Effects of Zingiber officinale on Superoxide Dismutase, Glutathione Peroxidase, Catalase, Glutathione and Malondialdehyde Content in HepG2 Cell Line

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Abstract
Ginger (Zingiber officinale) is one of the most heavily consumed dietary substances in the world. Some studies have shown that ginger may possess antioxidant and antitumor properties. The objective of this study is to measure activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH) and malondialdehyde (MDA) content in human liver cancer cell line (HepG2) and normal liver cell line (WRL-68) treated with Zingiber officinale extract. HepG2 and WRL-68 cell lines were grown in Eagle Minimum Essential Medium of Earle (EMEM) supplemented with 10% fetal calf serum and incubated at 37°C with 5% CO2. Activities of SOD, GPx, CAT, and GSH and MDA contents were measured spectrophotometrically. The results of this study showed that in untreated cultures, high SOD (2 fold higher than normal) activity was found in HepG2 cell line when compared to normal cell line. There were no significant differences in GPx and CAT activities, GSH and MDA contents between the untreated liver cancer and normal cell lines. Ginger extracts at 500 µg/ml, reduced SOD activity significantly by 72.32% in HepG2 cell line when compared to untreated cell lines. Ginger extract also reduced significantly GPx activities by 77.16 %, 87.35 % and 71.05 % in HepG2 cell line at 100, 200 and 500 µg/ml respectively, when compared to untreated culture. Ginger extracts at 200 and 500 µg/ml reduced catalase activities by 41.65 and 67.43 % in HepG2 cell line when compared to untreated culture respectively. Ginger extract had no effect on glutathione and MDA contents in normal (WRL-68) and in liver cancer (HepG2) cell lines at all concentration. This study showed that Zingiber officinale extract may exert its anticancer effect by replacing the function of SOD, GPx and catalase in removing superoxide radicals and hydrogen peroxide that cause oxidative damage to cells.

Keywords: antioxidant, anticancer, Zingiber officinale, HepG2, free radicals

Introduction
Oxidative damage by oxygen free radicals such as superoxide anions are known to be one of the factors involved in the mechanisms of diseases such as cancer [1,2]. Biological specimens contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. The formation of lipid hydroperoxides by oxidative lipid damage leads to dysfunction of membrane bound receptors. One such by product of lipid peroxidation is malonaldehyde (MDA). Oxygen free radicals are created through aerobic metabolism and are mostly removed by endogenous antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) [2]. Exogenous antioxidants such as vitamin C, E and β-carotene, prevent the cascade of oxidative reactions by combining with free radicals [1,3].

Tumor cells represent an example whereby oxygen toxicity is accomplished mainly due to deficiency of scavenger enzymes [SOD, GPx, CAT] [4]. Bize et al., [5] showed that both total SOD and manganese-containing superoxide dismutase (MnSOD) activities were lower in the tumor cell homogenates compared to normal liver. However high SOD activity has been found in human leukemia cell line [6]. In malignant cells, such as L 1210, S91 melanoma and Yoshida sarcoma, Hochstein and Cohen [7] showed a marked detoxification of H2O2 by glutathione peroxidase. However, a different behaviour of the two GPx isoenzymes were observed: while selenium-GPx activity decreased consistently with increasing proliferation rate of the tumor, the non-selenium GPx enzyme did not follow this pattern [8]. CAT activity has generally been found to be low in tumor cells, while tumor cells have varying levels of GPx activity [9]. Glutathione (GSH) is an essential electron donor to GPx in the reduction of hydroperoxides. GSH works synergistically with the other cellular antioxidants to neutralize and thereby prevent or diminish oxidative stress [1,3]. A deficiency
of hepatic GSH and its antioxidant partners or an increase in toxic free radical species, may contribute to the progression of liver disease such as alcoholic liver disease, viral hepatitis and liver cancer [10].

Ginger is an excellent remedy for digestive problems, such as nausea, indigestion, intestinal infections and certain types of food poisoning [11]. Rhizome of this plant possesses a warm pungent taste and pleasant odour. Ginger rhizome contains two classes of constituents: (i) the essential oils which give the aroma, (ii) the main pungent principles: gingerols. The oil of ginger is a mixture of over 24 constituents, consisting of monoterpenes (phellandrene, camphene and borneol) and sesquiterpenes (zingiberine and hisabolene) etc [11]. Ginger oleoresin contains pharmacologically active compounds such as 6-gingerol, shogaol, zingerone, resin and phenols. It has also been shown to contain vitamin B, C and minerals such as calcium, magnesium, potassium, phosphorus and linoleic acid [12]. Ginger extract has been shown to inhibit Epstein – Barr virus activation [13]. Its active compounds 6-Gingerol and 6- paradol had inhibitory effects on the viability and DNA synthesis of human leukemia cell line, HL-60 [14]. Essential oil of ginger significantly suppressed formation of DNA adducts by aflatoxin B-1 in microsomal enzyme mediated reaction [15]. Topical application of extract of ginger demonstrated significant protection against skin tumorigenesis [16]. Supplementation with ginger in rats fed a high fat diet, provided significant antioxidant effects, raising tissue.

The objective of this study is to determine baseline levels of SOD, GPx, CAT and GSH and MDA in liver cancer cell line, HepG2 and to observe the effects of the ginger extract (Zingiber officinale) on the antioxidant status of HepG2 cell line.

Materials and methods
Ginger extract
Ethanol extract of ginger was provided by Dr. Noor Azian Murad from Centre of Lipids and Engineering and Applied Research (CLEAR,University of Technology Malaysia) which was prepared as follows: 200 grams of the powder from fresh ginger rhizomes was extracted with ethanol (4 L ) for 9 h at room temperature. The solution was filtered and washed with n-hexane: water (1: 1). The ethanol extract was evaporated to dryness under vacuum and the resulting residue was partitioned between chloroform: water (1:1). The organic layer was collected and concentrated in vacuum. Oleoresin sample was tested to have a refractive index (RI) reading in the range of 1.5000 to 1.5220.

Cell culture and treatment
Human liver cancer (HepG2) and normal(WRL-68) cell lines were grown in Eagle Minimum Essential Medium of Earle ( EMEM, Flow lab, Australia) supplemented with 10 % fetal calf serum and incubated at 37° Celsius in a humidified atmosphere of 5 % CO2.

Enzyme assays
The 70 – 80 % confluent cells were washed twice with Phosphate Buffered Saline (PBS) , pH 7.2 and treated with 0.25% trypsin (PAA, Laboratories, GmBH, Austria), 2 mM EDTA (SIGMA, St. Louis, MO) in PBS for 10 min. The cell suspension was centrifuged for 10 minutes in a centrifuge (Damon, IEC, USA 600 rpm). Cell pellets were then lysed in 50 mM phosphate buffer pH 7.0, followed by sonication (Sonicator ultrasonic processor, XL,Heat Systems, USA) for 2 min on ice. The mixture was then centrifuged for 10 minutes at 10,000 x g and the supernatant was assayed for enzyme activities and protein concentration. SOD, GPx and CAT activities were measured by Beyer and Fridovich’s [18], Paglia and Valentine’s [19] and Aebi’s [20] methods respectively. The measurement of GSH content was carried out using 5,5’ dithiobis-2- nitrobenzoic acid, DTNB (Sigma Chemical Co., USA) by Griffith’s method [21]. Measurement of MDA formation was carried out through its reaction with thiobarbituric acid, TBA (Sigma Chemical Co., USA) using the method of Ohkawa [22].

Superoxide dismutase (SOD) activity measurement
20 µl of supernatant was added to a reaction mixture consisting of 0.033 mM EDTA, 3.3 mM methionine, 0.33 µg/ml riboflavin and 0.01 mM KCN (all purchased from Sigma Chemical Co., USA). The cuvette containing the reaction mixture was placed in a box illuminated with 20 W neon lamp for 10 min. Results were calculated by subtracting the changes in absorbance of the blank from the sample and dividing A by 0.012 to obtain the McCord - Fridovich units of activity.

Glutathione peroxidase (GPx) activity measurement
Both the blank and the system cuvettes contained 0.1 M KPO4 buffer (pH 7.0), 2 µM EDTA, 10 units/ml glutathione reductase, 4mM sodium azide, (Sigma Chemical Co., USA), 200 mM NADPH (Calbiochem) and supernatant of cell lysate. In addition, the system cuvette contained 1.0 mM glutathione,GSH (Sigma Chemical Co., USA). After 10 minutes of pre incubation at 37° C, the reaction was started by the addition of 1.0 mM H2O2 (Sigma Chemical Co., USA) to the blank and systems cuvettes. An additional blank assay in which the buffer was substituted for the supernatant was performed in order to correct the non enzymatic oxidation of GSH and NADPH by H2O2.
Catalase (CAT) activity measurement

CAT activity was determined by the method described by Aebi [20] using an ultraviolet spectrophotometer (UV-160A, Shimadzu, Japan). The decomposition rate of the substrate $H_2O_2$ was monitored at 240 nm. A molar absorptivity of 43.6 L/mol·cm was used to calculate the activity. One unit is equal to 1 µmol of $H_2O_2$ decomposition/min.

Glutathione (GSH) measurement

GSH reacts with 5,5'-dithiobis-2-nitrobenzoic acid, DTNB (Sigma Chemical Co., USA) which produces a yellow colored compound, 5'-thio-2-nitrobenzoic-acid (TNB). The mixed disulphide, between GSH and TNB that is concomitantly produced is reduced by glutathione reductase to recycle the GSH and to produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. Concentration of TNB was measured at 412 nm wavelength (UV-160A, Shimadzu, Japan).

Malondialdehyde (MDA) measurement

200 µl of supernatant samples were mixed with 200 µl of 8.1% SDS, 1.5 ml of 0.8% 2-thiobarbituric acid, TBA and 1.5 ml of 20% acetic acid (all purchased from Sigma Chemical Co., USA). After heating at 95°C for 60 minutes (Memmert, Germany) the reaction mixture was cooled in tap water and a mixture consisting of 1 ml of distilled water and 5 ml of n-butanol : pyridine (15 : 1) was added, mixed and incubated 10 min at 4°C. The mixture was then centrifuged at 200 g for 15 min at 4°C. The absorbance of the resulting organic layer was measured spectrophotometrically (UV-160A, Shimadzu, Japan) at 532 nm and compared to with an external standard of MDA.

Statistical Analysis

Results refer to mean ± standard deviation and are average of three values per experiment; each experiment was repeated at least three times. Statistical evaluations were assessed using the Student’s $t$ test, and $p < 0.05$ was considered significant.

Results

Figure 1 represents the status of SOD activities in normal (WRL-68) and liver cancer cell lines (HepG2) with and without treatment of ginger extract. In untreated normal (WRL-68) and liver cancer (HepG2) cell lines, SOD activities were 77.81 ± 3.8 unit/mg protein and 157.37 ± 22.8 unit/mg protein respectively. SOD activity was significantly reduced ($p < 0.05$) by 26.6% and 72.32% in HepG2 cell line when 200 µg/ml and 500 µg/ml ginger extracts were introduced in the cultures respectively. Changes observed in normal cultures were not significant.

Figure 1: Status of superoxide dismutase (SOD) activities in hepatoma cell line (HepG2) and in normal cell line (WRL-68) with and without ginger extract. Data are presented as mean ± standard deviation, SD from triplicate wells. *SOD activities were significantly lower ($p < 0.05$) in HepG2 cells at 200 and 500 µg/ml of ginger extract compared to untreated hepatoma cell line, while the changes seen in normal culture were not significant ($p > 0.05$) at all concentrations of ginger when compared to the untreated normal cells. # SOD activities were significantly higher in HepG2 cells ($p < 0.001$) when compared to normal cell line without ginger treatment.

Figure 2: Status of glutathione peroxidase (GPx) activities in HepG2 and in normal cell lines (WRL-68) with and without treatment of ginger extract. Data are presented as mean ± SD from triplicate wells. *GPx activities were significantly reduced ($p < 0.05$) in HepG2 cells at all concentrations of ginger extract when compared to the untreated culture. **GPx activities were significantly reduced ($p < 0.05$) in normal cell (WRL-68) at 200 µg/ml ginger extract compared to the untreated culture.
Figure 2 represents the status of GPx activity in normal (WRL-68) and liver cancer (HepG2) cell lines with and without treatment of ginger extract. In untreated WRL-68 and HepG2 cell lines, GPx activities were $0.651 \pm 0.18$ unit / mg protein and $0.529 \pm 0.09$ unit/mg protein respectively. GPx activities were significantly reduced ($p < 0.05$) by 77.16%, 87.35% and 71.05% in HepG2 cell line at 100, 200 and 500 µg/ml of ginger extract respectively. In normal culture, GPx activity was significantly reduced ($p < 0.05$) at 200 µg/ml ginger extract when compared to the untreated culture.

Figure 3 represents the status of CAT activities in normal (WRL-68) and liver cancer (HepG2) cell lines with and without treatment of ginger extract. In untreated WRL-68 and HepG2 cell lines, CAT activities were $0.86 + 0.035$ unit/mg protein and $1.00 + 0.19$ unit/mg protein respectively. CAT activities were significantly reduced ($p< 0.05$) at 200 and 500 µg/ml of ginger extract in HepG2 cell lines when compared to the untreated culture. In normal cell line (WRL-68), CAT activities were not significantly reduced ($p >0.05$) at all concentration of ginger extract, compared to the untreated culture.

Figure 4 depicts the status of glutathione (GSH) content in normal (WRL-68) and liver cancer (HepG2) cell lines with and without treatment of ginger extract. GSH levels were 36.91 ± 0.79 µM and 36.95 ± 0.82 µM in normal and in liver cancer cell (HepG2) lines respectively. Addition of ginger extract had no effect on both normal and liver cancer cell lines.

Figure 5 depicts the status of MDA in HepG2 and in normal (WRL-68) cell lines with and without treatment of ginger extract. In untreated WRL-68 and HepG2 cell lines, MDA levels were 73.4 ± 0.75 µM and 73.94 ± 0.37 µM respectively. Changes in MDA levels were not significant both in HepG2 and WRL-68 cell lines at all concentrations of ginger extract when compared to the respective untreated culture.
Discussion

The identification of plant-derived compounds (phytochemicals) having the capacity to interfere with carcinogenic processes has been received with interest lately. Ginger extracts (Zingiber officinale) contain three main compounds gingerol, shogaol and paradol which exhibit antioxidant activity. Our earlier study [23] has shown the anti-tumor effect of Zingiber officinale by inhibiting proliferation and inducing apoptosis in human hepatoma cell line, HepG2. In an attempt to elucidate the baseline levels of antioxidant enzyme status in HepG2 cell line, we measured the activities of SOD, GPx, CAT, and GSH and MDA contents. We had also explored the possible antitumour and antioxidant effects of ginger extract, Zingiber officinale in HepG2 cell line by measuring the endogenous antioxidant levels in this cell line when treated with ginger extract. It has been highly suggested that in the process of carcinogenesis excessive accumulation of reactive oxygen species may play an important role in causing oxidative damage. In an attempt to defend the situation, antioxidant enzymes (SOD, GPx, CAT) may be elevated or reduced in these cells [1-7]. This study showed that SOD activity was higher in hepatoma cell line (HepG-2) by 2 fold compared to control (WRL68), which may reflect the higher superoxide radicals in the former. This is comparable to the study of Lee et al., [24] whereby SOD activity in HepG-2 cell line was 2.8 fold higher than normal cell line. However, we noted that other antioxidant enzymes, CAT and GPx activities in HepG2 cell line were not significantly different when compared to normal cell line. GSH and MDA contents were also not significantly different in hepatoma cell line when compared to the respective control. An increase of 4.3-fold, 2.9-fold and 1.4-fold of CAT, GPx activities and GSH content respectively in control, and GSH and MDA contents. We had also explored the possible antitumour and antioxidant effects of ginger extract, Zingiber officinale in HepG2 cell line by measuring the endogenous antioxidant levels in this cell line when treated with ginger extract. It has been highly suggested that in the process of carcinogenesis excessive accumulation of reactive oxygen species may play an important role in causing oxidative damage. In an attempt to defend the situation, antioxidant enzymes (SOD, GPx, CAT) may be elevated or reduced in these cells [1-7]. This study showed that SOD activity was higher in hepatoma cell line (HepG-2) by 2 fold compared to control (WRL68), which may reflect the higher superoxide radicals in the former. This is comparable to the study of Lee et al., [24] whereby SOD activity in HepG-2 cell line was 2.8 fold higher than normal cell line. However, we noted that other antioxidant enzymes, CAT and GPx activities in HepG2 cell line were not significantly different when compared to normal cell line. GSH and MDA contents were also not significantly different in hepatoma cell line when compared to the respective control. An increase of 4.3-fold, 2.9-fold and 1.4-fold of CAT, GPx activities and GSH content respectively in hepatoma cells was reported by Lee et al., [24]. However, low levels of CAT, and GSH have been observed in chronic liver disease such as alcoholic liver disease, viral hepatitis and liver cancer [10]. Such observations could be due to modulations of the defense system in the tumour cells in relation with their growth and maintenance [24]. Bannister et al., [25] had noted 90 - 70 % decrease in the level of antioxidant enzymes, SOD and CAT respectively and an absence of GPx activity in human hepatoma cell line Hep3B. He has implicated such findings with excessive accumulation of oxygen free radicals and DNA damage in hepatoma cells, considered to be important in carcinogenic mechanism.

This study also showed that the antitumour effects of ginger extract may be exhibited by influencing the status of antioxidant enzymes in HepG2 cells. SOD, GPx and catalase activities which were higher in untreated hepatoma cells, were reduced significantly when ginger extract at concentrations of 200-500 µg/ml was introduced into the culture. Ginger extract could be taking over the role of antioxidants SOD, GPx and catalase in eliminating the superoxide radicals and hydrogen peroxide accumulation in HepG2 hepatoma cell line. However, ginger extract had no effect on the levels of GSH and MDA contents of HepG2 cells at all concentrations.

In summary, this study suggests the possible chemoprotective role of ginger extract (Zingiber officinale) by eliminating superoxide radicals and hydrogen peroxide in the liver cancer cell line HepG2 thus replacing the activities of SOD, GPx and CAT. Ginger extract had no effects on the activities of SOD,GPx and CAT in the normal cell line. Future studies should include the mechanism by which ginger extract acts as exogenous antioxidants in scavenging free radicals.

Acknowledgements

This work was generously supported by short term grant of Medical Faculty of UKM, FF:088.2003.

References