumin, an active component of turmeric inhibits the growth of human colon cancer cells independent of COX-2 expression (Hanif et al., 1997). Lee et al. (2009) showed that thiosulfinates from *Allium tuberosum L*. inhibited cell proliferation and activated both the caspase-dependent and caspase-independent apoptotic pathways in colon cancer cells (Lee et al., 2009). Other study demonstrated that luteolin induced G_1 and G_2/M cell-cycle arrest and promoted apoptosisthrough down regulation of several antiapoptotic proteinsin colon cancer cells.

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Emerging evidence has demonstrated that many natural products isolated from plant sources possess antitumor properties (Sakamoto et al., 1991). Since nature has provided many effective anticancer agents, plant derived drug research has made significant progress in anticancer therapies. Many components of medicinal or dietary plants have been identified as possessing potential chemopreventive properties capable of inhibiting, retarding or reversing the multi-stage carcinogenesis process (Surh et al., 1998; Surh et al., 2002). Plants of the ginger family (*Zingiber officinale*) are one of the most heavily consumed dietary substances in the world (Surh, 1999). It is one of the most widely used spices and has been used in traditional oriental medicines for centuries. Its extract and major pungent compounds have been shown to exhibit a variety of biological activities (Wei et al., 2005). The oleoresin from rhizome of ginger contains pungent ingredients including gingerol, shogaol and zingerone (Surh et al., 1998). Ginger's active components have been reported to exhibit cancer-preventive activity in several experimental carcinogenesis models including skin carcinogenesis (Murillo et al., 2008; Katiyar et al., 1996). Ginger supplementation suppresses colon carcinogenesis in the presence of procarcinogen (DMH) (Manju and Nalini, 2005). Recent study showed that [6]-gingerol a major phenolic compound derived from ginger, inhibited TRAIL-induced NF-κB activation by impairing the nuclear translocation of NF-κB, suppresses cIAP expression and increased TRAIL-induced caspase-3/7 activation in gastric cancer cells. On the other hand, [6]-shogaol alone reduced viability by damaging microtubules, arrested cell cycle in G_2/M phase and reduced viability in a caspase-3/7-independent manner in gastric cancer cells (Kazuhiro, 2007). Lee et al. (1998) showed that [6]-gingerol exerted inhibitory effects on the cell viability and DNA synthesis while inducing apoptosis of human promyelocytic leukemia HL-60 cells (Lee and Surh, 1998). Ginger was also shown to have anticancer effect by inducing apoptosis in rat liver cancer cells via up-regulation of the expression of pro-apoptotic protein, caspase-8 and downregulation of the expression of anti-apoptotic protein Bcl-2 (Yasmin et al., 2008). Exposure of Jurkat human T cell leukemia cells to various ginger constituents galanals A and B (isolated from the flower buds of Japanese ginger) resulted in apoptosis mediated through the mitochondrial pathway (Miyoshi et al., 2003). β-Elemene, an anticancer drug extracted from the ginger plant, triggered apoptosis in non-small-cell lung cancer cells through a mitochondrial release of the cytochrome *c*-mediated apoptotic pathway (Wang et al., 2005).

We have selected *Z. offinale* in this study to determine its mechanism as a potential chemopreventive agent against colon cancer cells HCT 116 and HT 29. Our results demonstrated that the inhibition of colon cancer cell growth by ginger involves both interference with cell cycle progression and apoptotic event.

MATERIALS AND METHODS

Ginger extract

Z. officinale (ginger) crude extract was obtained by ethanol extraction as provided by Noor Azian Murad from Center for Lipids Engineering Applied Research (CLEAR), Universiti Teknologi Malaysia. The sequence of pre-treatment for ginger extract include: peeling, slicing, washing of ginger rhizomes followed by bleaching, blanching, drying and grinding prior to solvent extraction. The sample calculation to obtain the moisture loss percent based on the initial and final weight of the sample is given by the equation:

$$\% \text{ moisture loss} = \frac{\text{Initial weight} - \text{current sample weight} \times 100\%}{\text{Initial weight}}$$

The dried ginger was fibrous and tough and therefore grinding of samples was done manually by pounding using 'mortar'. The pounded samples were then shredded manually. The ground ginger was dried prior to extraction using rotary evaporator in ethanol (1 L) for 6 h. The solvent was removed under vacuum at 500 mbar in the first hour, followed by 400 and 300 mbar in the next two hours to yield oleoresin, a brown viscous liquid (9.80 g, 4.9%). Refractive index reading of pure oleoresin is 1.5100.

Cell culture and treatment

HCT 116 and HT 29 cell lines were obtained from the American Type Culture Collection (Rockville, MD USA) and were cultured in RPMI 1640 medium (Flowlab, Australia) supplemented with 10% Fetal Calf Serum (FCS) (PAA Laboratories GmbH), 100 U/ml of penicillin and streptomycin (Thermo Scientific) at 37°C in 5% CO_2. Rates of cells proliferation, apoptosis and cell cycle were performed when cells reached 70% confluence density. Ginger extract was dissolved in 0.01% DMSO and added to cell lines after an overnight incubation.

Cell viability assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

For cell viability assay, $2 \cdot 10^4$ cells (HCT 116 and HT 29)/well were plated in 100 µl of RPMI 1640 media. HCT 116 and HT 29 cells were incubated overnight at 37°C in humidified atmosphere of 5% CO_2 for cells attachment. Ginger extract was added at various concentrations ranging from 0, 10, 50, 100, 200, 500, 800, 1000 and 1500 ∝g/ml after 24 h incubation. After 24 h incubation, MTT solution (2 mg/ml) was added to the plate at the final concentration of 0.5 mg/ml. The resulting MTT-products were determined by measuring the absorbance at 550 nm with ELISA reader. Each point represents the mean of triplicate experiments.

$$\% \text{ viability} = \frac{\text{Optical density of sample}}{\text{Optical density of control}} \times 100$$

Cell cycle assay

The HCT 116 and HT 29 cells were seeded into 25 cm^2 flask (1 x 10^5 cells/ml) and were incubated with 200, 500 and 800 µg/ml ginger extract for 24 h. Following treatment, cells were trypsinized and incubated for 30 min at room temperature in staining solution consisting of propidium iodide (PI; 50 µg/ml), sodium citrate (0.1%), Triton X-100 (0.1%) and DNase-free RNase (20 µg/ml). Stained cells were then analyzed for DNA content by flow cytometer within

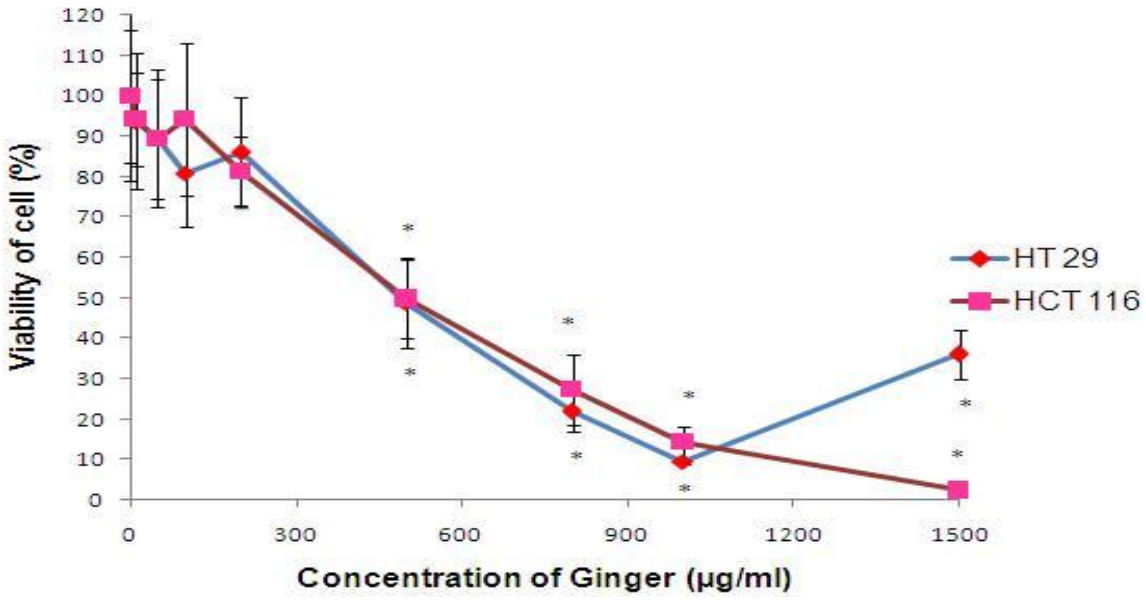

Figure 1. Effect of ginger extract on the proliferation of HCT 116 and HT 29 cells. Cells were treated with different concentration of ginger extract, incubated at 37°C and harvested after 24 hour. Cell viability was determined by the MTT assay. Data represent the mean ± S.D. (n = 3).

3 h of staining. Percentages of cells in each phase were calculated using Cell Modfit software programs (Becton Dickson, Canada).

Apoptosis–annexin V-FITC Staining

Apoptosis was detected with an annexin V-FITC kit (Beckton-Dickinson, Canada). All adhering and floating cells were harvested after incubation for 24 h with 200, 500 and 800 µg/ml ginger extract. Cells were collected, washed with ice-cold PBS and centrifuged. The cell pellet was resuspended in ice-cold binding buffer (100 mM HEPES/ NaOH, pH 7.5 containing 1.4 M NaCl and 25 mM $CaCl_2$) at a density of 1 x 10^6 cells per ml. Five hundred ∝l of this cell suspension was transferred to a 5 ml culture tube, to which 5 ∝l of Annexin V-FITC conjugate and 10 µl of propidium iodide were added. The cells were gently vortexed and incubated for 10 min at room temperature in the dark. The fluorescence of the cells was immediately determined by flow cytometry (Becton Dickson, Canada). The results were analysed using Cell Modfit software programs (Becton Dickson, Canada).

Statistical analysis

The experiments were repeated at least 3 times and the results were expressed as mean ± S.D. Statistical evaluation was done using the ANOVA (SPSS 16.0) where $p < 0.05$ was considered significant.

RESULTS

Figure 1 showed that ginger extract significantly inhibited the proliferation of both colon cancer cells in a dose-dependent manner. Proliferation of HCT 116 cells decreased when treated with ginger extract resulting in a 50% reduction at 496 ± 34.2 µg/ml, while HT 29 cells showed significant decrease in proliferation with 50% reduction at 455 ± 18.6 µg/ml of ginger extract.

To elucidate the mechanisms involved in the reduction in cell number by ginger, we examined whether such reduction was associated with cytostatic effect due to changes in cell cycle progression. Figures 2a and 2b summarized the relative percentages of HCT 116 and HT 29 cells in each phase of the cell cycle, following a 24-h treatment with varying ginger extract concentrations (200, 500 and 800 µg/ ml). The cell cycle alterations were concentration dependent. As shown in Figures 2a and 2b, in the absence of ginger extract, 48.82% HCT 116 cells and 54.22% of HT 29 were in the G_0/G_1-phase. However, treatment with ginger extract arrested both cells at G_0/G_1-phase: 63.15% for HCT 116 and 70.98% for HT 29 at 200 µg/ml. Interestingly, we found a unique characteristic whereby higher doses of ginger extract (800 µg/ml) did not arrest cells at G_0/G_1-phase but instead they were arrested at G_2/M-phase. The G_0/G_1 and G_2/M arrests were accompanied by corresponding reduction in the percentages of cells in the S-phase as shown in Figure 2a and 2b. Reduction in DNA synthesis was dependent on the dose of ginger extract: 15.06, 9.52 and 8.66%, when treated with 200, 500 and 800 µg/ml of ginger extract for HCT 116 cells and 8.81, 14.63 and 3.21% for HT 29 cells when treated with the same concentrations of ginger extract, respectively.

Figure 3a showed apoptotic effect of ginger extract on both HCT 116 and HT 29 cells using double staining method FITC-conjugated annexin V and PI. Ginger treatment increased the number of early and late apoptotic cells, respectively. Ginger extract at increasing concentrations induced higher rate of poptosis

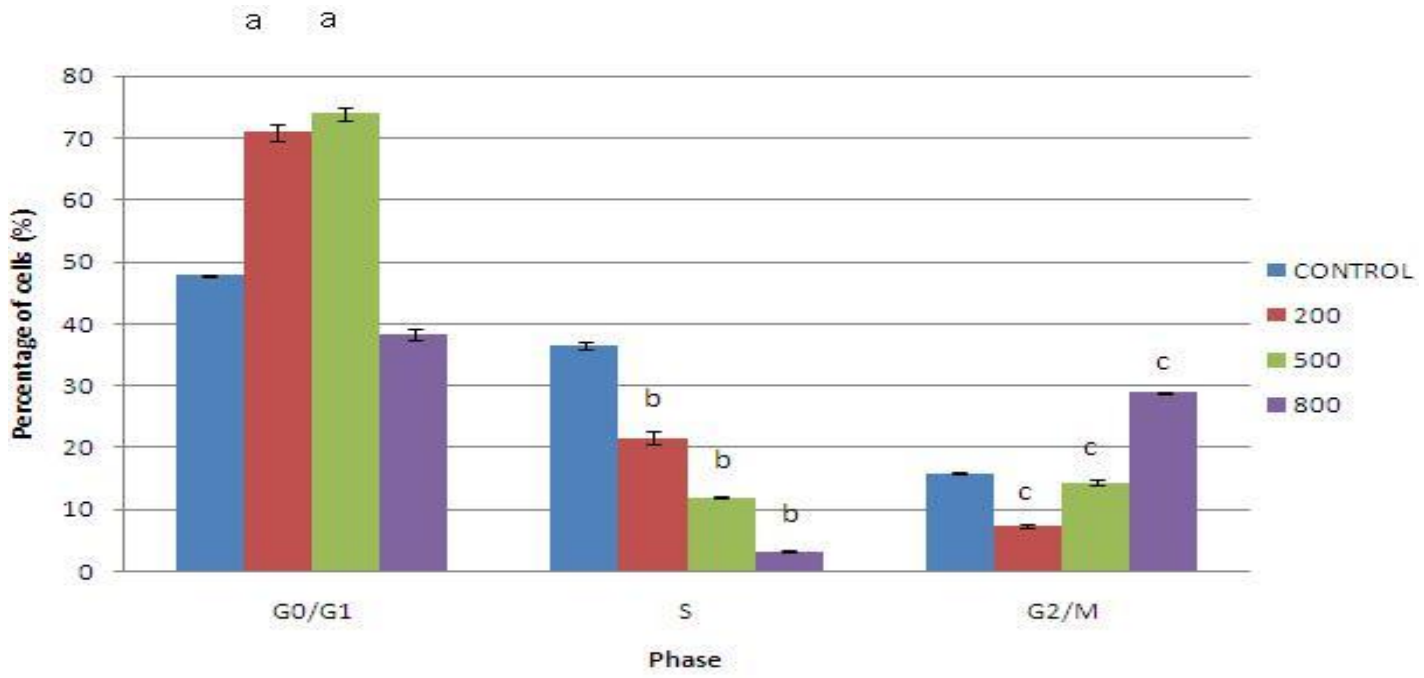

Figure 2a. Cell cycle profile of HCT 116 colon cancer cells in the presence of ginger extract. After a 24 h incubation with varying ginger extract concentrations. DNA content was evaluated with propidium iodide (PI) staining and fluorescence measured and analyzed. The values are the means ± SD of three independent experiments. a; significant compared to G0/G1 phase of control cell, b; significant compared to S phase of control cell, c; siginificant compared to G2/M phase of control cells..

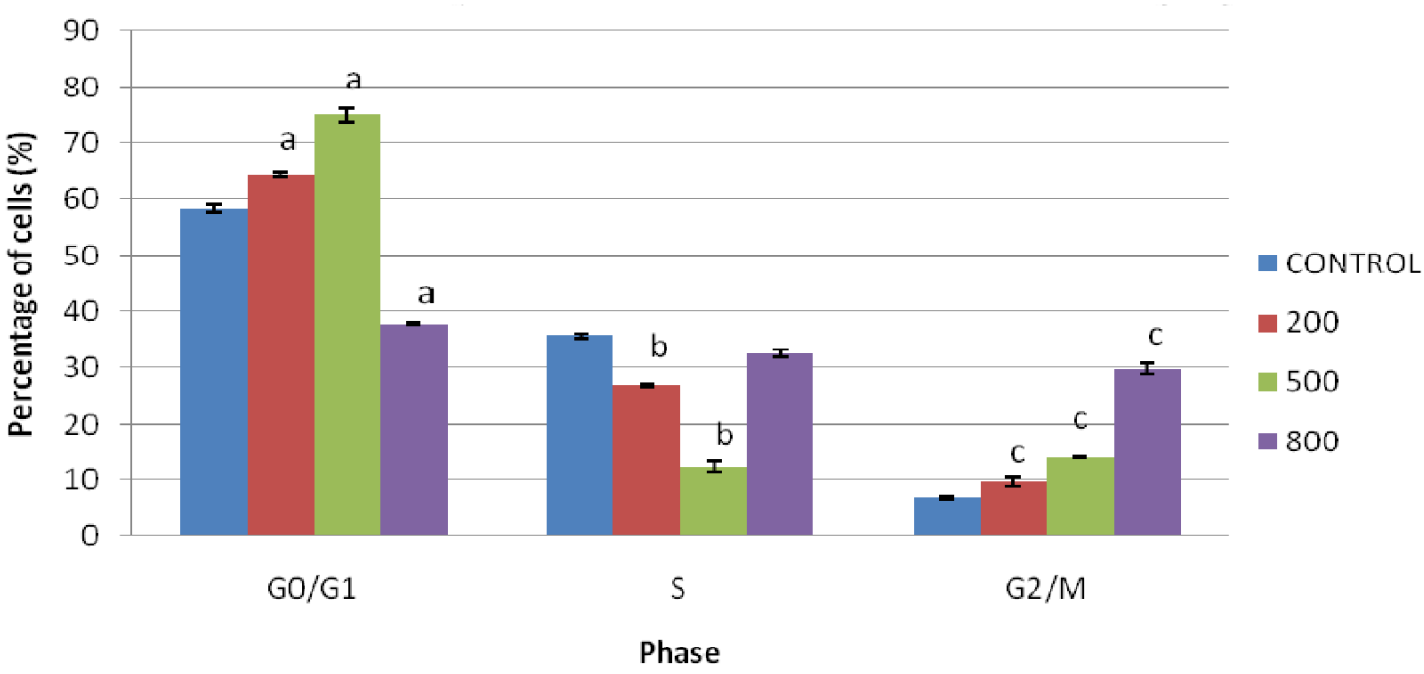

Figure 2b. Distribution of cell cycle phases of HT 29 cells after treatment with ginger extract. After a 24 hr incubation with varying ginger extract concentrations (0, 200, 500 and 800 µg/ml), DNA content were evaluated with propidium iodide staining and fluorescence measured and analyzed. The values are the means ± SD of three independent experiments. a; significant compared to G0/G1 phase of control cell, b; significant compared to S phase of control cell, c; siginificant compared to G2/M phase of control cells.

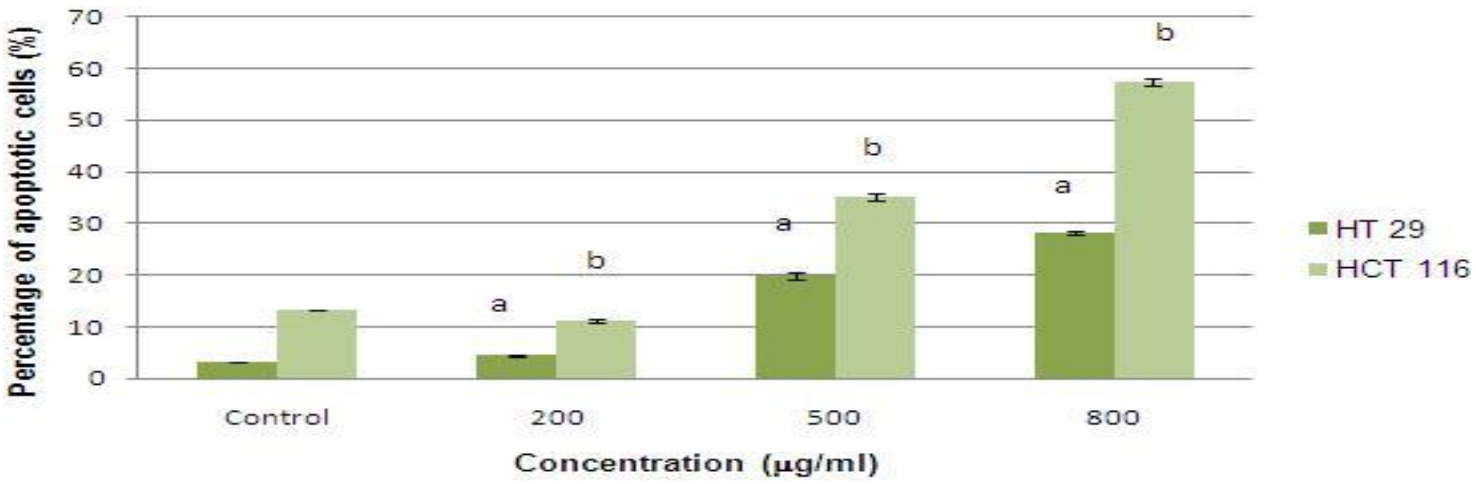

Figure 3a. Induction of apoptosis by ginger extract in human colon cancer cell lines, HT 29 and HCT 116. Percentages of apoptotic cells after a 24 h incubation with varying ginger extract concentrations. a: significant compared to control HT 29, b: significant compared to control HCT 116.

significantly ($p < 0.05$) compared to control for both HCT 116 and HT 29 cells as shown by cytograms of apoptosis for HCT 116 (Figure 3b) and HT 29 cells (Figure 3c).

DISCUSSION

There has been substantial interest in the search for potential chemopreventive agents in the past years for treatment against many cancers. Understanding how dietary components regulate proliferation and cell survival could play a critical role in the development of new agents that can prevent and treat cancer with lo w toxicity (Lee and Surh, 1998). Cancer chemoprevention was defined as the utilization of chemically active compoundsto reverse, suppress and prevent progression of disease from pre-invasive cancer to frank malignancy (Sporn et al., 1976).

Numerous diet-derived agents are included among promising agents and agent combination that are being evaluated clinically as chemopreventive agents for major cancer targets including breast, prostate, colon and lung cancers (Kellof et al., 2000). Ginger (*Z. officinalis* Roscoe, Zingiberaceae) is not only widely used as a dietary condiment but it has also been extensively utilized as a traditional oriental medicine (Taraphdar et al., 2001). Gingerols (that is, 6-gingerol, 8-gingerol and zingerone) and [6]-paradol have been identified as the main active ingredients of ginger and are responsible for the antioxidant activity and its characteristic pungent taste (Gruenwald et al., 2000; Keum et al., 2002). Antioxidative capacity of ginger has been associated with the ability of ginger to inhibit carcinogenesis by reducing oxidative stress and inducing apoptosis (Shukla and Singh, 2007; Katiyar, 1996). Bode and his co-workers, reported that [6]-paradol exerted its primary inhibitory effect on cell transformation through the induction of apoptosis (Bode et al., 2001). [6]-Paradol and other structurally related derivatives induced apoptosis in oral squamous carcinoma cell line in a dose- dependent manner through a caspase-3-dependent mechanism (Keum at al., 2002). Ginger has been shown to inhibit cell growth in several tumor cells including liver (HepG2) (Yasmin et al., 2008), gastrointestinal (Yoshimi et al., 1992) and breast (Nagasawa et al., 2002). Ginger was also found to possess an anti-tumor promoting potential as determined by inhibition of phorbol ester-induced inhibition of Epstein- Barr virus (EBV) activation in Raji cells (Koshimizu et al., 1988).

The present study provides evidence that ginger may act as a potent growth inhibitory compound in human colon adenocarcinoma cells and supports the possibility of a chemopreventive potential in colon cancer cells. Our study showed that HT 29 cells were more sensitive to ginger treatment with an IC_{50} much lower [455 µg/ml] than HCT 116 cells [496 µg/ml]. The cytotoxic effect could be as a result of the active component, gingerol that has been reported to inhibit the growth of HCT 116 human colon colorectal and liver $HepG_2$ cancer cells (Bode, 2003; Harliansyah et al., 2007). Azoxymethane-induced intestinal carcinogenesis in rats was significantly suppressed by dietary administration of gingerol (Yoshimi et al., 1992).

We also reported in this study that the inhibition mechanism of colon cancer cells growth by ginger involved interference in both cell cycle progression and apoptosis. Ginger was able to induce cell cycle progression in G_0/ G_1 phase and apoptotic death in a dose-dependent manner in both cells. Deregulated cell cycle progression driven by activation of growth-stimulating oncogenes, is one of the

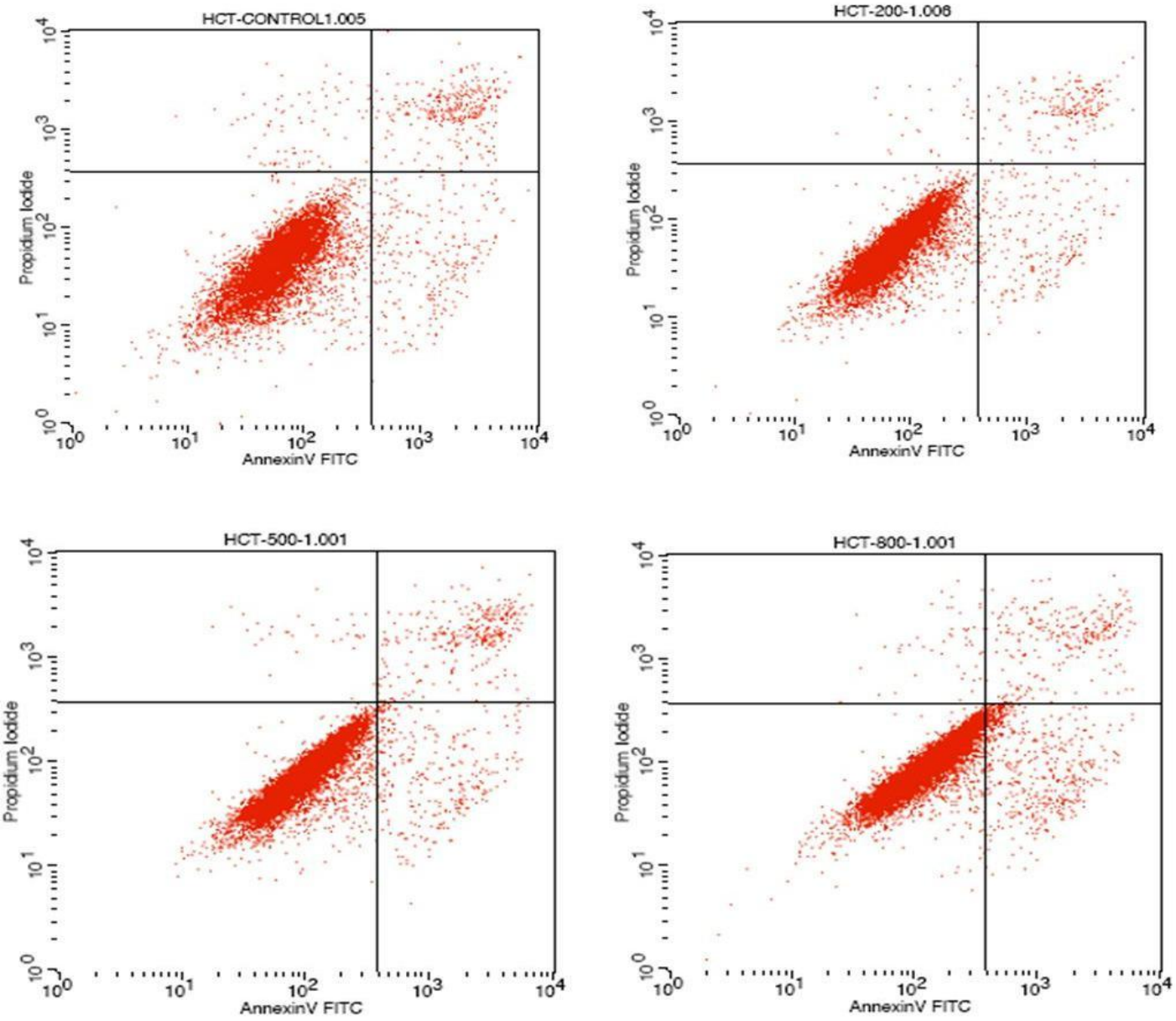

Figure 3b. Flow cytometric analysis of apoptosis induction by ginger in HCT 116 cells. Representative cytograms of apoptosis in HCT 116 cells incubated for 24 hours in the absence (A) or presence of 200 (B), 500 (C) and 800 µg (D) ginger. Within a cytogram, a quadrants 2 and 4 represent late and early apoptotic cells, respectively; quadrant 1, necrotic cells; quadrant 3, viable cells.

primary characteristics of cancer cells. Cell cycle progression is tightly controlled by the regulation of expression and activity of cyclin/cyclin-dependent kinase (CDK) complexes (Taraphdar et al., 2001). Dysregulation of the cell cycle check points and overexpression of growth-promoting cell cycle factors such as cyclin D1 and cyclin-dependent kinase (CDK) are associated with tumorigenesis (Deihl, 2002). Several dietary agents inclu-ding curcumin (Mukhopadhyay et al., 2002), resveratrol (Estrov et al., 2003), genistein (Li et al., 2005), dietary isothiocyanates (Jakubikova et al., 2005), apigenin (Takagaki et al., 2005) and silibinin (Tyagi et al., 2002) have been shown to block the deregulated cell cycle in cancers. In our study, ginger managed to arrest cell cycle at G_0/G_1-phase for both colon cancer cells at concentration below IC_{50}, whereas at higher concentrations, both cells were arrested by ginger extract at G_2/M phase. The cell cycle arrest observed could be due to inhibitory effect of ginger on cyclin dependent kinases and activation of cell cycle check points. Palozza et al. (2002) showed that arrest of the cell cycle and accumulation of cells in G_2/M phase in colon cancer cells was followed by the decreased expression of cyclin A, a protein known to regulate cdc2 kinase activity in G_2/M phase after

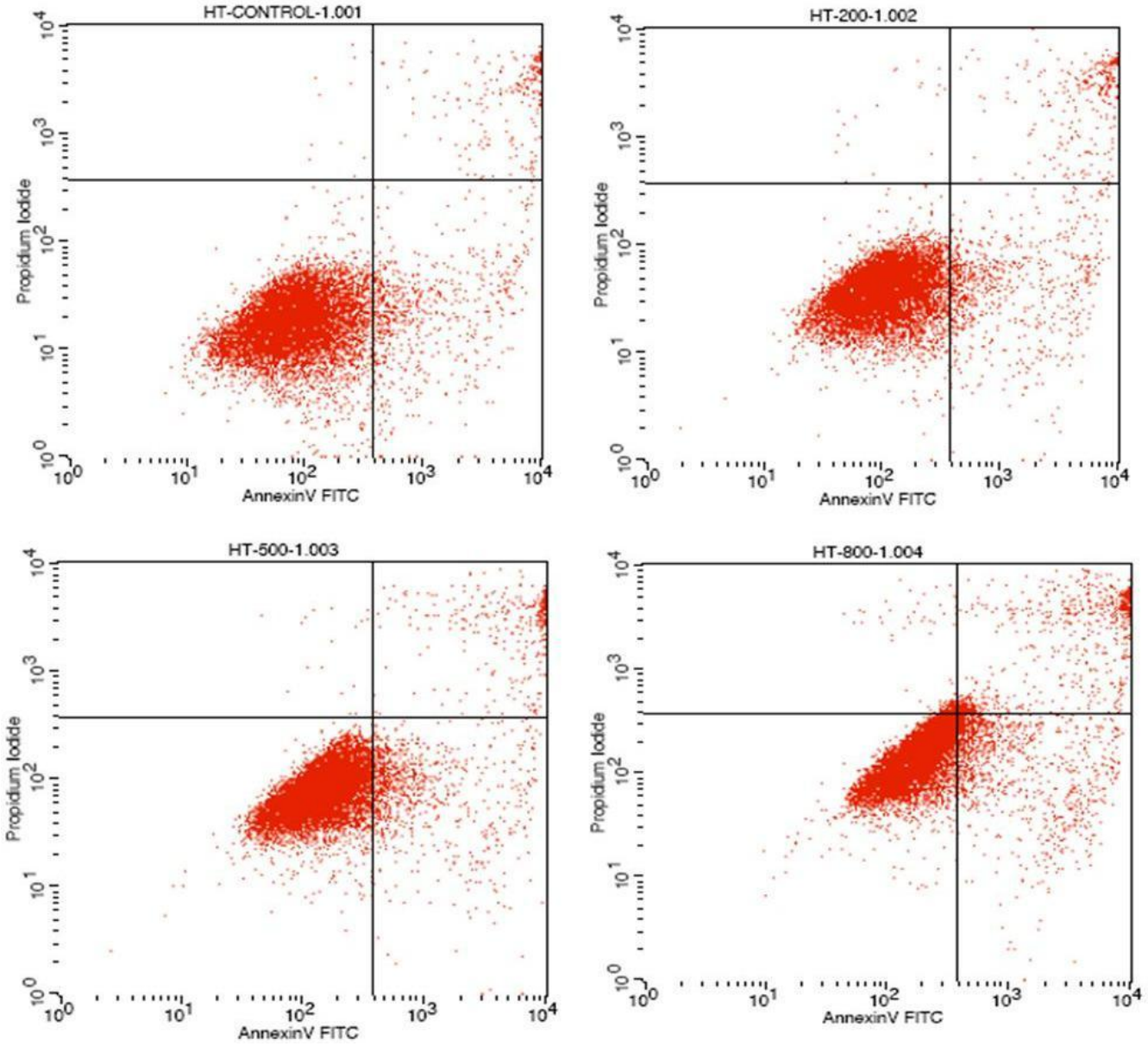

Figure 3c. Flow cytometric analysis of apoptosis induction by ginger in HT 29 cells. Representative cytograms of apoptosis in HT 29 cells incubated for 24 hours in the absence (A) or presence of 200 (B), 500 (C) and 800 µg/ml (D) ginger.

treatment with β-carotene. β-carotene also increased apoptosis through the downregulation of this protein.

In addition to regulation of the cell cycle, apoptosis plays an important role in the maintenance of tissue homeostasis whereby damaged cells are removed since impaired apoptosis contributes to development of cancer (Taraphdar et al., 2001). Compound that can elicit apoptosis is a good chemopreventive agent. In our study, we have shown that ginger at increasing concentration was not only able to inhibit DNA synthesis but also induced apoptosis especially at higher concentration and the percentage of cells that underwent apoptosis, increased dose dependently for both cells. Our observation of apoptosis in mutant p53- expressing HT 29 is similar to the study by Park et al. (2006) who found that [6]-gingerol induced apoptotic death in pancreatic cells. The percentage of late apoptotic cells was low compared to early apoptotic cells as detected by annexin V staining, suggesting that apoptosis occurred rather slow in colon cancer cells after treatment (Huang and Pardee, 1999). This is consistent with the observation by other researchers whereby colon cancer cells (HCT 116 and HT 29 cells) did not undergo apoptosis rapidly (Goldwasser et al., 1996). Apoptosis that occurred could be due to the down-regulation of NF-κB. Expression of several NF-κB regulated genes including Bcl-2, Bcl-XL,

cIAP, surviving, TRAF1 and TRAF2 have been reported to function primarily by blocking the apoptosis pathway (Aggarwal, 2004). Other than that, ginger also could induced apoptosis through mitochondrial pathway involving caspase-8, BID cleavage, cytochrome *c* release and caspase-3 activation like curcumin (Shishodia et al., 2005).

Conclusion

In this from our study, we have demonstrated that ginger extract inhibited the proliferation and induced apoptosis in HCT 116 and HT 29 colon cancer cells. The results suggest that ginger stimulates apoptosis through GoG_1 cell cycle arrest. Future our study will may thus be further investigate the possible use of ginger extract as a new alternative chemotherapeutic agent for human colon cancer.

Competing interests

To our knowledge, previous studies have shown the chemopreventive effect of *Z. officinale* in other cancer cells but less report evidence on colon cancer cells. Thus, our study represent among the first to show the mechanism of anticancer effect of *Z. officinale* in colon cancer cells HCT 116 and HT 29.

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