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Antiproliferative, Antioxidant and Apoptosis Effects of *Zingiber officinale* and 6-Gingerol on $HepG_2$ Cells

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Abstract: The present study was designed to compare the antioxidant, antiproliferative and apoptosis effects of ethanolic extract of ginger (*Zingiber officinale*) with its phenolic component (6)-Gingerol on human hepatoma cell line ($HepG_2$). We found that cytotoxic and antiproliferative effect of ginger extract and (6)-Gingerol could be associated with induction of apoptosis. Both ginger extract and (6)-Gingerol also showed remarkable antioxidant activities in comparison with ascorbic acid and N-acetyl-L-cysteine.

Key words: Antioxidant, antiproliferation, (6)-gingerol, $HepG_2$, *Zingiber officinale*

INTRODUCTION

Oxidative damage caused by free radicals is known to participate in the pathogenesis of several diseases such as cardiovascular, rheumatoid arthritis and cancer (Winyard *et al*., 2005). Extensive research in the last few years has revealed that regular consumption of certain fruits, vegetables and spices can reduce the risk of acquiring specific cancers (Aggarwal and Shishodia, 2006). Lee and Surh (1998) reported that (6)-Gingerol and (6)-Paradol were found to exert inhibitory effects on the viability and DNA synthesis of human leukemia (HL-60) cells. Increased vegetables and spices intake are linked to a reduction in the risk of acquiring several type cancers. Because these agents have been shown to suppress cancer cell proliferation, inhibit growth factor signaling pathways, induce apoptosis, suppress the expression of anti apoptotic proteins, inhibits cyclooxygenase, they may have untapped therapeutic value (Taraphdar *et al*., 2001; Surh, 2002).

Zingiber officinale Roscoe (ginger) is widely used all over the world as spice and condiments in daily cooking. Ginger has also been used as traditional oriental medicines to ameliorate inflammation, rheumatic disorder and gastrointestinal discomforts (Geiger, 2005). Crude ginger contains up to 5-8% oleoresin of which 25% of the oleoresin, consists mainly gingerol (Chrubasik *et al*., 2005). (6)-Gingerol has been associated with analgesic, anti-inflammatory, sedative, antipyretic and antibacterial effects in both *in vitro* and *in vivo* studies (Bhattacharjee, 2000).

Hepatocellular carcinoma (hepatoma) is one of the most common cancers in the world, with an annual incidence of approximately 1 million deaths, mainly in underdeveloped and developing countries (Pang *et al*., 2006). An imbalance between proliferation and apoptosis is strongly linked to the cause of most cancers including liver cancer (Farinati *et al*., 2001). The search for chemopreventive agents found in natural products or foods is gaining a lot of interest in cancer research (Gosslau and Chen, 2004).

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In the present study we compare the effects of ginger extract (*Zingiber officinale*) with its phenolic component (6)-Gingerol (component of ginger) in inhibiting proliferation and inducing apoptosis of hepatoma cells ($HepG_2$).

MATERIALS AND METHODS

Materials

Ginger (*Zingiber officinale*) extract was obtained by ethanol extraction as provided by Dr. Noor Azian Murad from Center for Lipids Engineering Applied Research (CLEAR), Universiti Teknologi Malaysia. (6)-Gingerol was purchased from WAKO, Japan. Eagle's Minimum Essential Medium (EMEM) and foetal bovine serum were obtained from Flow Lab, Australia. Trypsin was purchased from PAA, Laboratories, GmBH, Austria. The cell titer 96® AQ_{ueous} Non radioactive cell proliferation (MTS) assay kit was from Promega Corporation, Madison, WI, USA. All chemicals used were from Sigma (St. Louis, MO).

Methods

Cell Cultures

$HepG_2$ (ATCC.HB 8065, Rockville, MD, USA) were maintained in Eagle's minimum essential medium (Flow Lab, Australia) supplemented with 10% heat inactivated foetal bovine serum and 1% penicillin-streptomycin (Flow Lab, Australia). The cells were cultured as a monolayer in plastic 75 cm^2 tissue culture flash and grown at 37°C in humidified atmosphere of 5% CO_2. Cell's viability, proliferation and apoptosis were performed when the cells reached 70-80% confluence. Ginger extract and (6)-Gingerol (WAKO, Japan) were added to cells after 24 h incubation.

Sample Analysis

Antioxidant Activity in Cell Free System

The free radical-scavenging capacity of ethanolic extract from *Zingiber officinale* and (6) Gingerol were tested by its ability to bleach the stable 1,1-diphenyl-2-picryl-hydrazyl radical, DPPH (Sigma). The reaction mixture contained 1 mL of different concentrations of ginger extract (from 10 to 1000 μg mL^{-1}) or (6)-Gingerol (100, 200, 500, 1000 μg mL^{-1}) and 1 mL of freshly prepared 1 mM DPPH ethanolic solution. The resulting solution were left to stand for 30 min at room temperature, prior to being spectrophotometrically detected at 517 nm (Ito *et al*., 2005).

MTS Assay for Cell Viability

$HepG_2$ cells at a density of 2×10^4 cells mL^{-1} were plated in 96 well microtiter plates. After 24 h of incubation to allow for cell attachment, the cells were treated with 100 μL of varying concentrations of ginger extract and (6)-Gingerol (5, 10, 50, 100, 200, 500 and 1000 μg mL^{-1}) in complex medium and incubated again for 24 h at 37°C under 5% CO_2. Three hours after the addition of MTS solution (Promega, Madison, WI, USA) the amount of formazan formed was measured spectrophotometrically at 490 nm with microplate reader Versamax-Molecular, Devices B-02865. Fifty percent Inhibitory Concentration (IC_{50}) of ginger extract and (6)-Gingerol in $HepG_2$ cells were calculated from triplicate wells.

Cell Proliferation

Cellular proliferation of $HepG_2$ cells were measured using BrdU kit (Roche Diagnostics, Germany). $HepG_2$ cells were seeded into 96 well plates at a concentration of 2×10^4 cells mL^{-1} in EMEM. Cells were incubated with various dilutions of ginger extract and (6)-Gingerol in a 96-well plates at a final volume of 100 μL well for 24 h in a humidified atmosphere at 37°C. Ten microliter of BrdU labeling solution were added in cells and incubated for another 24 h at 37°C. 100 μL/well

anti-BrdU-POD working solution was added and incubated for 90 min at 25°C. After final rinsing, 100 µL/well substrate solution was added and incubated at 25°C until color development was sufficient for photometric detection using ELISA reader (Versamax-Molecular, Devices.B-02865) at 450 nm (reference wavelength; 690 nm).

Analysis of DNA Fragmentation for Apoptosis

$HepG_2$ cells grown at density of 2×10^6 cells 10 mL^{-1} were exposed to ginger extract and (6)-Gingerol at various concentrations (5, 10, 50, 100, 200, 500 and 1000 µg mL^{-1}) after 24 h incubation. Cellular DNA fragmentation was performed as per instruction in the ELISA kit (Roche Diagnostic, Germany). The absorbance of the samples was measured with ELISA reader (Versamax-Molecular, Devices.B-02865) at 450 nm (reference wavelength; 690 nm).

Analysis of Data

Statistically significant differences were assessed using the Student's t test.

RESULTS AND DISCUSSION

As shown in Fig. 1, both ginger extract and (6)-Gingerol revealed potent antioxidant activities. Percent scavenging activity of (6)-Gingerol was higher compared to the ethanolic extract of ginger at lower concentration (<100 µg mL^{-1}) but at higher concentration, both ginger extract and (6)-Gingerol at (500 µg mL^{-1}) exhibited up to 92.68±5.47 and 74.19±5.36%, respectively, of DPPH radical scavenging activity. The results showed that the order of potency of antioxidant activities as shown by DPPH radical scavenging capacity at concentration of 500 µg mL^{-1} is: diethyl dithiocarbamic (DDC) > ginger extract > Buthyl Hydroxyl Toluene (BHT) > (6)-Gingerol > N-Acetyl L-cysteine (NAc) > ascorbic acid. The isolation of bioactive compounds in the *Zingiber officinale* extracts in the future would help to ascertain the individual potency of the isolated compounds.

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions. Some spices or herbs contain bioactive phenolic substances with potent antioxidative and chemopreventive properties (Surh *et al*., 1998). The antioxidant activity of phenolic compounds is mainly due to their redox

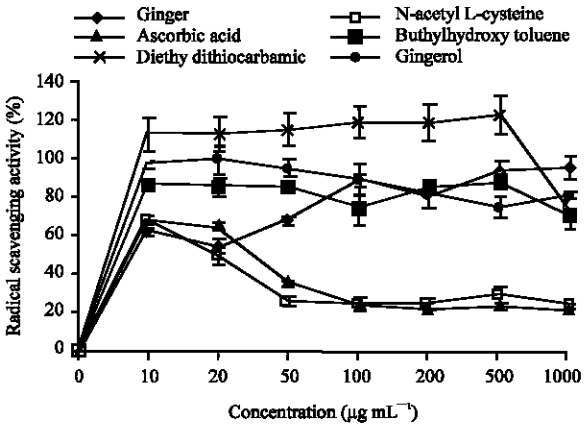

Fig. 1: Free radical scavenging activity of ginger extract. Results are mean±SD of three independent experiments

properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides. The phenolic compounds in many plant and vegetables, including ginger may contribute directly to antioxidative action. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g daily is ingested from a diet rich in fruits and vegetables (Gûlcin *et al*., 2002).

Tumors are disease with proliferation disorder and apoptosis obstacle. The inhibition of proliferation and induction of apoptosis are regulated by a network of signaling pathways and transcription factors, which are possible targets for a rational tumor therapy (Liu *et al*, 2004). Apoptosis is now recognized as an important mode of cell death in response to cytotoxic treatments. It has been well documented that the administration of many natural compounds with anti-tumor activities triggers the apoptotic death of cancer cells.

In this study, we found that ginger extract and (6)-Gingerol reduced viability of $HepG_2$ cells significantly ($p<0.01$), after 24 h treatment with varying concentrations between 5 to 1000 μg mL^{-1} with an IC_{50} of 358.71±17.12 and 431.70±10.44, respectively (Table 1). Ginger extract and (6)-Gingerol showed a dose dependent inhibition on the proliferation of $HepG_2$ cells with a corresponding induction of apoptosis (Table 2). Ginger extract showed a higher percentage of apoptosis compared to its phenolic component (6)-Gingerol at all concentrations which corresponds with its lower IC_{50}. Both ginger extract and (6)-Gingerol exhibited maximal induction of apoptosis at 500 μg mL^{-1}.

Ginger extract and (6)-Gingerol at concentration of 100 μg mL^{-1} and above, significantly affected the viability of $HepG_2$ cells, suggesting that the observed growth inhibition was caused by cytotoxic rather than a cytostatic effect of ginger and (6)-Gingerol. The results showed a decrease in the percentage of cell viability in a dose dependent effect for both ginger extract and (6)-Gingerol with concentrations ranging from 200-1000 μg mL^{-1}.

We further investigated the mechanism of apoptosis induced by ginger extract and (6)-Gingerol. The ability in inhibiting or in enhancing apoptosis by plant extracts depends on several factors such as; extract concentration, concerted action of multiple micronutrients, cell type and redox status (Palozza *et al*., 2004). $HepG_2$ cells are capable of undergoing apoptosis through the basic common signaling pathway. p53 and c-Myc play an important role in the apoptosis signaling pathway in $HepG_2$ cells treated with a number of apoptosis inducing compounds (Liu *et al*., 2002).

We found the percentage of apoptotic cells was increased in a dose dependent manner by treatment with ginger extract and (6)-Gingerol at concentrations ranging from 100-500 μg mL^{-1}, but percent apoptosis decreased at 1000 μg mL^{-1}. This could be due to the necrotic effect of high

Table 1: Cytotoxic activity of the ginger extract and (6)-Gingerol on $HepG_2$ cells

Treatment (μg mL^{-1})	Ginger extract (% of control)	(6)-Gingerol (% of control)
0	100.00	100.00
100	59.22±1.86	57.11±2.07
200	44.76±3.34	53.74±2.97
500	31.81±2.05	51.35±2.25
1000	29.39±3.81	11.65±2.52
IC50	358.71±17.12	431.70±10.44

Data were presented as mean±SD (n = 3)

Table 2: Effect of ginger extract and (6)-Gingerol on proliferation and apoptosis on $HepG_2$ cells

Extract (μg mL^{-1})	Rate of proliferation (% of control)	Apoptosis (% of control)
100 (Ginger)	79.63±8.42	49.26±4.17
200	66.92±5.44	54.38±7.64
500	58.71±7.37	73.58±7.19
1000	41.87±3.76	19.09±5.01
100 ((6)-Gingerol)	78.38±6.41	28.64±3.10
200	53.61±3.55	29.79±3.12
500	48.26±1.78	56.84±6.33
1000	40.79±1.73	12.73±2.55

Data were presented as mean±SD (n = 3)

concentration of ginger extract and (6)-Gingerol. Alternatively, over expression of Bcl-2 or Bcl-x can protect against chemotherapy induced release of mitochondrial cytochrome *c*, caspase activation and DNA fragmentation (Tong *et al*., 2004). This could be what was happening to $HepG_2$ cells at higher concentration of ginger extract. Apoptosis is a mechanistically driven form of cell death that is either developmentally regulated, or activated in response to specific stimuli or various forms of cell injury. In cancer biology, it is now evident that many cancer cells circumvent the normal apoptotic mechanisms to prevent their self destruction. Therefore, it would be advantageous in cancer chemotherapy and prevention to tip the balance in favor of apoptosis over mitosis (Yoo *et al*., 2002).

Although data from this study demonstrate that ginger was able to inhibit the growth and induce apoptosis of cancer cells *in vitro*, the *in vivo* anti tumor potentials of ginger remains to be determined.

CONCLUSIONS

The results of this study indicate that both *Zingiber officinale* extract and (6)-Gingerol have high DPPH radical scavenging activity. The anticancer effect of *Zingiber officinale* extract and (6)-Gingerol was demonstrated by inhibition of cellular proliferation and induction of apoptosis of hepatoma cells. The antiproliverative and apoptosis effect of ginger extract could be associated mainly with the action of its main phenolic component, (6)-Gingerol. However, ginger extract in its natural form has higher antioxidant, antiproliferation and apoptotic effect compared to (6)-Gingerol.

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