Sains Malaysiana 51(1)(2022): 217-237
http://doi.org/10.17576/jsm-2022-5101-18

Zingiber officinale and *Piper betle* Extracts Enhanced the Chemopreventive Effect against Colon Cancer Cells by Targeting Caspase-Mediated Apoptosis

(Ekstrak *Zingiber officinale* dan *Piper betle* Meningkatkan Kesan Kemohalang terhadap Sel Kanser Kolon dengan Menyasarkan Apoptosis Diperantara-Kaspase)

YASMIN ANUM MOHD YUSOF, SHAILAH ABDULLAH, NUR FATIN NABILAH MOHD SAHARDI, WAN ZURINAH WAN NGAH & SUZANA MAKPOL*

ABSTRACT

Food regimens and herbs have been a target of scientific research in treating cancer. This study aimed to elucidate the effect of Zingiber officinale *(ginger) and* Piper betle *(PB) alone or in combination on the colorectal cancer cell lines HCT116 and HT29. The chemopreventive effect of these extracts was determined by performing cell viability assay, cell cycle analysis, apoptosis analysis, and caspase 3 and 8 activity assays in HCT116 and p53-deficient HT29 cells. Ginger and PB extracts inhibited the proliferation of both cancer cell lines dose-dependently, but the combined extracts inhibited the growth of cancer cells synergistically with an IC_{50} PB plus ginger < IC_{50} PB < IC_{50} ginger. Similarly, the effect of the combined extracts on apoptosis of both cancer cell lines was higher than that of each individual extract. Consistent with the enhancement of caspase activity, both extracts increased the expression of BAX protein, while Bcl-2 protein was decreased. Flow cytometry analysis indicated that the combined extracts arrested a higher percentage of colon cancer cells at the G_0/G_1 phase with a concomitant decrease in cells in the S phase, indicating that the combined extracts inhibited human colon cancer cell growth by cell cycle arrest at the G_0/G_1 phase. The inhibitory effect of these combined extracts on cellular proliferation and the induction of apoptosis was synergistic, showing a significant combination index (CI) value < 1.0. The combined extracts of ginger and PB may serve as a potential chemopreventive agent against colon cancer through the induction of caspase-mediated apoptosis.*

Keywords: Apoptosis; cell cycle; colorectal cancer; Piper betle; Zingiber officinale

ABSTRAK

Industri makanan dan herba telah menjadi salah satu tarikan dalam kajian saintifik untuk merawat kanser. Objektif kajian ini dijalankan adalah untuk menentukan kesan Zingiber officinale *(halia) dan* Piper betle *(PB) secara individu atau gabungan terhadap titisan sel kanser kolorektum, HCT116 dan HT29. Kesan kemohalang kedua-dua ekstrak ini ditentukan melalui asai kebolehidupan, analisis kitaran sel, analisis apoptosis, asai aktiviti kaspase 3 dan 8 terhadap sel HCT116 dan sel HT29 yang kekurangan-p53. Ekstrak halia dan PB telah menghalang proliferasi untuk kedua-dua jenis sel kanser mengikut kepekatan dos, akan tetapi gabungan kedua-dua ekstrak telah menghalang pertumbuhan sel secara sinergistik dengan IC_{50} gabungan PB dan halia < IC_{50} PB < IC_{50} halia. Kesan apoptosis terhadap sel kanser bagi rawatan gabungan kedua-dua ekstrak juga lebih tinggi berbanding rawatan secara individu. Selari dengan peningkatan aktiviti kaspase, kedua-dua ekstrak telah meningkatkan ekspresi protein BAX dan menurunkan pengekspresan protein Bcl-2. Analisis aliran sitometri menunjukkan bahawa rawatan gabungan kedua-dua ekstrak telah menyebabkan peningkatan peratusan yang tinggi terhadap kitaran sel kanser kolon pada fasa G_0/G_1 dan pada masa yang sama menyebabkan penurunan peratusan sel pada fasa S, membuktikan bahawa gabungan kedua-dua ekstrak telah menghalang pertumbuhan sel kanser pada fasa G_0/G_1. Kesan perencatan gabungan kedua-dua ekstrak terhadap proliferasi sel dan induksi apoptosis adalah secara sinergistik dengan nilai indeks gabungan (CI) < 1.0. Oleh yang demikian, gabungan ekstrak halia dan PB mungkin mempunyai potensi sebagai agen kemohalang bagi melawan kanser kolon dengan cara menyasarkan apoptosis-diperantara kaspase.*

Kata kunci: Apoptosis; kanser kolorektal; kitaran sel; Piper betle; Zingiber officinale

Introduction

According to the World Health Organization GLOBOCAN Database, colorectal cancer is the third most commonly diagnosed cancer in men and the second most commonly diagnosed cancer in women worldwide, with 1,148,515 million new cases and almost 935,173 deaths in 2020. It is expected that the annual number of cancer cases will slowly increase over the next 2 decades (Sung et al. 2020).

Colon cancer occurs due to the transformation of normal colon epithelium into adenomatous polyps and then ultimately into invasive cancer. It involves mutations of the APC, K-ras and p53 genes, resulting in dysregulation of cell cycle progression, evasion of apoptosis, and the induction of genetic instability (Hanif et al. 1997; Lee et al. 2009; Mohd Yusof 2016; Sakamoto et al. 1991). Thus, inhibiting the growth and increasing the apoptosis of tumour cells are effective strategies for preventing cancer formation.

Among the measures and approaches for preventing cancer, modifying lifestyles and dietary regimens to include natural products with chemopreventive properties are promising (Sakamoto et al. 1991). Since nature has provided many effective anticancer agents, plant-derived drug research has made significant progress in anticancer therapies. Curcumin, an active component of turmeric, inhibits the growth of human colon cancer cells independent of cyclooxygenase-2 (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.

Plants of the ginger (*Zingiber officinale*, Zingiberaceae) family are one of the most heavily consumed dietary supplements around the world (Butt & Sultan 2011; Surh 1999). Its extract and major pungent components, such as 6-gingerol, 6-shogaol, and zerumbone, have been shown to exhibit a variety of biological activities ranging from antioxidant, anti-inflammatory, anti-proliferation, and inducer of apoptotic proteins in *in vitro* and animal cancer models (Butt & Sultan 2011; Mohd Yusof 2016). We have shown that ginger extract reduced the elevated expression of the inflammatory mediators NF-kB and TNF-α in liver cancer-induced rats (Habib et al. 2008). In addition, ginger induced apoptosis in rat liver cancer cells via upregulation of the expression of the proapoptotic protein caspase-8 and downregulation of the expression of the antiapoptotic protein Bcl-2 (Yusof et al. 2009). Exposure of Jurkat human T cell leukaemia cells to the 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), while β-elemene, an anticancer drug extracted from the ginger plant, triggered apoptosis in non-small cell lung cancer cells through mitochondrial release of the cytochrome *c*-mediated apoptotic pathway (Wang et al. 2005).

Piper betle (PB) leaves, a shade-loving, perennial evergreen climber of tropical origin, known in Malaysia as 'sireh', have a strong pungent aromatic flavour and are widely used as masticators in Asia. Some of the active compounds isolated from leaves and other parts of *Piper betle* are hydroxychavicol, hydroxychavicol acetate, allypyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A, and piperol B (Kumar et al. 2010). Studies in recent decades have associated its extract and active compounds with antiproliferative activity towards nasopharyngeal epidermoid carcinoma cells (Fathilah et al. 2010) and breast cancer cells T47D (Widowati et al. 2011). Other beneficial health effects of PB include antidiabetic, cardioprotective, anti-inflammatory, immunomodulatory, antiulcer, hepatoprotective and anti-infective effects (Shukla & Singh 2007).

Synergy achieved through the combination of herbs is an important tool in traditional Chinese medicine for the treatment of certain ailments, including cancer (Chung et al. 2004). Combining Pan Asian medicines and vitamins (PAM+V) with conventional therapy, compared with conventional therapy alone, reduced the risk of death from colon cancer in stage I by 95%, stage II by 64%, stage III by 29%, and stage IV by 75%. This study confirmed that combining PAM+V with conventional therapy improved survival compared with conventional therapy alone (Mcculloch et al. 2011). Wee et al. (2015) showed that the combination of gelam honey and ginger may serve as a potential therapy in the treatment of colorectal cancer through inhibition of the mTOR and Wnt/β catenin signalling pathways and induction of the apoptosis pathway.

Combining the knowledge of the anticancer properties of both ginger and PB extracts, we envisaged that there is a potential synergistic effect of these two dietary herbs as chemopreventive agents against colorectal cancer cell lines. Thus, this study aimed to elucidate the effect of ginger and PB alone or in combination on the colorectal cancer cell lines HCT116 and HT29 by focusing on cell viability, the cell cycle profile and apoptosis.

Materials and Methods

Ginger Extract

Zingiber officinale (ginger) extract was a gift from Professor Dr. Nor Azian Murad from Universiti Teknologi Malaysia. It was prepared by ethanol extraction according to the following sequence of pretreatment: peeling, slicing, washing of the 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 (1):

$$\% \text{ Moisture loss} = \frac{\text{Initial weight} - \text{Current sample weight}}{\text{Initial weight}} \times 100\% \quad (1)$$

The dried ginger was fibrous and tough; therefore, grinding of the samples was done manually by pounding using a 'mortar and pestle'. The pounded samples were then shredded manually. The ground ginger was dried in ethanol (1 L) prior to extraction using a rotary evaporator 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%). The refractive index reading of pure oleoresin is 1.5100.

Piper betle (PB) Extract

Piper betle (PB) leaf extract was obtained by aqueous extraction. The PB leaves (Ethno Resources Company, Kg. Melayu, Sungai. Buloh, Selangor, Malaysia) were washed and dried in an oven at 25 °C. The stalks were removed and kept for other purposes while the leaves were blended for 10 min. The slurry was then mixed in double distilled water (ddH_2O) at a proportion of 1:10 at 60 °C for 1 h. To obtain the aqueous extract, the cooled mixture was filtered through Whatman filter paper (UK). The filtrates were then freeze-dried with a FreeZone 12 litre Freeze Dry System Model 35XL (Missouri, USA).

Cell Culture and Treatment

HCT116 and HT29 cell lines were obtained from the American Type Culture Collection (Rockville, MD USA). Both cells lines are derived from human colorectal cancers that arose in men. Both cell lines were cultured in RPMI 1640 medium (Flowlab, Australia) supplemented with 10% foetal calf serum (FSC) (PAA Laboratories GmbH, Austria) and 100 U/mL penicillin and streptomycin (Thermo Scientific, USA) at 37 °C in 5% CO_2.

HT29 cells overproduce the p53 tumour antigen, and when cultured form well-differentiated adenocarcinoma consistent with colon cancer primary, grade I. HCT116 cells have a mutation in codon 13 of the KRAS proto-oncogene, with epithelial morphology and they are suitable for tumorigenesis research.

The rates of cell proliferation, apoptosis and the cell cycle were evaluated when the cells reached 70% confluence. The extracts of PB and ginger and their combination were added to the cell lines after overnight incubation.

Cell Viability Assay

For the cell viability assay, 2×10^4 cells per well were plated in 100 µL of RPMI 1640 media. The HCT116 and HT29 cells were incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2 to allow for cell attachment. The extracts of PB and ginger and their combination were added to the cell cultures at various concentrations ranging from 0 to 320 µg/mL after 24 h incubation. After further 24 h incubation, MTS solution (2 mg/mL) was added to the plate and incubated for 2 h at 37 °C. The resulting MTS products were determined by measuring the absorbance at 550 nm with a Molecular Devices Corporation VersaMax Microplate Reader (California, USA). Each point represents the mean of triplicate experiments.

Cell Cycle Analysis

HCT116 and HT29 cells were seeded into a 25 cm^2 flask after 24 h of treatment with the indicated concentrations of extracts of PB and ginger and their combination for 24 h. Following treatment, the 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). The stained cells were then analysed for DNA content by Becton Dickinson FACSCalibur™ Flow Cytometer (Ontario, Canada) flow cytometry. The percentages of cells in each phase were calculated using Cell Modfit software (Ontario, Canada).

Annexin V-FITC Staining

Apoptosis was detected with a Beckton Dickinson Annexin V-FITC kit (Ontario, Canada). The cells were collected at the indicated times, washed with ice-cold PBS and centrifuged; the cells were harvested by trypsinization, and any cells floating in the medium were

included. The cell pellet was resuspended in ice-cold binding buffer, and Annexin V-FITC and propidium iodide (PI) solution were added. The cells were incubated for 15 min in the dark followed by flow cytometry analysis using a Becton Dickinson FACSCalibur™ Flow Cytometer (Ontario, Canada).

ACTIVE CASPASE-3 ACTIVITY ASSAY

After 24 h of treatment with the extracts of ginger and PB and their combination (ginger: 200, 500 and 800 µg/mL; PB: 200, 450 and 800 µg/mL; combination: 50, 100 and 200 µg/mL), the cells were harvested by trypsinization and then washed twice with cold 1X PBS. The cells were then resuspended in BD Cytofix/Cytoperm solution and placed on ice for 20 min before a quick spin to isolate the pellet. The pellet was then washed twice with BD Perm/Wash buffer, resuspended in anti-caspase-3 antibody and incubated for 30 min at room temperature. After washing with BD Perm/Wash buffer, the pellet was resuspended in BD Perm/Wash buffer and analysed by a Becton Dickinson FACSCalibur™ Flow Cytometer (Ontario, Canada).

CASPASE-8 ACTIVITY ASSAY

After 24 h of treatment with the extracts of ginger and PB and their combination (ginger: 200, 500 and 800 µg/mL; PB: 200, 450 and 800 µg/mL; combination: 50, 100 and 200 µg/mL), the cells were harvested by trypsinization and then resuspended in 1X PBS. Then, 300 µL of the suspension was aliquoted into an Eppendorf tube, 1 µL of FITC-IETD-FMK was added, and the mixture was incubated in a 5% CO_2 incubator at 37 °C for 0.5-1 h. The cells were then centrifuged at 3,000 rpm for 5 min, and the supernatant was removed. The cells were resuspended in 0.5 mL of wash buffer and centrifuged at 3,000 rpm for 5 min. Finally, the cells were resuspended in 300 µL of wash buffer and placed on ice. The cells were then analysed by a Becton Dickinson FACSCalibur™ Flow Cytometer (Ontario, Canada) using the FL-1 channel.

TRAIL-INDUCED APOPTOSIS

To assess the effects of ginger and PB on TRAIL-mediated apoptosis, the cells were plated as described above and allowed to adhere overnight. TRAIL (25 µM), ginger and PB at the indicated concentrations (ginger: 200, 500 and 800 µg/mL; PB: 200, 450 and 800 µg/mL; combination: 50, 100 and 200 µg/mL) were added to the wells and incubated for 24 h.

WESTERN BLOTTING ANALYSIS

HCT116 and HT29 cells were cultured under standard culture conditions at a density of 1×10^6 cells/100 mm dish and then treated with either ginger, PB or their combination at concentrations of 0, 50, 100 and 200 µg/mL for 24 h. After treatment, the cells were harvested, washed with PBS, and lysed with RIPA buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Cell lysates were cleared by centrifugation, and the protein concentration in the lysates was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). For western blot analysis, the protein samples were separated on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The nonspecific sites on the membrane were blocked by incubation using blocking buffer (Tris-buffered saline containing 0.2% Tween-20 and 3% nonfat dried milk, pH 7.6). The membrane was incubated with the corresponding primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Antibody-bound proteins were detected using an enhanced chemiluminescent protein (ECL) detection system (Amersham Biosciences, Little Chalfont, UK).

HERB INTERACTION VALUES ANALYSIS

The interactions of two different herbs can be defined by the following conditions (Cassileth & Lucarelli 2003):

– Additivity: $dA/DA + dB/DB = 1$

– Antagonism: $dA/DA + dB/B > 1$

– Synergism: $dA/DA + dB/DB < 1$

where DA is the IC_{50} of herb A by itself, dA is the IC_{50} of the herb component in the fixed ratio combination, DB is the IC_{50} of herb B by itself, and dB is the IC_{50} of the herb component in the fixed ratio combination.

STATISTICAL ANALYSIS

The experiments were repeated at least 3 times, and the results are expressed as the mean ± SD. Statistical evaluation was performed using ANOVA (SPSS software version 20.0), where $p < 0.05$ was considered significant.

Results and Discussion

There has been substantial interest in the identification of chemopreventive agents in recent decades for 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 (Lee & Surh 1998). Cancer chemoprevention is defined as the utilization of chemically active compounds to reverse, suppress and prevent the progression of disease from preinvasive cancer to frank malignancy (Sporn et al. 1976). Numerous diet-derived compound agents are considered promising and they are being evaluated clinically as chemopreventive agents for major cancer targets, including breast, prostate, colon and lung cancers (Kelloff et al. 2000).

The antiproliferative effects of the ginger and PB extracts alone and in combination are shown in Figure 1. Ginger extract inhibited the growth of HCT116 and HT29 cells by 50% at 496±34.2 µg/mL and 455±18.6 µg/mL, respectively (Figure 1(A)), while PB inhibited the growth of HCT116 and HT29 cells by 50% at 400±15.29 µg/mL and 450± 14.56 µg/mL, respectively (Figure 1(B)). However, in combination, the ginger and PB extracts (50, 100 and 200 µg/mL) significantly inhibited the proliferation of both colon cancer cell lines in a synergistic manner by reaching the IC_{50} for HCT116 cells at 80 ± 12.3 µg/mL and the IC_{50} for HT29 cells at 100 ± 2.01 µg/mL of the combination extract (Figure 1(C)).

The findings of this study demonstrated that at low concentrations, a combination of dietary ginger *Zingiber officinale* and herb *Piper betle* was able to exert a synergistic antiproliferative effect on the growth of colon cancer cells, HT116 and HT29. Ginger is not only widely used as a dietary condiment but has also been extensively utilized as a traditional Chinese medicine (Mann 2011). Gingerols (that is, 6-gingerol, 8-gingerol and zingerone) and [2]-paradol have been identified as the main active ingredients of ginger and they are responsible for its antioxidant activity and its characteristic pungent taste (Nagasawa et al. 2002; Ngah & Yusof 2007; Yoshimi et al. 1992). The antioxidative capacity of ginger has been associated with the ability of ginger to inhibit carcinogenesis by reducing oxidative stress and inducing apoptosis (Katiyar et al. 1996; Shukla & Singh 2007). Bode et al. (2001) reported that [2]-paradol exerted its primary inhibitory effect on cell transformation through the induction of apoptosis. [6]-Paradol and other structurally related derivatives induced apoptosis in an oral squamous carcinoma cell line in a dose-dependent manner through a caspase-3-dependent mechanism (Keum et al. 2002). Ginger has been shown to reduce the formation of preneoplastic nodules in hepatocarcinogenesis-induced rats and inhibit the growth of HepG2 hepatoma cells (Ngah & Yusof 2007; Yusof et al. 2009), gastrointestinal (Yoshimi et al. 1992) and breast cancer cells (Nagasawa et al. 2002). Ginger was also found to possess antitumour potential, as determined by inhibition of phorbol ester-induced Epstein-Barr virus (EBV) activation in Raji cells (Koshimizu et al. 1988). The present study provides evidence that ginger extract was able to inhibit the growth of colon cancer cells and arrest the cells at the G0/G1 stage. A higher concentration of ginger also arrested the cells at the G2/M stage. Our findings suggest that ginger extract may act as a potent growth inhibitory compound in human colon adenocarcinoma cells and supports its chemopreventive potential in colon cancer cells.

A well-known herb in Asia, *Piper betle,* has been shown to possess antioxidant, immunomodulatory, anti-inflammatory, and antiproliferative effects. Numerous studies have documented these health benefits of *Piper betle*, which ultimately resulted in many cancer studies, including the prevention of skin cancer, specifically in a mouse model, where the tumours were significantly reduced in size (Shah et al. 2016). Although *Piper betle* was found to be associated with oral cancer, this was a case of correlation and not causation. The herb is commonly consumed within certain cultures, particularly Asiatic cultures, in combination with tobacco, areca nut, and slaked lime. All of these are known carcinogens. However, it was proven that *Piper betle* does not contain any mutagenic or carcinogenic elements (Kudva et al. 2018).

Moreover, our study showed that HCT116 cells were much more sensitive to combination treatment with an IC_{50} that was much lower (80 µg/mL) than HT29 cells (100 µg/mL). The synergistic cytotoxic effect could result from the active components of ginger and *Piper betle*, gingerol, and chavicol. Both compounds are phenolic compounds with high antioxidant activity. It has been reported that gingerol inhibits the growth of HCT116 human colon colorectal (Yoshimi et al. 1992) and liver HepG2 cancer cells (Bode et al. 2001). Chavicol, on the other hand, has been found to inhibit the growth of PaCa-2 and PANC-1 pancreatic cancer cells (Majumdar & Subramanian 2019) and AW13516 and AW8507 human oral cancer cells (Atiya et al. 2017).

In this study, we also found that the mechanism of inhibition of colon cancer cells by the combination

extract involved interference in both cell cycle progression and apoptosis. Figures 2(A)-2(F) show the percentages of HCT116 and HT29 cells in each phase of the cell cycle following 24 h treatment with combination doses of ginger and PB extract (at 50, 100, and 200 µg/mL). Treatment with various concentrations of ginger extract significantly increased the percentage of HCT116 cells in G_0/G_1 phase and decreased the percentage of cells in S and G2/M phase ($p<0.0.5$) (Figure 2(A)). A similar increase in the percentage of HT29 cells in G_0/G_1 phase and decreased S phase cells ($p<0.05$) were observed after treatment with various concentrations of ginger extract (Figure 2(B)). Treatment with various concentrations of PB extract increased the percentage of HCT116 cells in G_0/G_1 phase and decreased the number of cells in S and G_2/M phase ($p<0.05$) (Figure 2(C)). For HT29 cells, treatment with 450 µg/mL PB extract significantly increased the percentage of cells in G_0/G_1 ($p<0.05$) and decreased the percentage of S phase cells ($p<0.05$) (Figure 2(D)). However, at 600 µg/mL, the PB extract decreased the percentage of G_0/G_1 phase cells and increased the percentage of S phase cells ($p<0.05$). Combined treatment with ginger and PB extract significantly increased the percentage of HCT116 cells in the G_0/G_1 phase and decreased the percentage of S and G2/M phase cells ($p<0.05$) (Figure 2(E)). For HT29 cells, treatment with the combination of ginger and PB extracts at all concentrations caused a significant increase in the G_0/G_1 phase cells and significantly decreased the S phase cells ($p<0.05$) (Figure 2(F)).

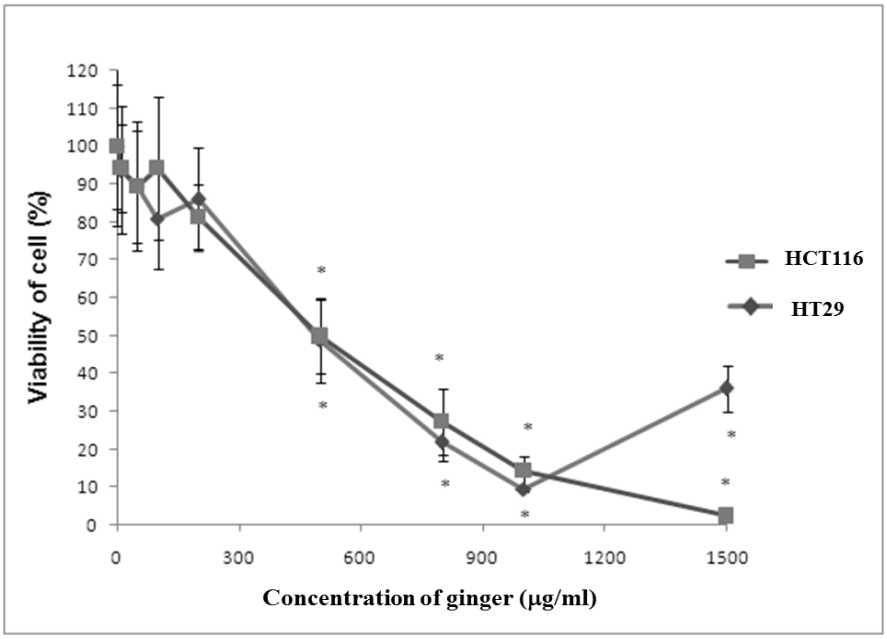
In addition, treatment with various concentrations of ginger extract significantly increased the percentage of apoptotic cells in both HCT116 and HT29 cells ($p<0.05$) (Figure 3(A)). A similar increase was observed in HT29 cells treated with various concentrations of PB extract ($p<0.05$) (Figure 3(B)). However, for HCT116 cells, only PB at a concentration of 800 µg/mL significantly increased the percentage of apoptotic cells ($p<0.05$). For HCT116 cells, combined treatment with ginger and PB extracts at various concentrations significantly increased the percentage of apoptotic cells ($p<0.05$) (Figure 3(C)). However, for HT29 cells treated with the combination of ginger and PB extracts, a significant increase in the percentage of apoptotic cells was observed at concentrations of 100 and 200 µg/mL ($p<0.05$).

Moreover, caspase-3 expression was significantly increased in both HCT116 and HT29 cells treated with various concentrations of ginger extract ($p<0.05$) (Figure 4(A)). A similar increase in caspase-3 expression was observed in HT29 cells treated with various concentrations of PB extract ($p<0.05$) (Figure 4(B)). Both HCT116 and HT29 cells showed a significant increase in caspase-3 expression when treated with the combination of ginger and PB extracts at various concentrations ($p<0.05$) (Figure 4(C)).

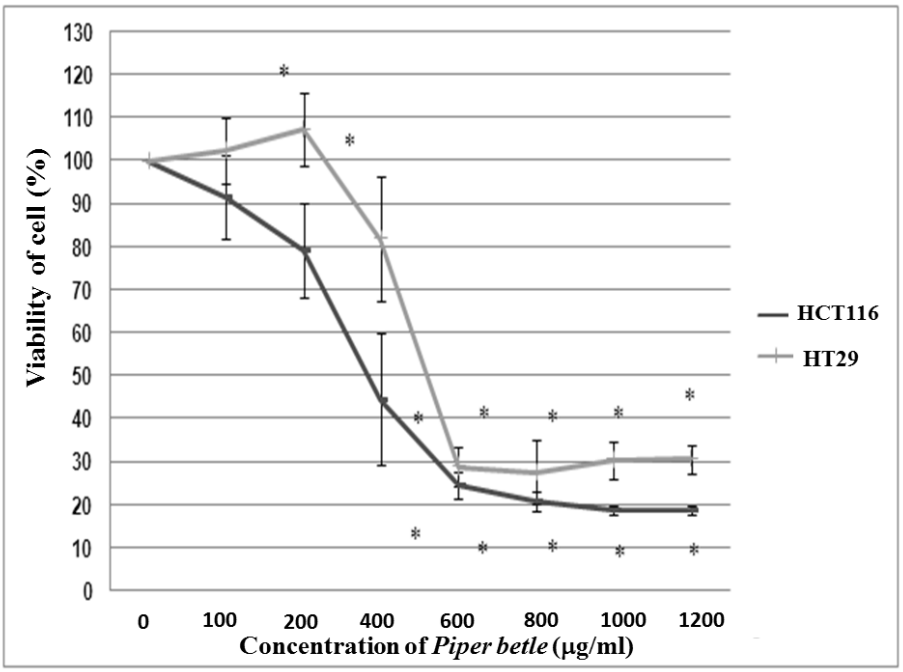
The expression of caspase-8 was significantly increased in both HCT116 and HT29 cells treated with various concentrations of ginger extract ($p<0.05$) (Figure 5(A)). A similar increase in caspase-8 expression was observed in HCT119 cells treated with various concentrations of PB extract ($p<0.05$) (Figure 5(B)), while caspase-8 was only increased in HT29 cells treated with 800 µg/mL PB ($p<0.05$). The combination of ginger and PB extracts at various concentrations increased the expression of caspase-8 in HT29 cells, but only at the highest concentration did it cause a significant increase in caspase-8 activity in HCT119 cells ($p<0.05$) (Figure 5(C)).

No significant change was observed in the expression of BAX protein in either HCT116 or HT29 cells treated with either ginger or PB extracts alone or in combination (Figure 6(A)). The expression of Bcl-2 was significantly decreased in HT29 cells treated with ginger alone or ginger in combination with PB ($p<0.05$) (Figure 6(B)). A similar decrease in Bcl-2, however, was not observed in HT119 cells treated with either ginger or PB.

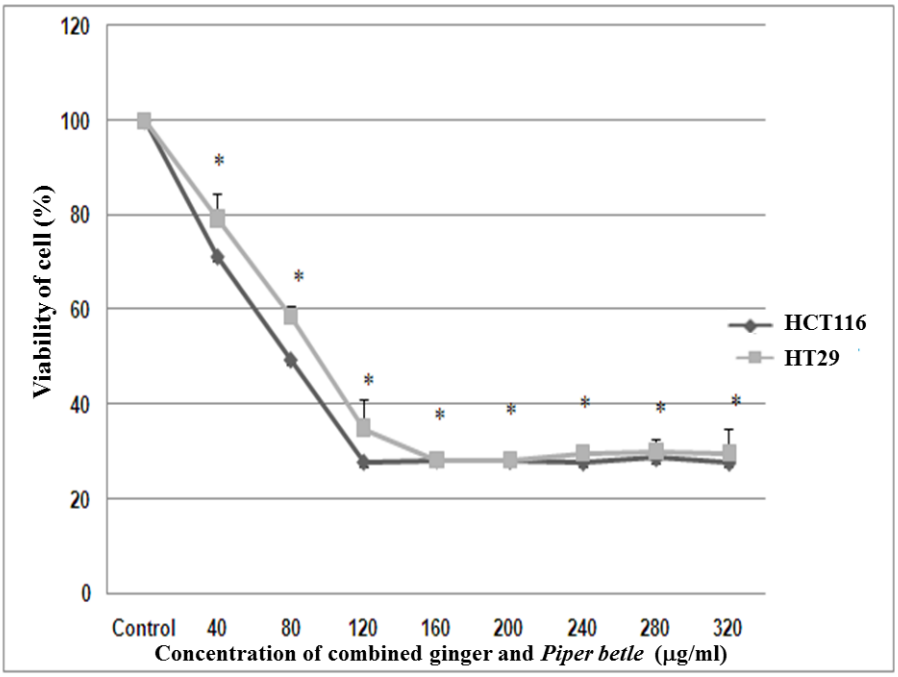
The expression of TP53 protein was significantly increased in HCT119 cells treated with ginger but it was decreased when treated with PB and the combination of ginger plus PB ($p<0.05$) (Figure 6(C)). HT29 cells, however, did not express any TP53 in either untreated control or ginger- or PB-treated cells. This could be due to their overexpression of tumour antigen p53 and not the normal p53 antigen we analysed. Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a potent stimulator of apoptosis, and tumour cells are significantly more sensitive to TRAIL-induced apoptosis than normal cells. We observed that the combination of ginger and PB treatment caused a significant reduction in the rate of TRAIL-induced apoptosis in both HT116 and HT29 cells; however, ginger treatment alone significantly increased the rate of TRAIL-induced apoptosis in HT29 cells ($p<0.05$) (Figure 6(D)). This shows that TRAIL induced significant amounts of apoptosis in combination with ginger extract in HT29 colon cancer cells compared to its combination with PB.


Viability of cell (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
0
300
600
900
1200
1500
Concentration of ginger (µg/ml)
HCT116
HT29

(A)

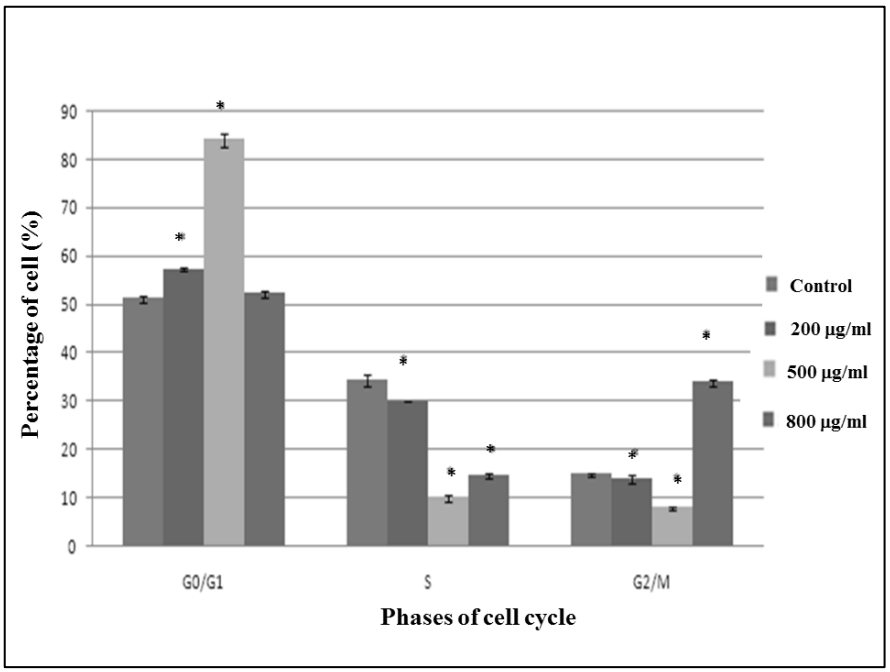

Viability of cell (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
100
200
400
600
800
1000
1200
Concentration of *Piper betle* (µg/ml)
HCT116
HT29

(B)

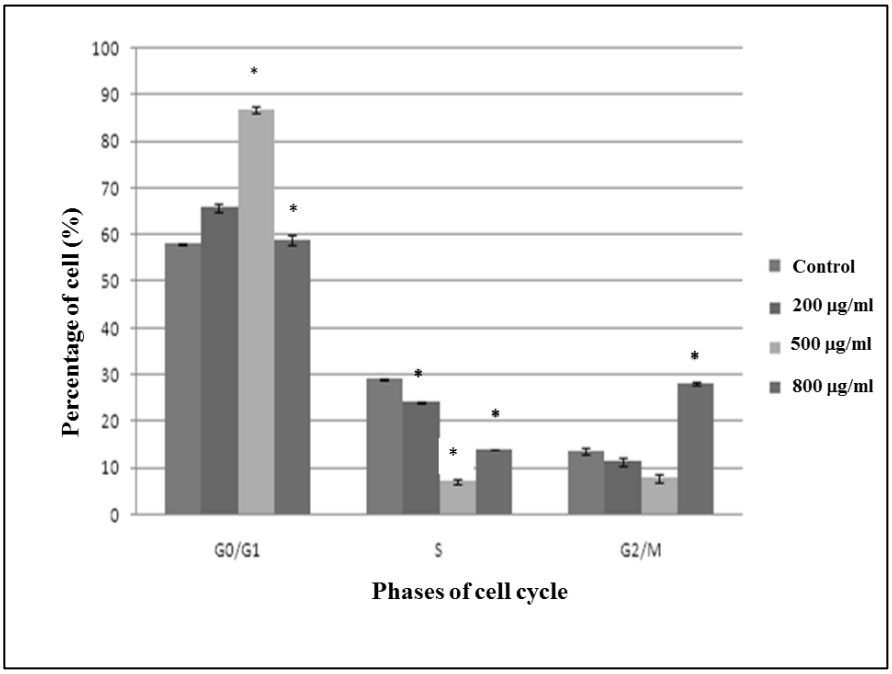


(C)

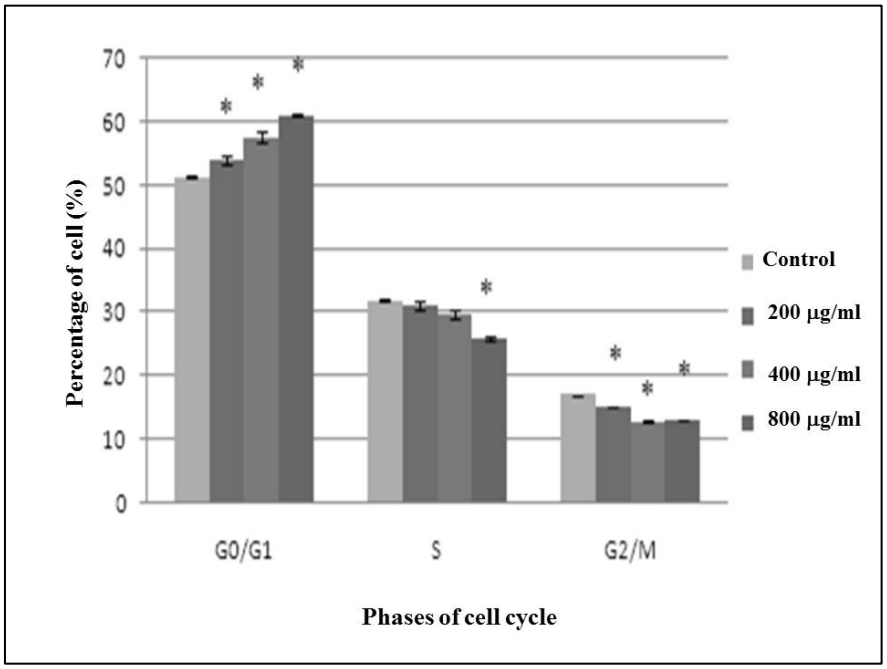
FIGURE 1. Cell viability analysis of colon cancer cells HCT116 and HT29. Effect of ginger (A), *Piper betle* (B), and combination of ginger and *Piper betle* (C) on the viability of HT29 and HCT116 colon cancer cells. Data are presented as the means ± SD, n=3. *p<0.05 significantly different compared to control



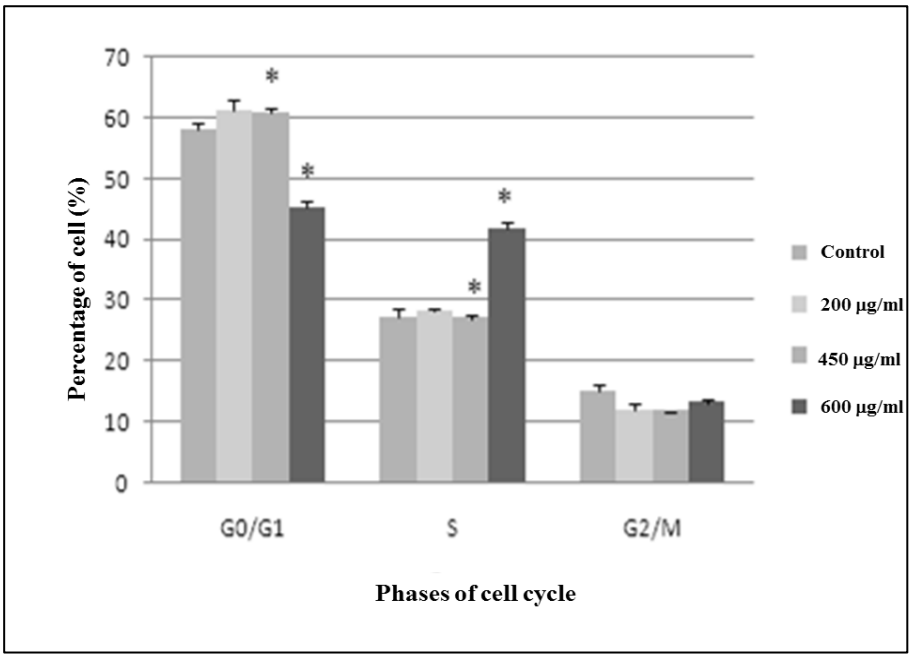
(A)


100
90
80
70
60
50
40
30
20
10
0
Percentage of cell (%)
G0/G1
S
G2/M
Phases of cell cycle
Control
200 µg/ml
500 µg/ml
800 µg/ml

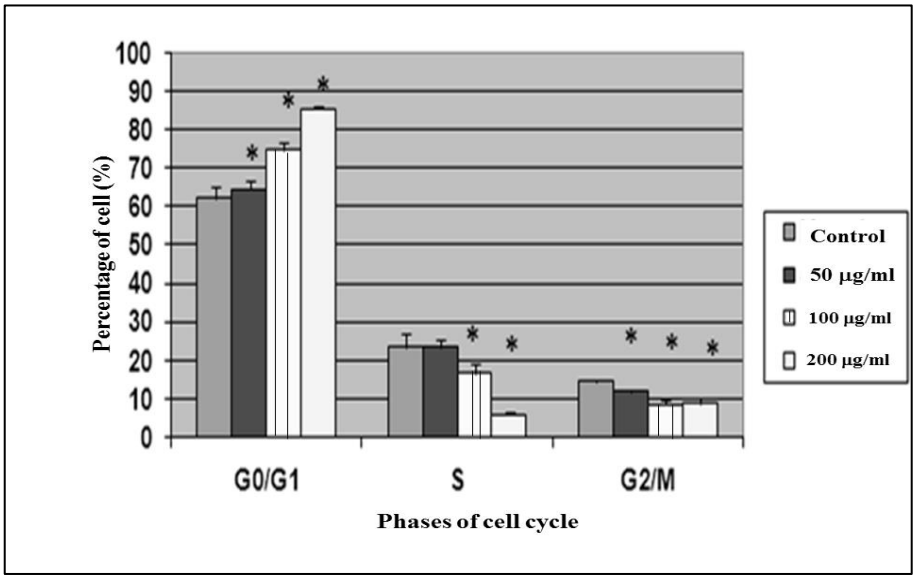
(B)


70
60
50
40
30
20
10
0
Percentage of cell (%)
G0/G1
S
G2/M
Phases of cell cycle
Control
200 µg/ml
400 µg/ml
800 µg/ml

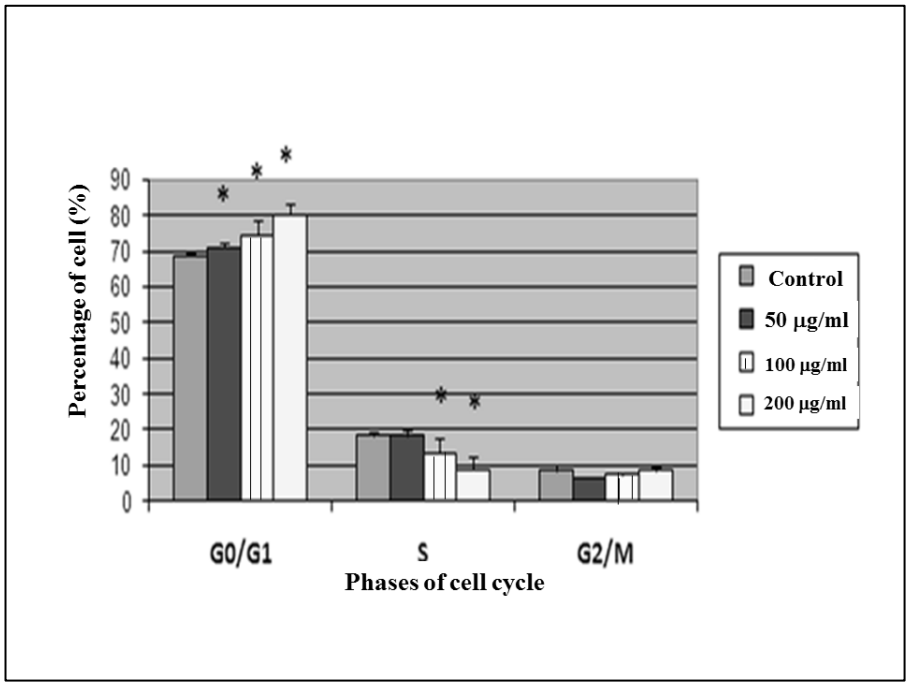
(C)


70
60
50
40
30
20
10
0
Percentage of cell (%)
G0/G1
S
G2/M
Phases of cell cycle
Control
200 µg/ml
450 µg/ml
600 µg/ml

(D)

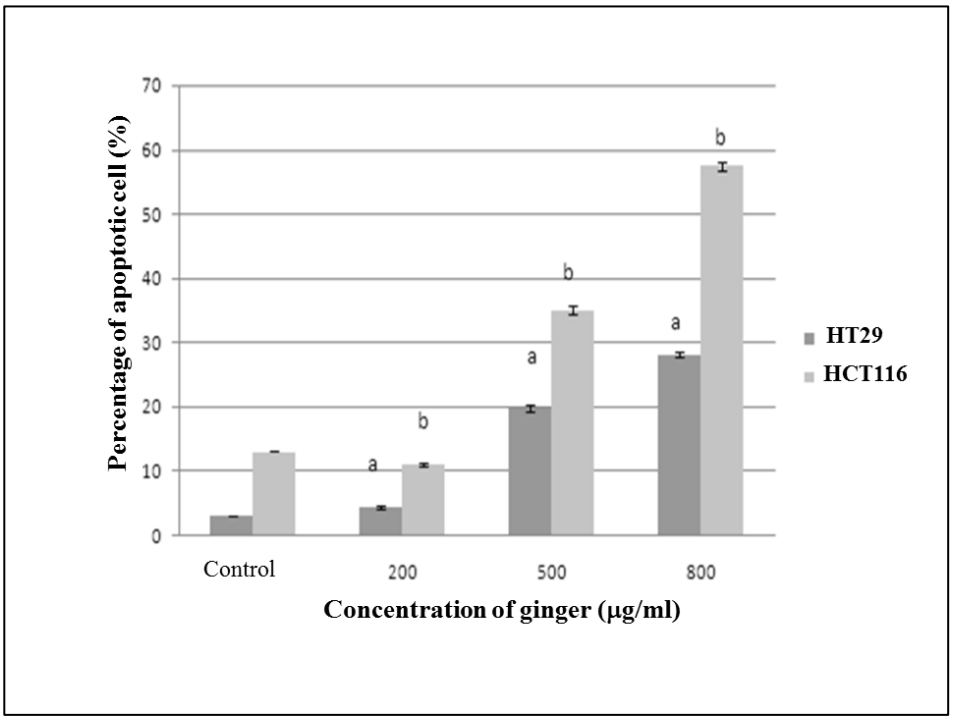

100
90
80
70
60
50
40
30
20
10
0
Percentage of cell (%)
G0/G1
S
G2/M
Phases of cell cycle
Control
50 µg/ml
100 µg/ml
200 µg/ml

(E)

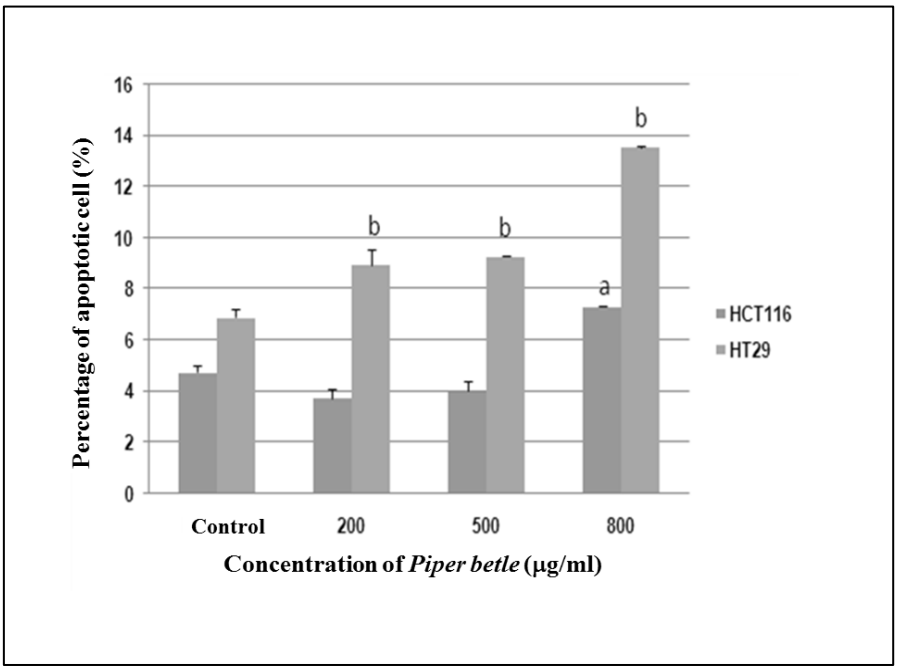


(F)

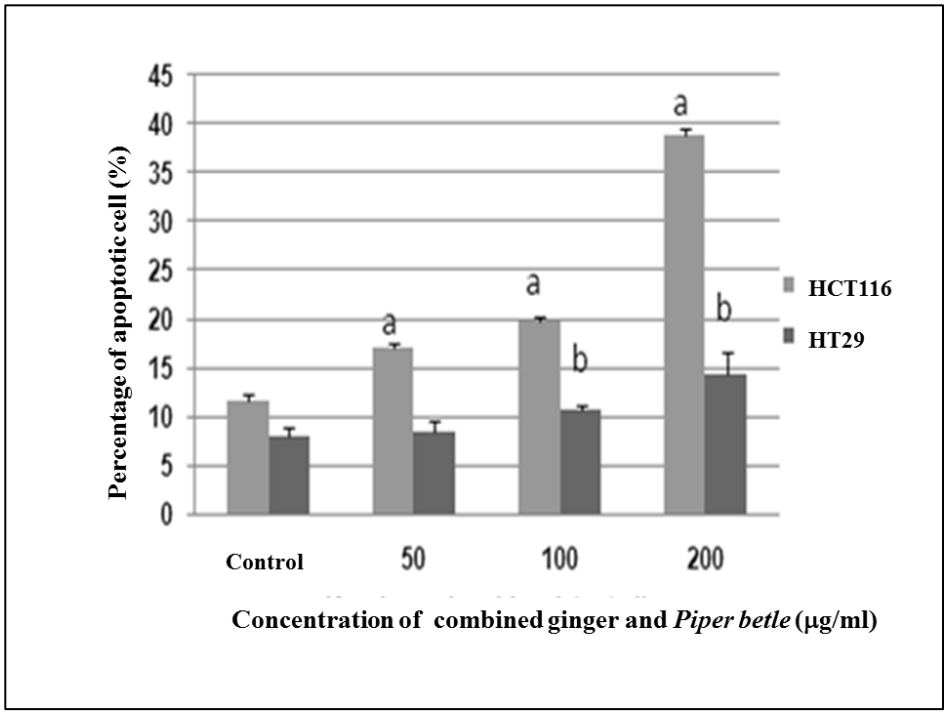
FIGURE 2. Cell cycle profile of colon cancer cells HCT116 and HT29. Effect of ginger on cell cycle of HCT116 (A), HT29 (B); effect of *Piper betle* on cell cycle profile of HCT116 (C), HT29 (D); and effect of combination of ginger and *Piper betle* on cell cycle profile of HCT116 (E), HT29 (F). Data are presented as the means ± SD, n=3. *p<0.05 significantly different compared to untreated control



(A)

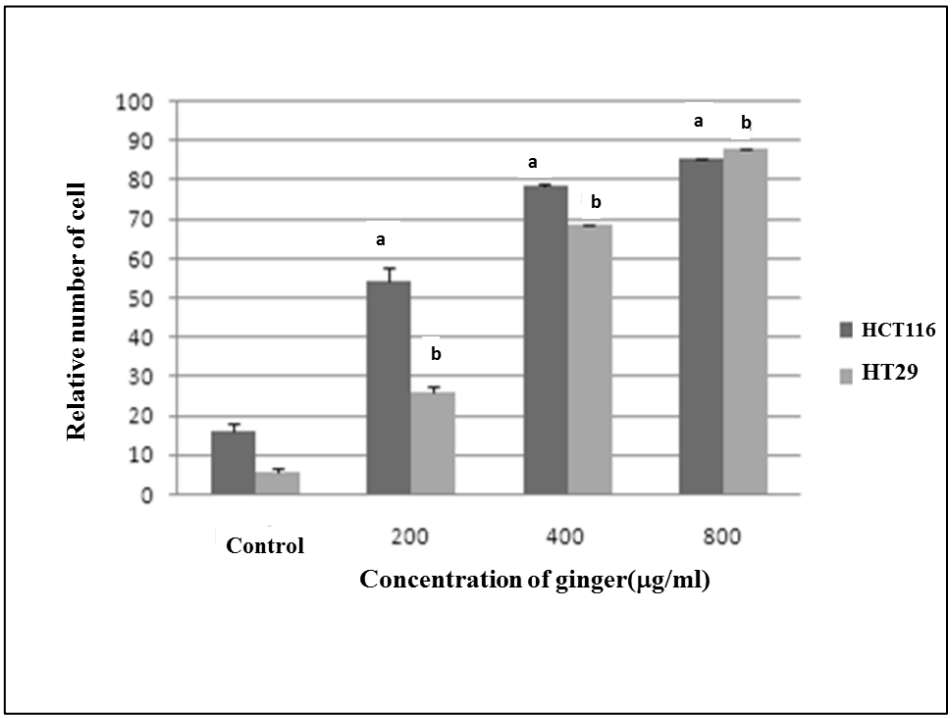


(B)

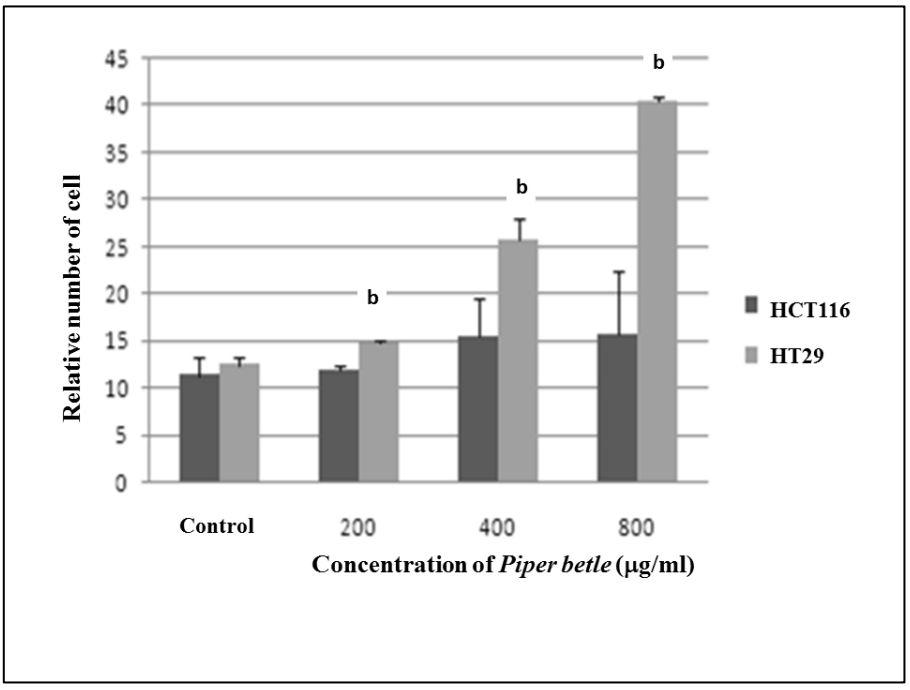


(C)

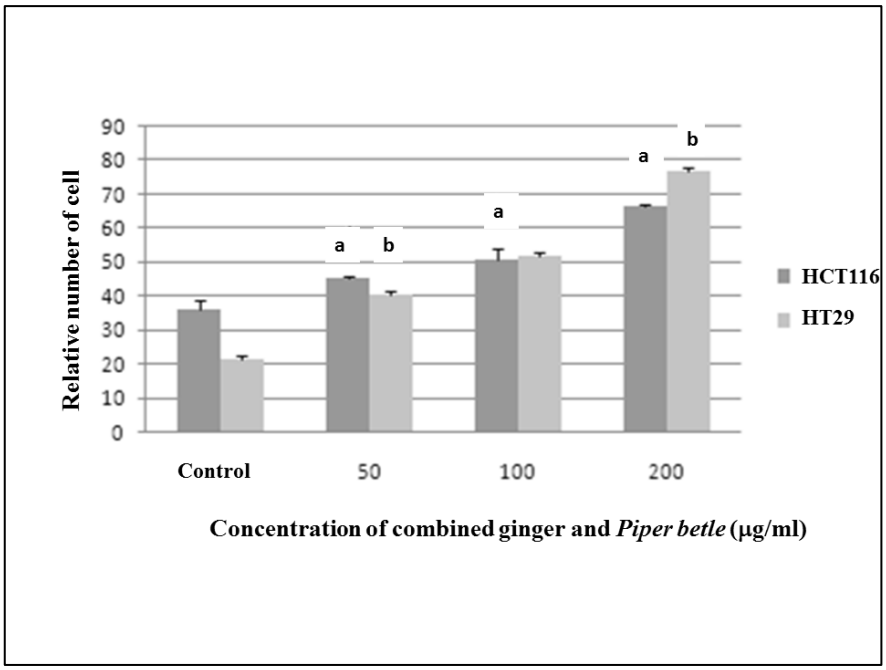
FIGURE 3. Apoptotic changes detected by Annexin V-FITC in colon cancer cells HCT116 and HT29. Effect of ginger (A), *Piper betle* (B), and combination of ginger and *Piper betle* (C) on the percentage of apoptotic cells of HCT116 and HT29. Data are presented as the means ± SD, n=3. [a]$p<0.05$ significantly different compared to HCT116 control, [b]$p<0.05$ significantly different compared to HT29 control


Relative number of cell
100
90
80
70
60
50
40
30
20
10
0
a
b
a
b
a
b
Control
200
400
800
Concentration of ginger(μg/ml)
HCT116
HT29

(A)

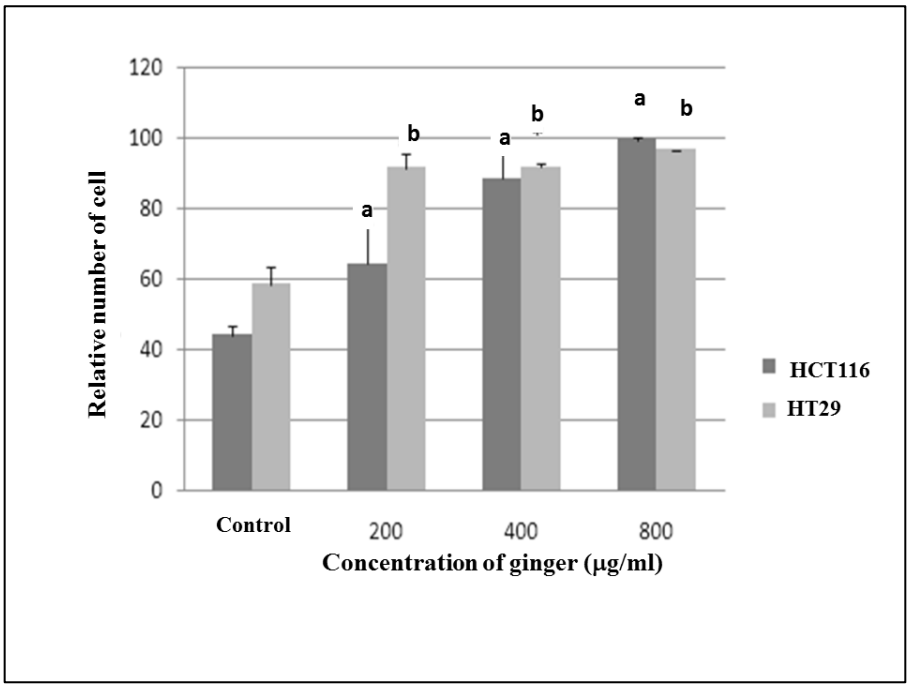

Relative number of cell
45
40
35
30
25
20
15
10
5
0
b
b
b
Control
200
400
800
Concentration of *Piper betle* (μg/ml)
HCT116
HT29

(B)

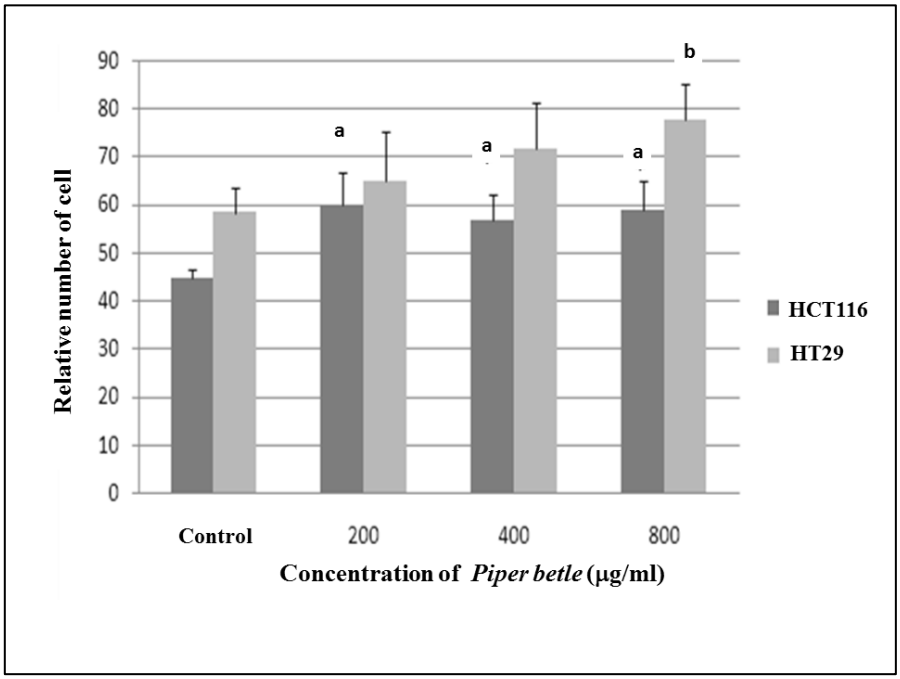


(C)

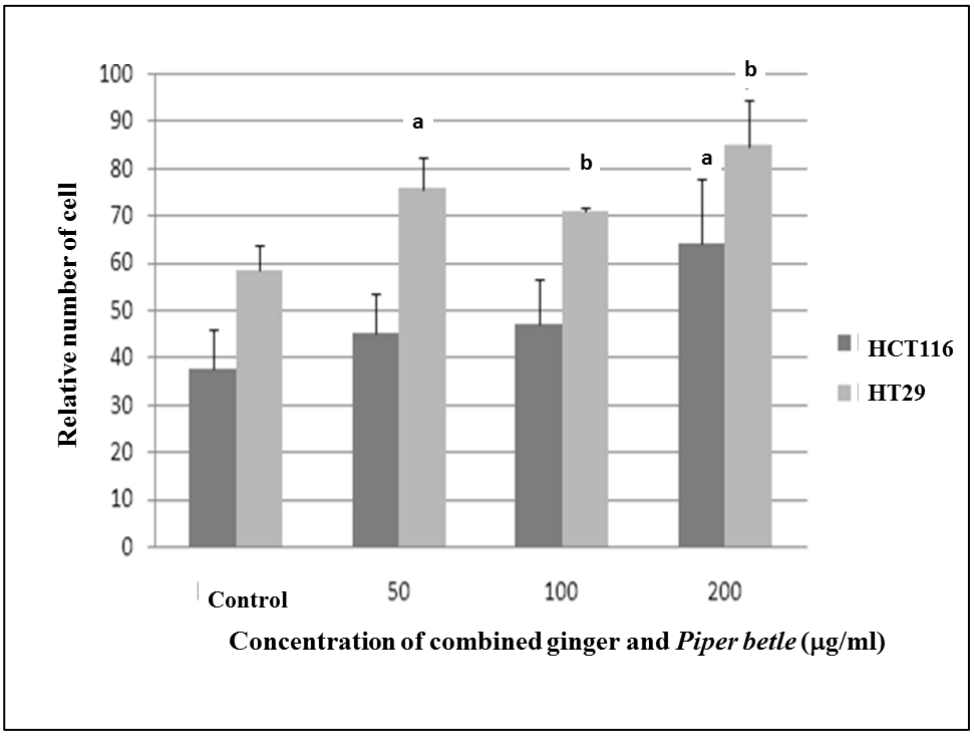
FIGURE 4. Expression of caspase-3 in colon cancer cells HCT116 and HT29. Effect of ginger (A), *Piper betle* (B), and combination of ginger and *Piper betle* (C) on the expression of caspase-3 in HCT116 and HT29. Data are presented as the means ± SD, n=3. [a]p<0.05 significantly different compared to HCT116 control, [b]p<0.05 significantly different compared to HT29 control



(A)

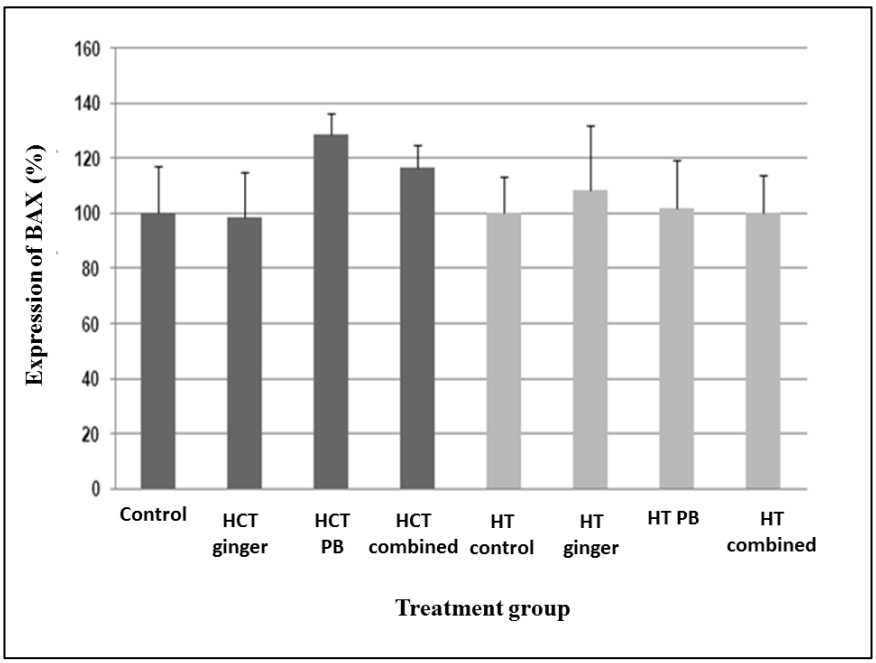


(B)

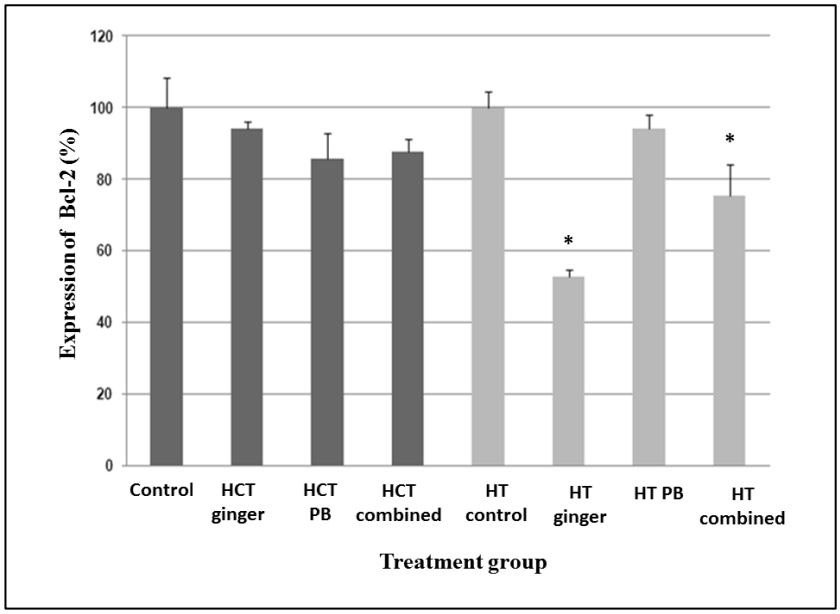


(C)

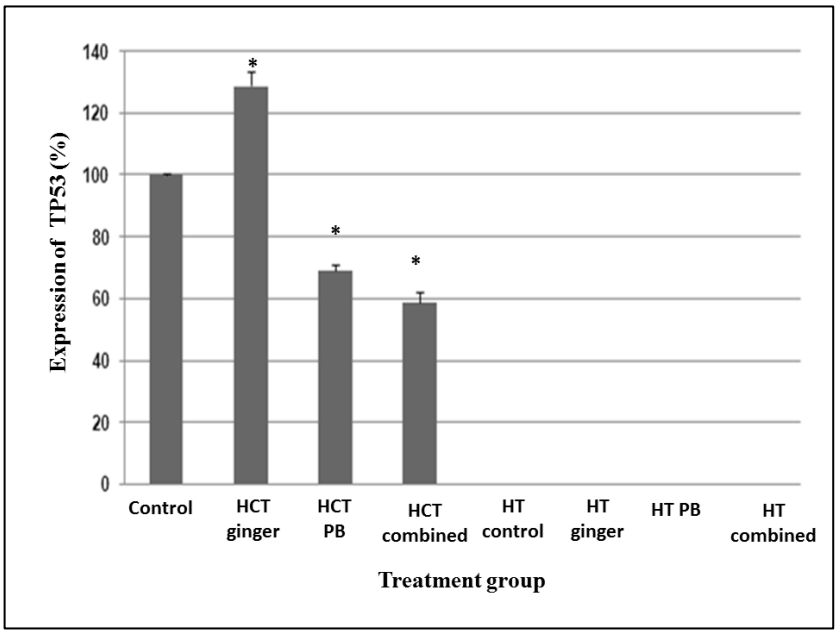
FIGURE 5. Expression of caspase-8 in colon cancer cells HCT116 and HT29. Effect of ginger (A), *Piper betle* (B), and combination of ginger and *Piper betle* (C) on the expression of caspase-8 in HCT116 and HT29. Data are presented as the means ± SD, n=3. [a]$p<0.05$ significantly different compared to HCT116 control, [b]$p<0.05$ significantly different compared to HT29 control


160
140
120
100
80
60
40
20
0
Expression of BAX (%)
Control
HCT ginger
HCT PB
HCT combined
HT control
HT ginger
HT PB
HT combined
Treatment group

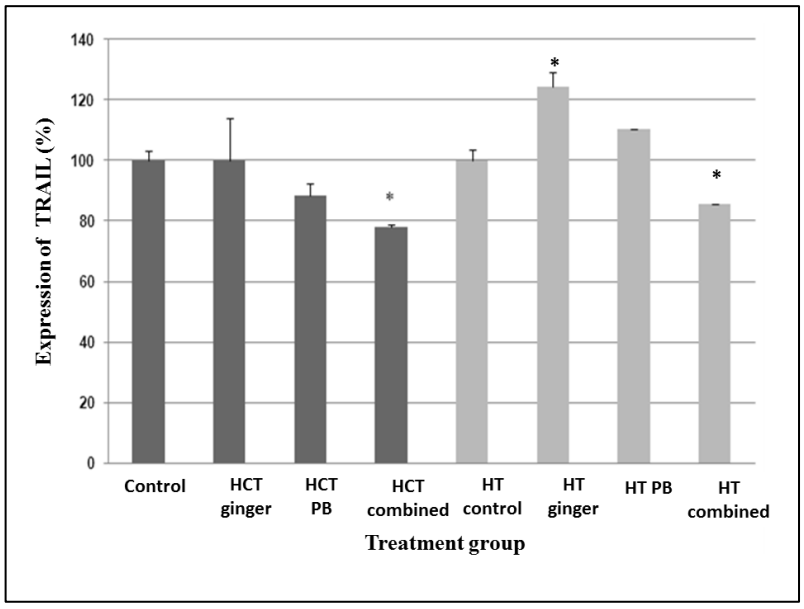
(A)


120
100
80
60
40
20
0
Expression of Bcl-2 (%)
*
*
Control
HCT ginger
HCT PB
HCT combined
HT control
HT ginger
HT PB
HT combined
Treatment group

(B)



(C)



(D)

FIGURE 6. Expression of pro- and anti-apoptotic proteins in colon cancer cells HCT116 and HT29. Effect of ginger, *Piper betle*, and combination of ginger and *Piper betle* on the expression of BAX (A), Bcl-2 (B),TP53 (C) and TRAIL-induced apoptosis (D) in HCT116 and HT29. Data are presented as the means ± SD, n=3. $^{*}p<0.05$ significantly different compared to control

From these results, ginger and PB were able to block cell cycle progression in G0/G1 phase and apoptotic death in a dose-dependent manner in both cell lines. Dysregulation of cell cycle progression driven by the activation of growth-stimulating oncogenes is one of the primary characteristics of the formation of cancer cells. Cell cycle progression is tightly controlled by the regulation of the expression and activity of cyclin/cyclin-dependent kinase (CDK) complexes (Taraphdar et al. 2001). Dysregulation of cell cycle checkpoints and overexpression of growth-promoting cell cycle factors such as cyclin D1 and cyclin-dependent kinase (CDK) are associated with tumorigenesis (Diehl 2002). Several dietary agents, including 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 the cell cycle at G_0/G_1 phase for both colon cancer cell lines at concentrations below the IC_{50}. The cell cycle arrest observed could be due to the inhibitory effect of ginger on cyclin-dependent kinases and the activation of cell cycle checkpoints. Palozza et al. (2002) showed that arrest of the cell cycle and accumulation of cells in G_2/M phase in colon cancer cells were followed by decreased expression of cyclin A, a protein known to regulate cdc2 kinase activity in G_2/M phase after treatment with β-carotene. β-Carotene was shown to increase apoptosis through the downregulation of cdc2 kinase. In this study, we found that more cells were arrested at the G_0/G_1 phase after treatment with the combination extract compared to treatment with ginger and PB alone.

In addition to the 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 the development of cancer (Taraphdar et al. 2001). Compounds that can elicit apoptosis are good chemopreventive agents. In our study, we showed that combination treatment at increasing concentrations was not only able to inhibit DNA synthesis but also induced apoptosis, especially at higher concentrations, and the percentage of cells that underwent apoptosis increased dose-dependently for both cancer cell lines. Our observation of apoptosis in mutant p53-expressing HT 29 cells is similar to the findings reported by Park et al. (2006) and Md Yusof et al. (2019), who found that [6]-gingerol induced apoptotic death in pancreatic cells and colorectal cancer 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 slowly in colon cancer cells after treatment (Huang & Pardee 1999). This is consistent with the observation by other researchers that colon cancer cells (HCT116 and HT29 cells) did not undergo apoptosis rapidly (Goldwasser et al. 1996). Our findings showed that the apoptotic pathway is caspase-dependent, as indicated by a significant increase in caspase 3 and 8 levels in both colon cancer cell lines after treatment with ginger and PB, especially with the combination extracts.

Other studies have reported the role of the extrinsic apoptotic pathway, as shown by Park et al. (2008) and Zhang et al. (2008), who found that sorafenib and vorinostat interact with each other to kill renal, hepatocellular and pancreatic carcinoma cells. The mechanism is via activation of the CD95 extrinsic apoptotic pathway, concomitant with drug-induced reduction of c-FLIP-s expression via RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) signalling to eIF2α.

Conclusion

Our study provided more evidence that ginger and PB extracts inhibited human colon cancer cell growth by cell cycle arrest at the G_0/G_1 phase, leading to the induction of caspase-mediated apoptosis. This effect was enhanced by combination treatment with both extracts, indicating the potential chemopreventive properties of ginger and PB against colon cancer.

Acknowledgements

This work was supported by the Universiti Kebangsaan Malaysia Dana Cabaran Perdana Grant (grant number: AP-2017-009/3).

References

Atiya, A., Sinha, B.N. & Lal, U.R. 2017. Bioactive phenylpropanoid analogues from *Piper betle* L. var. birkoli leaves. *Natural Product Research* 31(22): 2604-2611.

Bode, A.M., Ma, W.Y., Surh, Y.J. & Dong, Z. 2001. Inhibition of epidermal growth factor-induced cell transformation and activator protein 1 activation by [6]-gingerol. *Cancer Research* 61(3): 850-853.

Butt, M.S. & Sultan, M.T. 2011. Ginger and its health claims: Molecular aspects. *Critical Reviews in Food Science and Nutrition* 51(5): 383-393.

Cassileth, B.R. & Lucarelli, C.D. 2003. *Herb-Drug Interactions in Oncology*. Hamilton, London: BC Decker Inc.

Chung, V.Q., Tattersall, M. & Cheung, H.A. 2004. Interactions of a herbal combination that inhibits growth of prostate cancer cells. *Cancer Chemotherapy and Pharmacology* 53(5): 384-390.

Diehl, J.A. 2002. Cycling to cancer with cyclin D1. *Cancer Biology & Theraphy* 1(3): 226-231.

Estrov, Z., Shishodia, S., Faderl, S., Harris, D., Van, Q., Kantarjian, H.M., Talpaz, M. & Aggarwal, B.B. 2003. Resveratrol blocks interleukin-1β–induced activation of the nuclear transcription factor NF-κB, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. *Blood* 102(3): 987-995.

Fathilah, A.R., Sujata, R., Norhanom, A.W. & Adenan, M.I. 2010. Antiproliferative activity of aqueous extract of *Piper betle* L. and *Psidium guajava* L. on KB and HeLa cell lines. *Journal of Medicinal Plants Research* 4(11): 987-990.

Goldwasser, F., Bae, I., Fornace, A.J. & Pommier, Y. 1996. Differential GADD45, p21C1P1/WAF1, MCL-1 and topoisomerase II gene induction and secondary DNA fragmentation after camptothecin-induced DNA damage in two mutant p53 human colon cancer cell lines. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics* 8(7-8): 317-323.

Habib, S.H.M., Makpol, S., Hamid, N.A.A., Das, S., Ngah, W.Z.W. & Yusof, Y.A.M. 2008. Ginger extract (*Zingiber officinale*) has anti-cancer and anti-inflammatory effects on ethionine-induced hepatoma rats. *Clinics* 63(6): 807-813.

Hanif, R., Qiao, L., Shiff, S.J. & Rigas, B. 1997. Curcumin, a natural plant phenolic food additive, inhibits cell proliferation and induces cell cycle changes in colon adenocarcinoma cell lines by a prostaglandin-independent pathway. *Journal of Laboratory and Clinical Medicine* 130(6): 576-584.

Huang, L. & Pardee, A.B. 1999. β-lapachone induces cell cycle arrest and apoptosis in human colon cancer cells. *Molecular Medicine* 5(11): 711-720.

Jakubikova, J., Bao, Y. & Sedlak, J. 2005. Isothiocyanates induce cell cycle arrest, apoptosis and mitochondrial potential depolarization in HL-60 and multidrug-resistant cell lines. *Anticancer Research* 25(5): 3375-3386.

Katiyar, S.K., Agarwal, R. & Mukhtar, H. 1996. Inhibition of tumor promotion in sencar mouse skin by ethanol extract of *Zingiber officinale* rhizome. *Cancer Research* 56(5): 1023-1030.

Kelloff, G.J., Crowell, J.A., Steele, V.E., Lubet, R.A., Malone, W.A., Boone, C.W., Kopelovich, L., Hawk, E.T., Lieberman, R., Lawrence, J.A. & Ali, I. 2000. Progress in cancer chemoprevention: Development of diet-derived chemopreventive agents. *The Journal of Nutrition* 130(2): 467S-471S.

Keum, Y.S., Kim, J., Lee, K.H., Park, K.K., Surh, Y.J., Lee, J.M., Lee, S.S., Yoon, J.H., Joo, S.Y., Cha, I.H. & Yook, J.I. 2002. Induction of apoptosis and caspase-3 activation by chemopreventive [6]-paradol and structurally related compounds in KB cells. *Cancer Letters* 177(1): 41-47.

Koshimizu, K., Ohigashi, H., Tokuda, H., Kondo, A. & Yamaguchi, K. 1988. Screening of edible plants against possible anti-tumor promoting activity. *Cancer Letters* 39(3): 247-257.

Kudva, A.K., Rao, S., Rao, P., Periera, R., Bhandari, G., Mathew, J.M., Ashwini, K., Pais, M.L., Swamy, M.K. & Baliga, M.S. 2018. *Piper betle* Linn. in cancer: past, present, and future. In *Anticancer Plants: Properties and Application*, edited by Akhtar, M. & Swamy, M. Singapore: Springer pp. 327- 347.

Kumar, N., Misra, P., Dube, A., Bhattacharya, S., Dikshit, M. & Ranade, S. 2010. *Piper betle* Linn. a maligned Pan-Asiatic plant with an array of pharmacological activities and prospects for drug discovery. *Current Science* 2010: 922-932.

Lee, E. & Surh, Y.J. 1998. Induction of apoptosis in hl-60 cells by pungent vanilloids,[6]-gingerol and [6]-paradol. *Cancer Letters* 134(2): 163-168.

Lee, J.H., Yang, H.S., Park, K.W., Kim, J.Y., Lee, M.K., Jeong, I.Y., Shim, K.H., Kim, Y.S., Yamada, K. & Seo, K.I. 2009. Mechanisms of thiosulfinates from *Allium tuberosum* L.-induced apoptosis in HT-29 human colon cancer cells. *Toxicology Letters* 188(2): 142-147.

Li, Y.M., Zhou, B.P., Deng, J., Pan, Y., Hay, N. & Hung, M.C. 2005. A hypoxia-independent hypoxia-inducible factor-1 activation pathway induced by phosphatidylinositol-3 kinase/Akt in HER2 overexpressing cells. *Cancer Research* 65(8): 3257-3263.

Majumdar, A.G. & Subramanian, M. 2019. Hydroxychavicol from *Piper betle* induces apoptosis, cell cycle arrest, and inhibits epithelial-mesenchymal transition in pancreatic cancer cells. *Biochemical Pharmacology* 166: 274-291.

Mann, A. 2011. Biopotency role of culinary spices and herbs and their chemical constituents in health and commonly used spices in Nigerian dishes and snacks. *African Journal of Food Science* 5(3): 111-124.

McCulloch, M., Broffman, M., Van Der Laan, M., Hubbard, A., Kushi, L., Abrams, D.I., Gao, J. & Colford Jr., J.M. 2011. Colon cancer survival with herbal medicine and vitamins combined with standard therapy in a whole-systems approach: Ten-year follow-up data analyzed with marginal structural models and propensity score methods. *Integrative Cancer Therapies* 10(3): 240-259.

Md Yusof, K., Makpol, S., Fen, L.S., Jamal, R. & Ngah, W.Z.W. 2019. Suppression of colorectal cancer cell growth by combined treatment of 6-gingerol and γ-tocotrienol via alteration of multiple signalling pathways. *Journal of Natural Medicines* 73(4): 745-760.

Miyoshi, N., Nakamura, Y., Ueda, Y., Abe, M., Ozawa, Y., Uchida, K. & Osawa, T. 2003. Dietary ginger constituents, galanals A and B, are potent apoptosis inducers in Human T lymphoma Jurkat cells. *Cancer Letters* 199(2): 113-119.

Mohd Yusof, Y.A. 2016. Gingerol and its role in chronic diseases. *Adv. Exp. Med. Biol*. 929: 177-207.

Mukhopadhyay, A., Banerjee, S., Stafford, L.J., Xia, C., Liu, M. & Aggarwal, B.B. 2002. Curcumin-induced suppression of

cell proliferation correlates with down-regulation of cyclin D1 expression and CDK4-mediated retinoblastoma protein phosphorylation. *Oncogene* 21(57): 8852-8861.

Nagasawa, H., Watanabe, K. & Inatomi, H. 2002. Effects of bitter melon (*Momordica charantia* L.) or ginger rhizome (*Zingiber offifinale* Rosc) on spontaneous mammary tumorigenesis in SHN mice. *The American Journal of Chinese Medicine* 30(02n03): 195-205.

Ngah, W.Z.W. & Yusof, Y.A.M. 2007. Antiproliferative, antioxidant and apoptosis effects of *Zingiber officinale* and 6-Gingerol on Hepg2 cells. *Asian Journal of Biochemistry* 2(6): 421-426.

Palozza, P., Serini, S., Maggiano, N., Angelini, M., Boninsegna, A., Di Nicuolo, F., Ranelletti, F.O. & Calviello, G. 2002. Induction of cell cycle arrest and apoptosis in human colon adenocarcinoma cell lines by β-carotene through down-regulation of cyclin A and Bcl-2 family proteins. *Carcinogenesis* 23(1): 11-18.

Park, M.A., Zhang, G., Martin, A.P., Hamed, H., Mitchell, C., Hylemon, P.B., Graf, M., Rahmani, M., Ryan, K., Liu, X. & Spiegel, S. 2008. Vorinostat and sorafenib increase ER stress, autophagy and apoptosis via ceramide-dependent CD95 and PERK activation. *Cancer Biology & Therapy* 7(10): 1648-1662.

Park, Y.J., Wen, J., Bang, S., Park, S.W. & Song, S.Y. 2006. [6]-Gingerol induces cell cycle arrest and cell death of mutant p53-expressing pancreatic cancer cells. *Yonsei Medical Journal* 47(5): 688-697.

Sakamoto, S., Kudo, H., Kuwa, K., Suzuki, S., Kato, T., Kawasaki, T., Nakayama, T., Kasahara, N. & Okamoto, R. 1991. Anticancer effects of a Chinese herbal medicine, juzen-taiho-to, in combination with or without 5-fluorouracil derivative on DNA-synthesizing enzymes in 1, 2-dimethylhydrazine induced colonic cancer in rats. *American Journal of Chinese Medicine* 19(03n04): 233-241.

Shah, G.A., Shah, T.I. & Telang, S. 2016. Anti-proliferative efficacy of *Piper betle* leaf extracts against B16F10 melanoma in an *in-vivo* experimental model. *World Journal of Pharmacy and Pharmaceutical Sciences* 5: 835-843.

Shukla, Y. & Singh, M. 2007. Cancer preventive properties of ginger: A brief review. *Food and Chemical Toxicology* 45(5): 683-690.

Sporn, M.B., Dunlop, N.M., Newton, D.L. & Smith, J.M. 1976. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Federation Proceedings* 35(6): 1332-1338.

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F. 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 71: 209-249. https://doi.org/10.3322/caac.21660

Surh, Y.J. 1999. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 428(1-2): 305-327.

Takagaki, N., Sowa, Y., Oki, T., Nakanishi, R., Yogosawa, S. & Sakai, T. 2005. Apigenin induces cell cycle arrest and p21/WAF1 expression in a p53-independent pathway. *International Journal of Oncology* 26(1): 185-189.

Taraphdar, A.K., Roy, M. & Bhattacharya, R.K. 2001. Natural products as inducers of apoptosis: Implication for cancer therapy and prevention. *Current Science* 2001: 1387-1396.

Tyagi, A.K., Singh, R.P., Agarwal, C., Chan, D.C. & Agarwal, R. 2002. Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth Inhibition, G2-M arrest, and apoptosis. *Clinical Cancer Research* 8(11): 3512-3519.

Wang, G., Li, X., Huang, F., Zhao, J., Ding, H., Cunningham, C., Coad, J.E., Flynn, D.C., Reed, E. & Li, Q.Q. 2005. Antitumor effect of beta-elemene is non-small-cell lung cancer cells is mediated via induction of cell cycle arrest and apoptotic cell death. *Cellular and Molecular Life Sciences* 62(7-8): 881-893.

Wee, L.H., Morad, N.A., Aan, G.J., Makpol, S., Ngah, W.Z.W. & Yusof, Y.A.M. 2015. Mechanism of chemoprevention against colon cancer cells using combined Gelam honey and Ginger extract via mTOR and Wnt/β-catenin pathways. *Asian Pacific Journal of Cancer Prevention* 16(15): 6549-6556.

Widowati, W., Mozef, T., Risdian, C., Ratnawati, H., Tjahjani, S. & Sandra, F. 2011. The comparison of antioxidative and proliferation inhibitor properties of *Piper betle* L., *Catharanthus roseus* [L] G.Don, *Dendrophtoe petandra* L., *Curcuma mangga* Val. extracts on T47D cancer cell line. *International Research Journal of Biochemistry and Bioinformatics* 1(2): 22-28.

Yoshimi, N., Wang, A., Morishita, Y., Tanaka, T., Sugie, S., Kawai, K., Yamahara, J. & Mori, H. 1992. Modifying effects of fungal and herb metabolites on azoxymethane-induced intestinal carcinogenesis in rats. *Japanese Journal of Cancer Research* 83(12): 1273-1278.

Yusof, Y., Ahmad, N., Das, S., Sulaiman, S. & Murad, N. 2009. Chemopreventive efficacy of ginger (*Zingiber officinale*) in ethionine induced rat hepatocarcinogenesis. *African Journal of Traditional, Complementary and Alternative Medicines* 6(1): 87-93.

Zhang, G., Park, M.A., Mitchell, C., Hamed, H., Rahmani, M., Martin, A.P., Curiel, D.T., Yacoub, A., Graf, M., Lee, R. & Roberts, J.D. 2008. Vorinostat and sorafenib synergistically kill tumor cells via FLIP suppression and CD95 activation. *Clinical Cancer Research* 14(17): 5385-5399.

Yasmin Anum Mohd Yusof
Faculty of Medicine and Defense Health
Universiti Pertahanan Nasional Malaysia
Kem Sungai Besi
57000 Kuala Lumpur, Federal Territory
Malaysia

Shailah Abdullah, Nur Fatin Nabilah Mohd Sahardi, Wan Zurinah Wan Ngah & Suzana Makpol*
Department of Biochemistry, Level 17 Preclinical Building
Universiti Kebangsaan Malaysia Medical Center
Jalan Yaacob Latif, Bandar Tun Razak
56000 Cheras, Kuala Lumpur, Federal Territory
Malaysia

*Corresponding author; email: suzanamakpol@ppukm.ukm.edu.my

Received: 22 April 2020
Accepted: 19 April 2021