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ORIGINAL ARTICLE

Ginger Extract (*Zingiber officinale Roscoe*) Triggers Apoptosis in Hepatocarcinogenesis Induced Rats

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ABSTRAK

Ekstrak halia telah dilaporkan oleh kumpulan kami sebelum ini yang ianya mempunyai kesan antikanser dan antioksidan dengan menurunkan beban tumor dan peroksidasi lipid pada tikus teraruh kanser. Kajian ini melihat pada pengekspresan protein pro-apoptosis kaspase-8 dan protein anti-apoptosis Bcl-2 pada tikus teraruh kanser. Tiga puluh ekor tikus Wistar dibahagi kepada lima kumpulan berdasarkan pada diet yang diberi, iaitu i) kawalan (makanan asas), ii) minyak zaitun iii) ekstrak halia (100 mg/kg berat badan) iv) diet kurang kolin + etionin dalam minuman, CDE (untuk mengaruh kanser) dan v) tikus teraruh kanser, CDE + ekstrak halia. Tikus dibunuh pada minggu ke-lapan dan tisu hepar dikeluarkan untuk pengesanan protein pro-apoptosis dan anti-apoptosis, kaspase-8 dan Bcl-2 melalui kaedah imunohistokimia. Pengamatan H&E menujukkan terdapat banyak sel oval yang mengesahkan terdapat kanser hepar pada kumpulan yang menerima diet CDE. Terdapat 91.6% (11/12) sampel dari kumpulan CDE yang menunjukkan kehadiran sel oval dan pengeksresan Bcl-2. Tetapi apabila kumpulan CDE dirawat dengan ekstrak halia, sel oval dan peng-ekspresan Bcl-2 menurun kepada 8.4% (1/12). Bagi kaspase-8 pula, 41.7% (5/12) sampel dari kumpulan CDE menunjukkan pewarnaan positif dan meningkat ke 100% (12/12) apabila dirawat dengan ekstrak halia. Penemuan dari kajian ini menunjukkan ekstrak halia mempunyai kesan antikanser dengan mengaruh apoptosis melalui peningkatan protein pro apoptosis, kaspase-8, dan penurunan protein anti-apoptosis Bcl-2.

Kata kunci: tikus teraruh kanser, ekstrak halia, apoptosis, kaspase-8, Bcl-2

ABSTRACT

Ginger extract has been reported previously by our group to exhibit anticancer and antioxidant effects by reducing tumour burden and lipid peroxidation respectively in hepatocarcinogenesis induced rats. The current study examined the expression of pro-apoptotic protein caspase-8 and anti-apoptotic protein Bcl-2 in hepatocarcinogenesis treated rats. Thirty normal male Wistar rats were divided into 5 groups based on the diet given: i) control (normal rat chow), ii) olive oil, iii) ginger extract (100mg/kg body weight), iv) choline deficient diet + ethionine, CDE (to induce liver cancer) and v) CDE+

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ginger extract. Rats were killed at week 8, and liver tissues were excised for immunohistochemical study to identify pro-apoptotic and anti-apoptotic proteins, caspase-8 and Bcl-2. The observation on H&E staining confirmed the CDE diet induced liver cancer as indicated by the presence of numerous oval cells. Identification of Bcl-2 expression showed that 91.6% (11/12) of the samples from the CDE group revealed positive staining while treatment with ginger extract however inhibited the expression with only 8.4% (1/12) samples showing positive staining for Bcl-2. As for caspase-8 protein, 41.7% (5/12) of the samples from CDE group showed positive staining, which increased to 100% (12/12) with ginger extract treatment. Our findings suggest that ginger extract has an anticancer effect by inducing apoptosis in liver cancer cells via up-regulation of the expression of pro-apoptotic protein, caspase-8 and down-regulation of the expression of anti-apoptotic protein Bcl-2.

Key words: hepatocarcinogenesis-induced rats, ginger extract, apoptosis, caspase-8, Bcl-2

INTRODUCTION

Chemoprevention is a promising strategy to prevent cancer by using natural or synthetic substance. Many studies have successfully shown the effectiveness of diets having chemopreventive effects against the growth of cancer cells. White and green tea ameliorated colon cancer by inducing apoptosis and modulation of certain genes (Santana-Rios et al. 2002, Orner et al. 2003). Curcumin in tumeric induced apoptosis by activating caspases in malignant glioma cells (Gao et al. 2005). *Zingiber officinale* or commonly called ginger is widely used for medicinal purposes since thousands of years ago in Chinese, Arabs, Romans and Indian medicine (Ross 2002). In our previous study, ginger extract was shown to reduce lipid peroxidation and tumour burden in hepatocarcinogenesis-induced rats. Other studies had reported that ginger exerted its anti-tumour properties by inhibiting proliferation and inducing apoptosis in HL-60 leukemia cells (Surh & Lee 1998) and Jurkat human T cell leukemia cells through down-regulation of anti-apoptotic protein Bcl-2 and enhancement of pro-apoptotic protein Bax (Miyoshi et al. 2003).

Cancer is caused by an imbalance in the rate of proliferation and apoptosis or cell death. Apoptosis is a form of programmed cell death characterized by morphological changes in cells executed by cysteine-aspartate proteases (caspases) and regulated by the Bcl-2 family of proteins involved in the signal transduction pathways (Coultas & Strasser 2003, Hanson *et al*. 2008). It is the preferential way of targetting and removing cancer cells. Dietary compounds that can trigger apoptosis would be a potential use in cancer chemoprevention.

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide with nearly half a million of new cases annually (Wei 2006). In Malaysia, HCC is the eleventh most common cancer among all malignant diseases (Gerard & Halimah 2003). The attributable risk factors of HCC are chronic hepatitis B and C infections, cirrhosis, exposure to aflatoxin and certain chemicals such as polyamine hydrocarbon (PAH), diethylnitrosamine (DEN) and 2-acetylaminofluoren (AAF) (Schafer & Sorrell 1999). Currently, the treatments of HCC include surgical re-

section, liver transplantation, drug and radiation therapy (Forner et al 2006). All these procedures posed a lot of risk and side effects. The search for an alternative and safe compound or chemopreventive agent would benefit patients and reduce the unwanted side effects and risk factors. Ginger is an indispensable ingredient of Asian foods and this report concerns the chemopreventive efficacy of ginger in an ethionine induced liver cancer carcinogenesis model by examining the expression of pro- and anti-apoptotic proteins, caspase-8 and Bcl-2 respectively.

MATERIALS AND METHODS

Animals, Chemicals and Treatment

Thirty male Wistar albino rats aged between 3-4 months and weighing 200-250 g were supplied by the Animal Care Unit of Universiti Kebangsaan Malaysia (UKM) Medical Center. The study was approved by the Animal Ethics of the Faculty of Medicine, UKM. Animals were kept in a polycarbonate cage provided with food and water *ad libitum*. They were maintained under standard conditions of temperature and humidity with an alternating light and dark cycle. Rats were randomized into 5 groups of 6 animals each. The first group and the second group served as the control group and were fed with normal rat chow (Gold Coin, Malaysia) and rat chow plus olive oil respectively. The latter served as a control for gavage method and the delivery of ginger. Rats in group 3 received ginger extract at 100 mg/kg body weight by gavage method. Ginger extract was prepared by ethanol extraction and kept at 4^0C. It was dissolved in olive oil and force-fed to the rats. Rats in group 4 were fed with choline deficient diet (ICN Biochemicals, USA) plus 0.1% ethionine (Sigma Chemical Co., USA) in drinking water, known as CDE. This is the model to induce the production of oval cells, which are the precursor cells of liver cancer (Akhurst et al. 2001). Rats in group 5 received ginger as in group 3 plus CDE diet. All rats were killed at 8 weeks and the liver tissues were excised after perfusion and embedded in paraffin blocks for immunohistochemical staining.

Liver perfusion and preparation of paraffin blocks

The rats were sacrificed using ether. All equipments for dissection were sterilized using 70% alcohol before use. The rats were anasthesized intraperitoneally with Zoletil 50 (0.1 ml/100g body weight), followed by heparin (25, 000 U/ml) injection to the inferior vena cava. The portal vein was then canulated using an intravenous catheter, size 16G (2.25 inches) for the perfusion procedure. The liver was then perfused in PBS, pH 7.4 for 1 minute at a 10ml/min flow rate at room temperature, followed by 1:1 ratio of 4% para-formaldehyde and 0.1% glutaraldehyde for 3 minutes. Then the liver was perfused back in PBS for another 2 minutes. A portion of the perfused liver was then immersed in 10% formalin for fixation before embedding in paraffin.

Preparation of tissue sections

The paraffin-embedded tissues were cut at 3 µm thick with a rotatory microtome (Leica, Germany). The tissue sections were placed on poly-L-Lysine (Sigma-Aldrich Co. USA) treated slides with 1:10 dilution. The slides were then dried overnight and stored at room temperature until used for staining.

Hematoxylin & eosin (H&E) staining

The sections were deparaffinized and hydrated with sequential washes in xylene and alcohol. Nuclei were stained by immersing in Mayer's hematoxylin solu-

tion (Lab Vision Corp., UK) for 8 minutes and rinsed under running tap water. The slides were then dipped in 1% acid alcohol to remove excess hematoxylin followed by immersion in 2% sodium acetate. Slides were rinsed in running water followed by eosin staining for 5 min to stain the cytoplasm. Finally, slides were dehydrated through a series of graded alcohols and mounted with dibutylpthalate xylene (DPX).

Immunohistochemistry for detection of Bcl-2 and caspase-8

Paraffin sections (3 μm thick) were cut from liver specimens. Sections were deparaffinized and rehydrated by sequential immersion in xylene, a series of alcohol concentrations (100, 95, 80 and 70%), and in running water. Slides were preincubated in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Slides were washed in Tris-HCl-buffered saline (TBS) before incubating with bovine serum albumin and biotin as a blocking step to reduce nonspecific staining. For detection of Bcl protein, slides were then immersed in target retrieval solution (pH9.9) (DAKO, U.S.A.) at 98^{o}C in a water bath for 20 minutes. For caspase-8, the target retrieval solution (pH9.9) (DAKO, U.S.A.) was heated in microwave oven for 20 minutes at 98^{o}C. Slides were left at room temperature for 20 minutes before washing in Tris-buffered saline (TBS) for 3 changes at 3 minutes each. Tissues were then incubated with the primary antibody. For evaluation of Bcl-2 expression, tissues were incubated with monoclonal mouse anti-human Bcl-2 (DAKO, Denmark) at 1:50 dilution for 30 minutes while for the evaluation of caspase-8, tissues were incubated with rabbit polyclonal to human Caspase-8 (Abcam, UK) at 1:100 dilution for 1 hour. After several washings, slides were then incubated with secondary antibody conjugated with biotin and streptavidin labeled with horseradish peroxidase (LSAB kit, DAKO, Denmark) for 30 minutes. The slides were then treated with diamino-benzidine, DAB (DAKO, U.S.A.) for 20 minutes before counterstaining with haematoxylin for visualization of antigen. TBS was used in place of the primary antibody for the negative control. Human tonsil and gastric tissues were used as positive controls for Bcl-2 and caspase-8 respectively.

Immunostaining analysis

Immunoreactivity evaluation was based on staining intensity and percentage of positive staining of Bcl-2 and caspase-8. Staining intensity was divided into 3 categories: 3+ indicates strongly positive, 2+ indicates moderately positive, and 1+ indicates slightly positive. The percentage of positive staining was determined from 1% to 100% of cells stained positively at 10 different fields observed under 400X magnification of light microscope. Twelve slides (2 slides from each rat) were prepared from a total of six rats to represent each group.

Statistical analysis

Descriptive analysis was used to compare the expression of apoptotic protein in different sample groups.

RESULTS

H&E staining for oval cells expression

The control groups (normal rat chow, normal rat chow + olive oil) and rats treated with ginger showed normal rat liver histology with hexagon shaped hepatocytes, round nucleus and distinct cytoplasm. The sinusoids were clearly visible under 400x magnifications with Kupffer cells located at the sinusoidal wall (Figure 1A). A similar morphology of

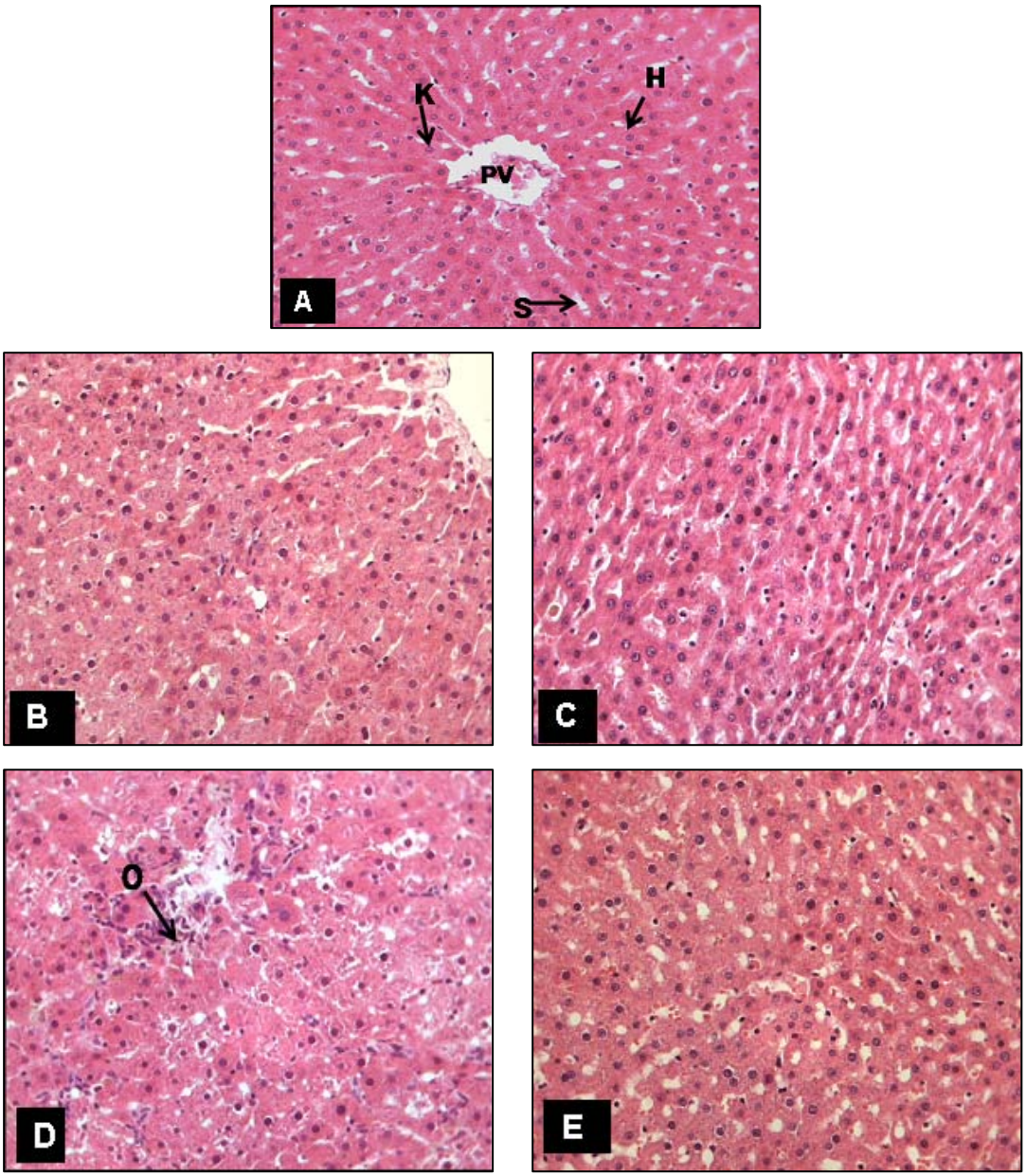

Figure 1: Effects of ginger extract on expression of oval cells. (A) H&E staining of control, rat chow alone, (B) olive oil + rat chow, (C) ginger extract, 100mg/kg body weight, (D) liver cancer induced group, CDE (arrows indicate the presence of oval cells in CDE group) and (E) CDE group treated with ginger extract. H: Hepatocyte, K: Kupffer cell, PV: Portal vein, S: Sinusoid O: Oval cell (Magnification x400).

Table 1: Effect of ginger supplementation on oval cells expression in control and liver cancer-induced group, CDE.

Group	Positive No. (%)[#]	Negative No. (%)[#]
Control week 0	0(0)	12(100)
Control week 8	0(0)	12(100)
Ginger*	0(0)	12(100)
Olive oil**	0(0)	12(100)
Cancer (CDE)***	11(91.6)	1(8.4)
CDE+ Ginger	0(0)	12(100)

[#] Indicates number of slides and percentage in parenthesis for positive or negative staining. 12 slides were prepared from each group. Two slides were obtained from each block that represent each rat of the same group.

* 100 mg/kg body weight

** Olive oil served as control for delivery of ginger by gavage method to rats

*** Choline deficient diet with 0.1% ethionine in drinking water

Table 2: Effect of ginger extract on Bcl-2 expression in control and CDE (liver cancer) groups

Group	Positive No. (%)[#]	Negative No. (%)[#]
Control week 0	0(0)	12(100)
Control week 8	0(0)	12(100)
Ginger*	0(0)	12(100)
Olive oil**	0(0)	12(100)
Cancer (CDE)***	11(91.6)	1(8.4)
CDE+ Ginger	0(0)	12(100)

[#] Indicates number of slides and percentage in parenthesis for positive or negative staining. 12 slides were prepared from each group. Two slides were obtained from each block that represent each rat of the same group.

* 100 mg/kg body weight

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liver cells was also observed in the ginger extract group (Figure 1C) and CDE group treated with ginger extract (Figure 1E). Abnormal liver cell morphology was observed in the liver-cancer induced group with irregular shaped hepatocytes and sinusoids (Figure 1D). Numerous oval-shaped cells with large nucleus and scanty cytoplasm located mainly near the periportal site were seen in the liver-cancer induced group (CDE).

Table 1 shows increased oval cell expression in CDE group (91.6% of sample) which was abrogated after treatment with ginger extract (100 mg/kg body wt).

Bcl-2 expression

Figure 2 represents patterns of Bcl-2 expression in control groups and in CDE group treated with ginger. Figure 2A shows positive staining for Bcl-2 protein in tonsil tissue.

There were no Bcl-2 expression observed in the control (and olive oil group, image not included) and ginger extract groups (Figures 2B and 2C). However, Bcl-2 expression was clearly observed in the CDE group (Figure 2D) with 91.6% of the cells positively stained (Table 2) with the following intensities: 66.6% cells with 1+ intensity and 25% cells with 2+ intensity (Table 3). Oval cells that were stained with Bcl-2 showed patchy expression in the cytoplasm with no expression observed in the nucleus (Figure 2D, 400x). Treatment of ginger inhibited the expression of Bcl-2 in the CDE group (Figure 2E).

Caspase-8 expression

Figure 3 represents patterns of caspase-8 expression in control and ginger groups and in CDE group treated with ginger. Figure 3A shows positive staining for caspase-8 in human gastric tissue. The expression of caspase-8 was observed in both CDE and CDE group treated with ginger extract (Figure 3D, 3E). No caspase-8 expression was observed in the control and ginger groups (Figures 3B, 3C). For CDE group, 41.7% of cells were stained with caspase-8 (Table 4)

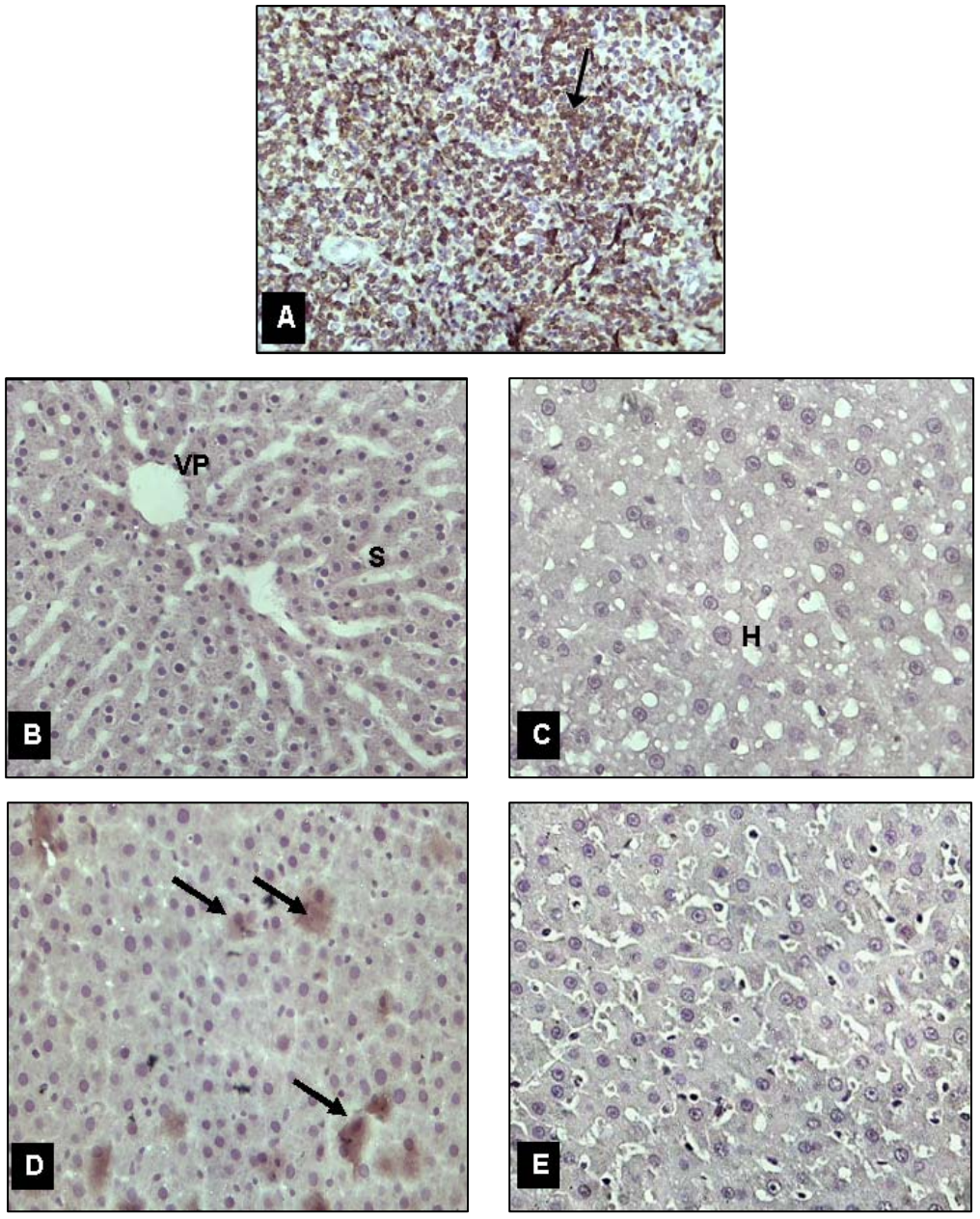

Figure 2: Effects of ginger extract (100 mg/kg body wt) on the expression of Bcl-2 in liver cancer induced group (CDE). (A) Positive immunohistochemical staining of Bcl-2 in germinal layer of human tonsil tissue, (B) Control, olive oil+ rat chow, (C) ginger extract, 100mg/kg body weight, (D) liver cancer induced group, CDE and (E) CDE group treated with ginger extract. Positive Bcl-2 expression is clearly seen in CDE group with patchy staining pattern (Magnification x400).

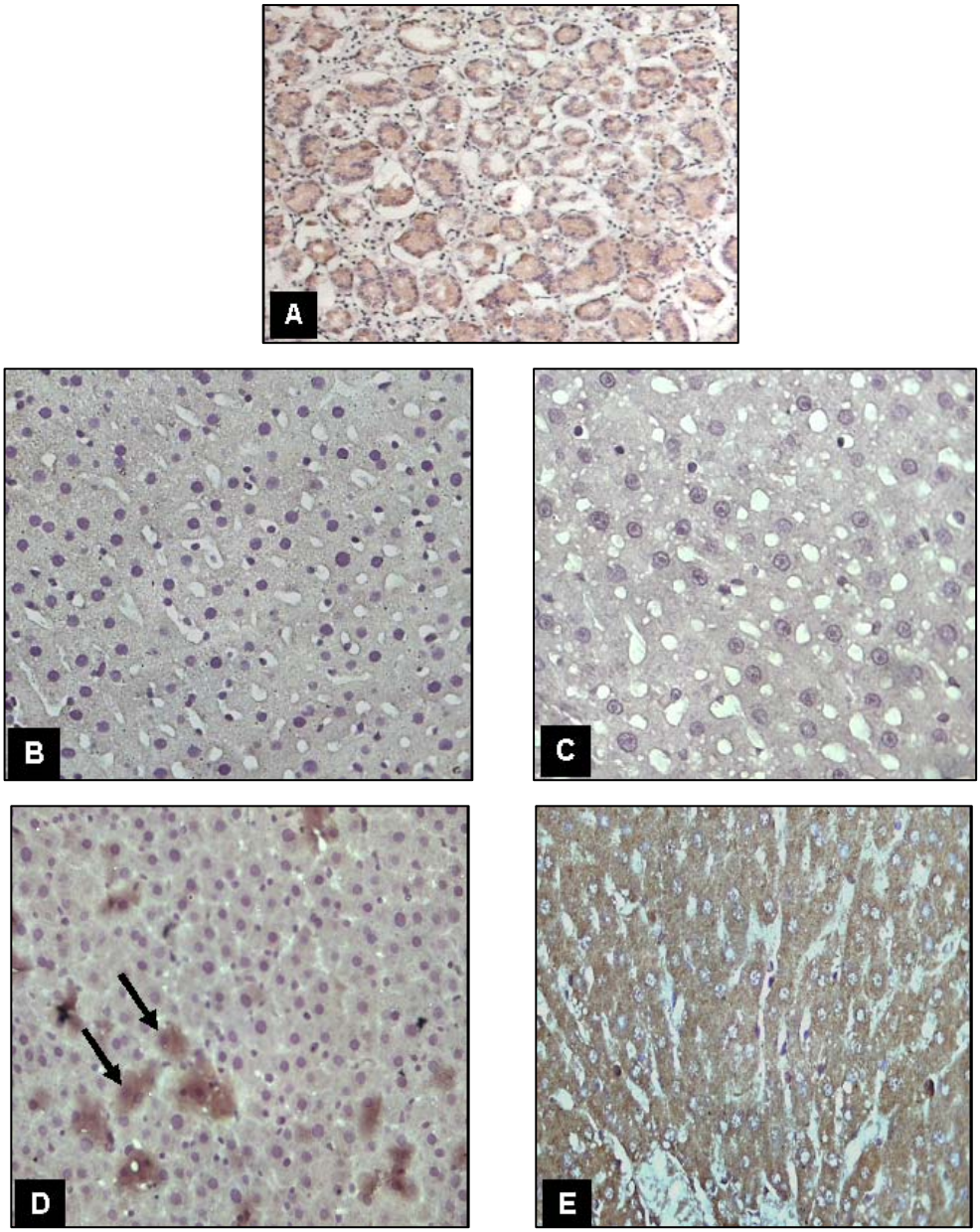

Figure 3: Effects of ginger extract on the expression of caspase-8 in CDE group. Positive immunohistochemical staining for caspase 8 in gastric gland of human gastric tissue, (B) olive oil+ rat chow, (C) ginger extract, 100mg/kg body weight, (D) liver cancer induced group, CDE, and (E) CDE group treated with ginger extract. Positive caspase-8 expression in CDE group with patchy staining (D) and homogenous staining in CDE + ginger group (E) were clearly seen. (Magnification x400).

Table 3: Intensity of Bcl-2 expression in CDE group (liver cancer) with supplementation of ginger extract

Intensity	Cancer induced group No. (%)
Positive	**11(91.6)**
3+	0(0)
2+	3(25)
1+	8(66.6)
Negative	**1(8.4)**

Staining intensity was divided into 3 categories: 3+ indicates strongly positive, 2+ indicates moderately positive, and 1+ indicates slightly positive. The percentage of positive staining was determined based on 1% to 100% of cells stained positive at 10 different fields observed under 400X magnification of light microscope.

Table 4: Effect of ginger extract on the caspase-8 expression in the control and CDE (liver cancer) groups

Group	Positive No. (%)#	Negative No. (%)#
Control week 0	0(0)	12(100)
Control week 8	0(0)	12(100)
Ginger*	0(0)	12(100)
Olive oil**	0(0)	12(100)
Cancer (CDE)***	5(41.7)	7(58.3)
CDE+ Ginger	12(100)	0(0)

# Indicates number of slides and percentage in parenthesis for positive or negative staining. 12 slides were prepared from each group. Two slides were obtained from each block that represent each rat of the same group.

* 100 mg/kg body weight

** Olive oil served as control for delivery of ginger by gavage method to rats

*** Choline deficient diet with 0.1% ethionine in drinking water

Table 5: Effect of ginger extract on the intensity of caspase-8 expression in the CDE group (liver cancer).

Intensity	CDE No. (%)	CDE+Ginger No. (%)
Positive	**5(41.7)**	**12(100)**
3+	0(0)	6(50)
2+	1(8.3)	3(25)
1+	4(33.4)	3(25)
Negative	**7(58.3)**	**0(0)**

with the following intensities: 33.4% cells with 1+ intensity and 8.3% cells with 2+ intensity (Table 5). However, for CDE group treated with ginger extract, 100% of the cells were stained positively for caspase-8 with the following intensities: 50% with 3+ intensity, 25% with 2+ intensity and 25% with 1+ intensity (Table 4). Caspase-8 was expressed in a patchy pattern in the cytoplasm of the oval cells of CDE group (Figure 3D). A homogenous staining of caspase 8 was observed in the CDE group treated with ginger extract (Figure 3E).

DISCUSSION

Most of the flavouring ingredients in Asian foods are rich in phytochemicals with medicinal properties which include turmeric, cloves, garlic, aniseed, mustard, saffron, cardamom and ginger (Sengupta et al. 2004). Some of these spice condiments have been supported by experimental models to have chemopreventive properties which have the ability to interfere with carcinogenic process. The purpose of chemopreventive agent in cancer prevention is to block or cause delay in onset of cancer, progression from precancerous lesion or recurrence after treatment (Tsuda et al. 2004).

Ginger has been used in time of immemorial for food condiments and medicinal use especially to aid digestion and treat stomach upset, diarrhoea and nausea. It was shown to have strong antioxidant properties attributed by the bioactive components such as 6-paradol, shogaol, zingerone and gingerol (Masuda et al. 1995, Aeschbach et al. 1994, Chang et al. 1994, Cao et al. 1993, Reddy & Lokesh 1992). Antioxidative capacity of ginger has been associated with the ability of ginger to inhibit carcinogenesis by reducing oxidative stress and inducing apoptosis (Shukla & Singh 2006, Katiyar et al. 1996, Manju & Nalini

2005, Rhode et al. 2007).

Our study clearly showed the efficacy of ginger having antitumour properties by inhibiting the proliferation of oval cells as evidenced by the reduced number of oval cells and the perpetuation of normal histological structure of liver tissues in liver cancer induced rats treated with ginger extract. Oval cell proliferation precedes neoplasia in many rodent models of hepatocellular carcinoma and in chronic liver disease of human studies (Ackhurst et al. 2001, Lowes et al. 1999). Prevention of this proliferative response can reduce the risk of subsequent carcinoma. This was supported by our previous findings which showed that ginger reduced oval cell proliferation and liver tumour formation in hepatocarcinogenesis induced rats (Mohd Habib et al. 2008). This property of ginger could be attributed to the presence of high phenolic compounds such as [6]-gingerol, and [6]-paradol. [6]-gingerol has been shown to suppress experimental metastases in tumour-bearing mice skin carcinogenesis probably via its anti-angiogenic activity (Kim et al. 2005) and inhibition of COX-2 expression along with suppressed NF-kB DNA binding activity (Kim et al. 2004). Since tumour promotion is closely linked to oxidative stress, a compound that exhibits antioxidant properties could act as an anticarcinogenic agent (Shukla & Singh 2006).

Some compounds present in ginger may exert cancer preventive effects by inducing apoptosis in cancerous or transformed cells. (Shukla & Singh 2006). Several studies have reported that compounds in ginger suppress proliferation of human cancer cells through induction of apoptosis accompanied by downregulation of anti-apoptotic protein Bcl-2 and enhancement of pro-apoptotic protein Bax expression (Lee & Surh 1998, Miyoshi et al. 2003). The diminished expression of anti-apoptotic Bcl-2 protein observed in CDE group treated with ginger correlated with inhibition of oval cell expression. This confirmed that ginger must have bioactive compounds to produce such chemopreventive effects. Our findings are supported by Reed et al. (1994), who have shown that Bcl-2 prevented the death of neoplastic cells via apoptosis. Vaux et al. (1988) reported that expression of Bcl-2 induced the tumour formation in mice. Our study showed patchy staining of Bcl-2 in oval cells and the results are supported by Frommel et al. (2000) who reported that Bcl-2 was only expressed in proliferative cells near the periportal site of liver (oval cells) but not in normal hepatocytes, Kupffer cells and bile duct epithelium.

Caspase-8 is an executor zymogen protein involved in apoptosis signaling pathway. Ishiguro et al (2007) proved that [6]-gingerol facilitated TRAIL-induced apoptosis by activation of caspase-3/7 activity in human gasric cancer cells. We observed caspase-8 staining in both CDE and CDE group treated with ginger, with enhanced staining observed in the latter. This observation confirmed that in cancer cells, apoptosis is a natural way of inhibiting its growth, and chemopreventive agents, having the ability of inducing apoptosis, enhance further the effect of apoptosis as seen with the effect of ginger extract.

In conclusion, our study agrees with other studies which provide substantial evidence that ginger extract are effective inhibitors of carcinogenic process exhibited by reduction of oval cells, Bcl-2 protein and induction of caspase-8 expression.

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