Journal of Medicinal Plants Research Vol. 5(14), pp. 3247-3255, 18 July, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Antioxidant potential and anticancer activity of young ginger (*Zingiber officinale* Roscoe) grown under different CO_2 concentration

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Accepted 3 May, 2011

In the present study, leaves and rhizomes extract from two Malaysian young ginger (*Zingiber officinale* Roscoe) varieties namely: Halia Bentong and Halia Bara grown under ambient (400 µmol/mol) and elevated (800 µmol/mol) CO_2 concentrations were studied for their antioxidant and *in vitro* anticancer activities against two human cancer cell lines (MCF–7 and MDA–MB–231). Antioxidant activities in both varieties determined using thiobarbituric acid (TBA) assays increased significantly with increasing CO_2 concentration from 400 to 800 µmol/mol. High antioxidant activity was observed in the rhizomes of Halia Bara grown under elevated CO_2 concentration. The results showed that CO_2 enriched Halia Bara exhibited the highest anticancer activity on MCF–7 cancer cells with IC_{50} values of 25.3 and 27.31 µg/ml respectively for rhizomes and leaves extract. IC_{50} values for MDA–MB–231 exhibition were 30 and 32.81 µg/ml, respectively for rhizomes extract of Halia Bara and Halia Bentong. Results showed that Halia Bentong and Halia Bara possessed anticancer and antiradical properties especially when grown under elevated CO_2 concentration. Antioxidant activities of ginger leaves and rhizomes could be increased or improved by using CO_2 enrichment in a controlled environment condition. Results also implied that these ginger varieties could be employed in ethno-medicine for the management of cancerous diseases.

Key words: Thiobarbituric acid assays, CO_2 enrichment, Halia Bentong, Halia Bara, breast cancer cell, MCF–7, MDA–MB–231.

INTRODUCTION

Cancer is a multi-step disease incorporating physical, environmental, metabolic, chemical and genetic factors, which play a direct and/or indirect role in the induction and deterioration of cancers. Diet with high consumption of antioxidant rich fruits and vegetables significantly reduces the risk of many cancer diseases suggesting that confident antioxidants could be effective agents for the inhibition of cancer spread. These agents present in the diet are a group of compounds with low toxicity, safe and generally accepted (Fresco et al., 2006). Isolated polyphenols from different plants have been considered in a number of cancer cell lines indicative of different evolutionary stages of cancer. Anticancer activities of flavonoids were described in previous studies (Ramos, 2007; Mavundza et al., 2010). Some tests showed antitumor properties of quercetin including the inhibition of cancer cell proliferation and migration (Lim et al., 2006). The isolated polyphenols from strawberry including kaempferol, quercetin, anthocyanins, coumaric acid and ellagic acid, were shown to inhibit the growth of human cancer cell lines for breast (MCF–7), oral (KB, CAL–27), colon (HT–29 and HCT–116), and prostate (LNCaP and DU–145) (Zhang et al., 2008; Damianaki et al., 2000). Similar results have also been reported in previous studies with wine extracts and isolated polyphenols (resveratrol, quercetin, catechin and

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epicatechin) (Kampa et al., 2000), and green tea polyphenols (epigallocatechin and epicatechin), (Weisburg et al., 2004). Arts et al. (2002) reported catechin ability to control postmenopausal cancer in woman. He found that catechin intake may protect against rectal cancer. Epicatechin and gallocatechan 3-gallate induce reduction in experimental lung tumour metastasis (77 and 46%). Epigallocatechin 3-gallate is an effective antiangiogenesis agent, which inhibits tumor cell invasion and proliferation (Ogasawara et al., 2007; Tang et al., 2007). It also inhibits growth of the NBT-II bladder tumor cells and breast cancer cell lines (Chen et al., 2004).

Manthey et al. (2001) reported that citrus flavonoids inhibited the growth of HL–60 leukemia cells. Kaempferol belongs to the flavonoids group. Luo et al. (2009) showed that kaempferol inhibited the growth of ovarian cancer cell lines (91%), and A2780/CP70 (94%) by concentration of 20 and 40 µM respectively. Quercetin may help to inhibit prostate cancer growth (Verschoyle et al., 2007). Inhibition of breast cancer cell lines (MCF–7 and MDA–MB–231) by quercetin was reported by Gibellini et al. (2010). In recent years, research about carcinogenic potential of quercetin has exhibited its promise as an anticancer agent. Likewise, *in vitro* and *in vivo* studies showed that quercetin was able to inhibit viability of leukemic cells, colon and ovarian carcinoma cell and especially human breast cancer cells (Davis et al., 2000; Gibellini et al., 2010). The Zingiberaceae family is well-known in Southeast Asia and many of its species are being used in traditional medicine, which is found to be effective in the treatment of several diseases. The five Zingiberaceous plants, *Curcuma longa* (turmeric), *Alpinia galanga* (greater galanga), *Kaempferia galangal* (proh hom), *Boesenbergia pandurata* (finger root) and *Zingiber officinale* (ginger) have been well investigated; they are perennial herbs that are widely cultivated in tropical regions of Asia, and have been commonly used as medicinal plants and spices in Asia. The rhizomes of these plants possess diverse biological activities, for instance, antimicrobial (Yamada et al., 1992; Hiserodt et al., 1998), antiulcer (Matsuda et al., 2003), anti-inflammatory (Araujo and Leon, 2001), antioxidant (Ghasemzadeh et al., 2010a), cytotoxic and antitumor (Itokawa et al., 1987; Murakami et al., 2004), antispasmodic (Ammon and Wahl, 1991), and antidepressant activities (Yu et al., 2002). *Boesenbergia rotunda* (Zingiberaceae) exhibited antibacterial, antifungal, anti-inflammatory, analgesic, antipyretic, antispasmodic, antitumor and insecticidal activities (Tewtrakul et al., 2003).

The rhizome of *Z. officinale* is generally used as a culinary spice in Malaysia, and also for the treatment of oral diseases, leucorrhoea, stomachb pain, stomach discomfort, diuretic, inflammation and dysentery. Shukla et al. (2007) reported cancer preventive properties of ginger and showed that this ability is related to [6]-gingerol. Kuokkanen et al. (2001) showed that the concentration of total phenolics was significantly increased in the birch leaves produced in the CO_2-enriched air, as has also been observed in the experiments of Lavola and Julkunen (1994), Williams et al. (1994), Kinney et al. (1997) and Ibrahim et al. (2011). Environmental conditions, cultural practice, and management approaches can impact the quality of food by their abilities to promote good health and well being. In fact, new management strategies are emerging that use ecophysiological factors to elevate phytochemical concentrations in food crops. Some ecophysiological conditions that are thought to have significant impact on enhancing the health-promoting phytochemicals in a number of plants include environmental conditions and cultural and management practices (Schreiner, 2005).

Thus, there is an increasing interest in using appropriate strategies and management practices to improve the quality of food crops by enhancing their nutritive and health-promoting properties. Information about anticancer and antioxidant activities of enriched ginger by elevated CO_2 concentration is scarce. On the other hand, the impacts of cultural conditions and CO_2 concentration on biopharmaceutical production in herbs have not been widely investigated and it needs to be understood, especially when the objective is the optimization of the herb chemistry. The aim of this study was to screen antioxidant potential and anticancer activities (*in vitro*) of two Malaysian young ginger varieties (*Z. officinale*) grown under different CO_2 concentration.

MATERIALS AND METHODS

Plant materials

Two varieties of *Z. officinale* Roscoe (Halia Bentong and Halia Bara) rhizomes were germinated for two weeks and then transferred to polyethylene bags which were filled with soilless mixture of burnt rice husk and coco peat in a ratio 1:1. The plants were transferred to CO_2 growth chamber (Conviron EF7, Canada) with two different CO_2 concentrations (400 µmol/mol, ambient; 800 µmol/mol, elevated CO_2 concentration).

Pure carbon dioxide (99.8% purity) was supplied from high concentration carbon dioxide cylinder and injected through a pressure regulator into the growth chamber. Irradiance (310 µmol/m^2/s), relative humidity and air temperature of chamber were controlled using integrated control, monitoring and data management system software (Dynamac Corp., Rockville, MD). Plants were harvested at 16 weeks and leaves and rhizomes separated and freeze dried and kept in –80ºC for future analysis. The location of experiment was Biosystem Laboratory, Engineering Faculty, University Putra Malaysia (UPM).

Extract preparation

Leaves and rhizomes (1 g) were powdered and extracted using methanol (50 ml), with continuous swirling for 1 h at room temperature using an orbital shaker. Extracts were filtered under suction, evaporated and crude extract stored at –20°C for further use.

Table 1. Antioxidant activity of ginger extractions grown under different CO_2 concentration as measured by the TBA methods.

CO_2 (µmol/mol)	Varieties	Plant parts	TBA
400	H. Bentong	Leaves	69.19±2.12^{de}
		Rhizomes	67.72±1.84^{e}
	H. Bara	Leaves	70.73±1.89^{cde}
		Rhizomes	67.88±0.64^{e}
800	H. Bentong	Leaves	71.41±2.72^{cd}
		Rhizomes	76.25±1.53^{ab}
	H. Bara	Leaves	73.39±1.31^{bc}
		Rhizomes	78.73±1.15^{a}

All analyses are the mean of triplicate measurements ± standard deviation. Means not sharing a common letter were significantly different at $P \leq 0.05$. Results of TBA expressed as percent.

Determination of antioxidant activity

Thiobarbituric acid (TBA) assay

Various concentrations of testing samples (10 to 500 µg/ml) are added to an aqueous solution (2 ml) containing 200 µl of tris buffer (pH 7.4), 300 µl of 1 M KCl, 400 µl of 1% SDS (sodium dodecyl sulfate), 10 µl of linolenic acid, 40 µl of 1.0 µM $FeCl_2$ and 20 µl of 0.5 µM H_2O_2 in a brown non transparent vial (to avoid any oxidation caused by UV irradiation). The sample vial is then incubated for 18 h at 37°C while being shaken. After the incubation, oxidation is terminated by adding 50 µl of 4% BHT in ethanol solution, and 2 ml of the TBA reagent (0.67% TBA) is added to the sample. The sample was heated at 80°C for 1 h and then cooled in an ice bath for 10 min. A blank sample was prepared following the same procedure without a test sample. The TBA-MA adduct formed is measured using a spectrophotometer at 532 nm. A known antioxidant such as BHT and α-tocopherol were used as a positive control in the assay (Kosar et al., 2008). The antioxidant activities were calculated as follows:

Antioxidant activity (%) = ((absorbance control – absorbance sample)/control) × 100

Cell culture and treatment

Human breast carcinoma cell lines (MCF–7 and MDA–MB-231) were cultured in 100 µl of RPMI 1640 (Roswell Park Memorial Institute medium) media containing 10% fetal bovine serum (FBS). MCF–7 and MDA–MB–231 cells were incubated overnight at 37°C in 5% CO_2 for cells attachment.

MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The experiment was conducted as follows: Briefly, cancer cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 100 µl RPMI. 24 h after seeding, the medium was removed and then the cells were incubated for 3 days with RPMI in the absence and the presence of various concentration of ginger extracts. Ginger extract was added at various concentrations ranging from 4.6875, 9.375, 18.75, 37.5, 75, 150 and 300 µg/ml. After incubation, 20 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent was added into each well. This plate was incubated again for 4 h in CO_2 incubator at 37°C. The resulting MTT–products were determined by measuring the absorbance at 570 nm using ELISA reader (Lau et al., 2004). Each point represents the mean of triplicate experiments. The sell viability was determined using the formula:

Viability (%) = (optical density of sample/optical density of control) ×100

IC_{50} values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell line.

Statistical analysis

The experimental results were expressed as mean ± standard deviation of three replicates. Where applicable, the data were subjected to two-way analysis of variance (ANOVA) and the differences among samples were determined by Duncan's multiple range test using the statistical analysis system (SAS, 1999) and MSTATC programs. *P*-value of ≤ 0.05 was regarded as significant.

RESULTS AND DISCUSSION

Determination of antioxidant activity of ginger

The results obtained from the preliminary analysis of antioxidant activity are shown in Table 1 and Figure 1. According to the data obtained, significant differences were observed among treatments for antioxidant activities. From the result, the antioxidant activity of leaves was higher than rhizomes extracts in both varieties that were grown under ambient CO_2 concentration. The results also had indicated that antioxidant activities increased significantly by elevated

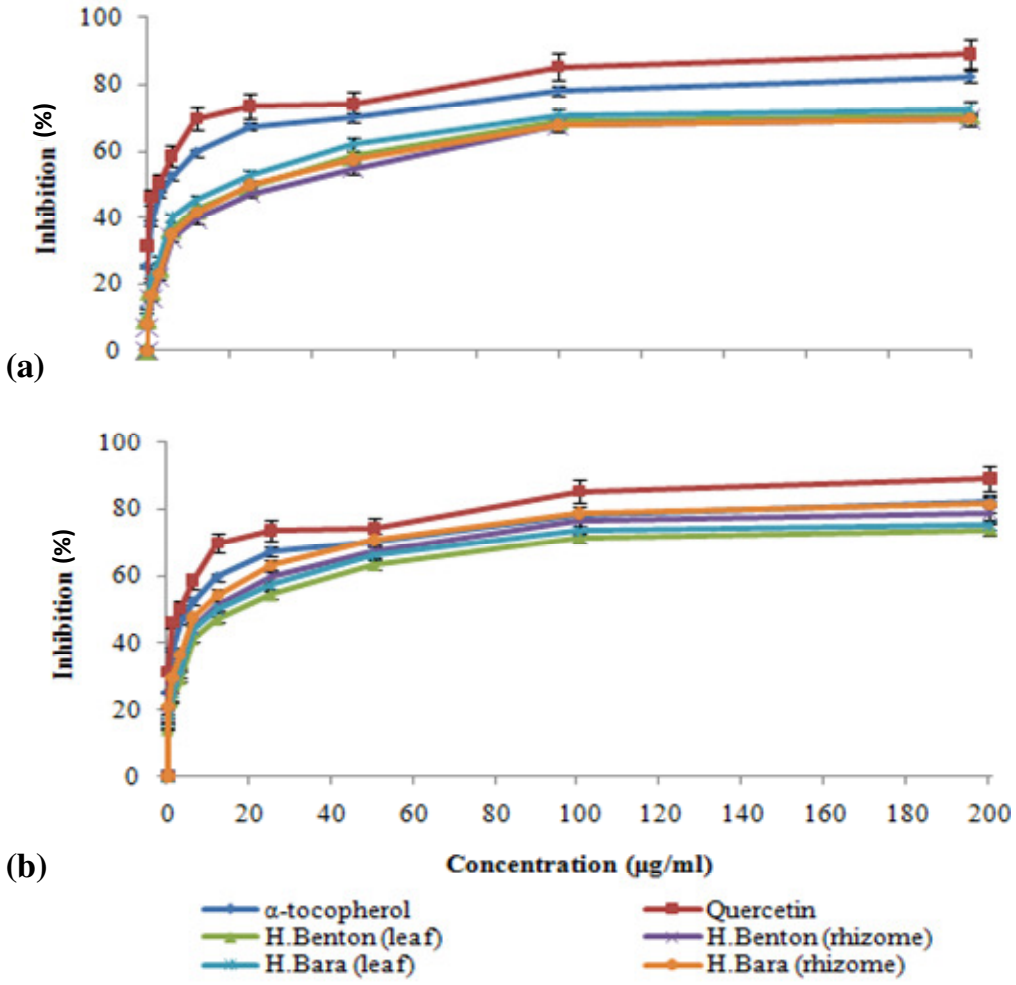

Figure 1. TBA activity of ginger varieties grown under different CO_2 concentration (a: 400 and b: 800 µmol/mol). Values are expressed in the mean of triplicate measurements ± SEM.

CO_2 concentration. Antioxidant activity was enhanced in rhizomes by elevated CO_2 concentration more than in leaves with highest value of TBA (78.73%) obtained from Halia Bara rhizomes. The leaves extract of Halia Bentong and Halia Bara in ambient CO_2 condition exhibited strong potential of free radical scavenging activity. According to the results TBA content of the Halia Bara leaves grown in ambient CO_2 concentration reached to 70.73%, while at the same extract concentration, that of the rhizomes was 67.88% (Table 1). In ambient CO_2 concentration differences between leaves and rhizomes in both varieties for TBA activity was not significant, while in elevated CO_2 concentration significant differences was observed between different parts of varieties. The results of the current study showed that TBA activity of the ginger parts extract were less than those of α-tocopherol (82.1%) and quercetin (94.7%), at 45 µg/ml when grown under ambient CO_2 (Figure 1). Halia Bara rhizomes grown under elevated CO_2 showed higher antioxidant activities compare to α-tocopherol at concentration of 47 µg/ml and above. Methanol extracts may include phenolic and hydroxy-phenolic compounds with acids, alcohols, sugars or glycosides, as reported by Kim et al. (2004). Many researchers had shown that high total flavonoids content increases antioxidant activity and there was a linear correlation between flavonoids content and antioxidant activity (Jung et al., 2007; Ghasemzadeh et al., 2010a, b). Silva et al. (2002) showed myricetin is expected to be the most efficient flavonoid antioxidant followed by quercetin.

It is well known that phenolic and flavonoid compounds act as hydrogen donors in that reaction mixture and therefore, the formation of hydroperoxides were decreased (Gebicka and Banasiak, 2009). The free radical scavenging of phenolic compounds was mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Huang et al., 2005). In the present study

Table 2. Anticancer activities (cell viability) of ginger extracts towards MCF–7 and MDA–MB–231 cell lines as determined by the MTT assay (at concentration 37.5 µg/ml).

CO_2 (µmol/mol)	Varieties	Plant parts	MCF–7	MDA–MB–231	Normal cell
400	H. Bentong	Leaves	59.55±2.55^{a}	63.36±1.85^{b}	96.75±1.18^{a}
		Rhizomes	57.66±1.68^{a}	69.41±2.3^{a}	94.28±1.04^{ab}
	H. Bara	Leaves	50.65±0.56^{b}	58.22±1.09^{c}	95.15±0.46^{ab}
		Rhizomes	57.14±1.74^{a}	66.60±2.31^{ab}	92.38±1.86^{bcd}
800	H. Bentong	Leaves	44.83±1.53^{c}	48.16±1.03^{d}	93.25±1.94^{bc}
		Rhizomes	49.07±1.04^{b}	44.35±1.86^{e}	90.15±2.02^{de}
	H. Bara	Leaves	40.47±1.46^{d}	43.12±1.99^{e}	91.07±0.67^{cde}
		Rhizomes	38.98±2.2^{d}	39.61±2.43^{f}	88.47±1.24^{e}
Positive control	Tamoxifen		24.6±1.7	26.29±2.1	---

All analyses are the mean of triplicate measurements ± standard deviation. Results expressed in percent of cell viability. Means not sharing a common letter were significantly different at $P \leq 0.05$.

leaves and rhizomes extracts of two Malaysian ginger varieties showed good potential of antioxidant activities.

Anticancer activity

As shown in Table 2, two ginger varieties parts (leaves and rhizomes) were found to express MCF–7 and MDA–MB–231 cancers inhibitory activity when tested at concentrations of 4.6875-300 µg/ml. The effects of different extracts on each cancer cell line are depicted in Figures 2 and 3. At a concentration of 37.5 µg/ml, however, most of extract exhibited strong anticancer activity towards MCF–7 and MDA–MB–231 cells. In this concentration, extract of Halia Bara rhizomes grown under elevated CO_2 concentration exhibit lowest MCF–7 and MDA–MB–231 cell viability respectively at 38.98 and 39.61%. MCF–7 and MDA–MB–231 treated with tamoxifen (positive control) showed 24.6 and 26.29% viability in same concentration (37.5 µg/ml). For MCF–7 cell, the anticancer activity of leaves extract in ambient CO_2 concentration was significantly stronger than that of the rhizomes extract in Halia Bara variety. For MDA–MB–231 cell, the anticancer activity of leaves extract in ambient CO_2 concentration was significantly stronger than that of the rhizomes extracts but with increasing of CO_2 concentration anticancer power increased significantly in rhizomes of both varieties. Of all extracts investigated Halia Bara rhizomes that were obtained from plants grown under elevated CO_2 concentration exhibited the strongest anticancer activities towards cancer cells and the IC_{50} values for MCF–7 and MDA–MB–231 cells were 25.3 and 30 µg/ml respectively (Table 3). While IC_{50} value of rhizomes extract of Halia Bara grown in ambient CO_2 for MCF–7 and MDA–MB–231 cells were 46.8 and 39.2 µg/ml, respectively. Accordingly, with increasing of CO_2 concentration, IC_{50} value decreased significantly in both varieties. Further-more, IC_{50} values of tamoxifen as a positive control for MCF–7 and MDA–MB–231 cells were 19.1 and 22.61 µg/ml, respectively.

Based on the several *in vivo* and *in vitro* studies, many mechanisms of anticancer action may be involved. These include cell cycle arrest, carcinogen inactivation, antiproliferation, inhibition of angiogenesis, induction of apoptosis and differentiation, antioxidation and reversal of multidrug resistance or a combination of these mechanisms (Davis et al., 2000; Gibellini et al., 2010). Flavonoids are among the best candidates for mediating the protective effect of diets rich in fruits and vegetables with respect to colorectal cancer. To gain additional information about their effects on cancer cells and their mechanisms of action, a series of related flavonoids was added to cultures of cancer cells. All flavonoid compounds increased growth inhibition and cell loss at concentrations of 1 to 100 mM, relative effectivity being quercetin > apigenin > fisetin> kaempferol (Verschoyle et al., 2007). Quercetin belong to the flavonoids group with powerful antioxidant activity (Davis et al., 2000). It is also a natural anti-histamine and antiinflammatory. Previous studies showed that quercetin may help to prevent cancer, especially prostate cancer (Verschoyle et al., 2007; Rietjens et al., 2005). Scambia et al. (1994) reported quercetin inhibited human breast cancer cells (MCF–7 and MDA–MB–231) significantly. Similar finding was reported by other studies (Davis et al., 2000). Du et al. (2010) explained mechanism of breast cancer inhibition by quercetin. In ginger quercetin is an abundant flavonoid compound (Khaki et al., 2010; Ghasemzadeh et al., 2010b, c). According to previous reports, antioxidant activity of quercetin was believed to have cytoprotective

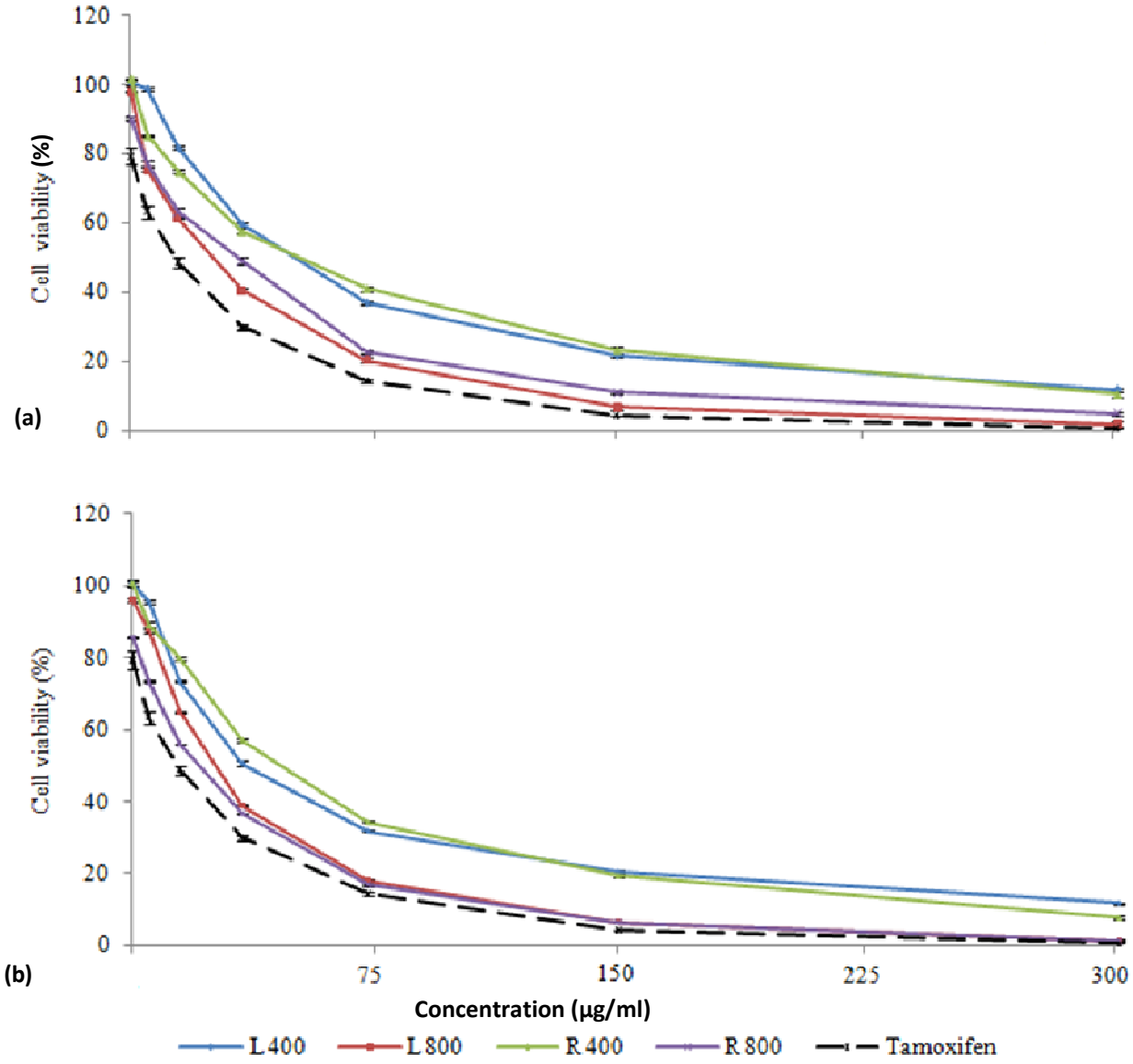

Figure 2. Dose-dependent anticancer of ginger extracts (a: Halia Bentong; b: Halia Bara) towards MCF–7 cell line as determined by the MTT assay. Values are expressed in the mean of triplicate measurements ± SEM. L and R respectively are leaves and rhizomes. 400 and 800 µmol/mol represent ambient and elevated CO_2 concentration.

role against oxidative stress. It seemed that quercetin does not only protect cells from free radical damage through antioxidant effect, but also motivate apoptotic cell death through pro-oxidant activity, and inhibits tumorigenesis. High concentration of quercetin was detected from Halia Bentong and Halia Bara leaves and rhizomes compared to other flavonoid compounds (Ghasemzadeh et al., 2010b). Then anticancer power maybe related to quercetin content in those varieties. Accordingly, flavonoid compounds could probably be responsible for the anticancer activity of *Z. officinale.* According to the American National Cancer Institute (NCI), the criterion of normal cell viability for the crude extracts of herbs is 76% (Itharat et al., 2004). That means the extract of herbs that shows cell viability earlier of this range is suitable for human consumption and is not harmful. Then, according to the data from Table 2, the concentration at 37.5 µg/ml of ginger extract did not have inhibition effect on normal cells viability. Suggesting this concentration is not harmful for human body. Increased concentration of flavonoids through CO_2 enrichment has the potential to enhance the production and quality of medicinal plants. Increases in the levels of phenolic and flavonoid components of *Populus tremuloides* by CO_2 enrichment method has been reported by Lindroth et al. (1993). The results of our previous study indicated that

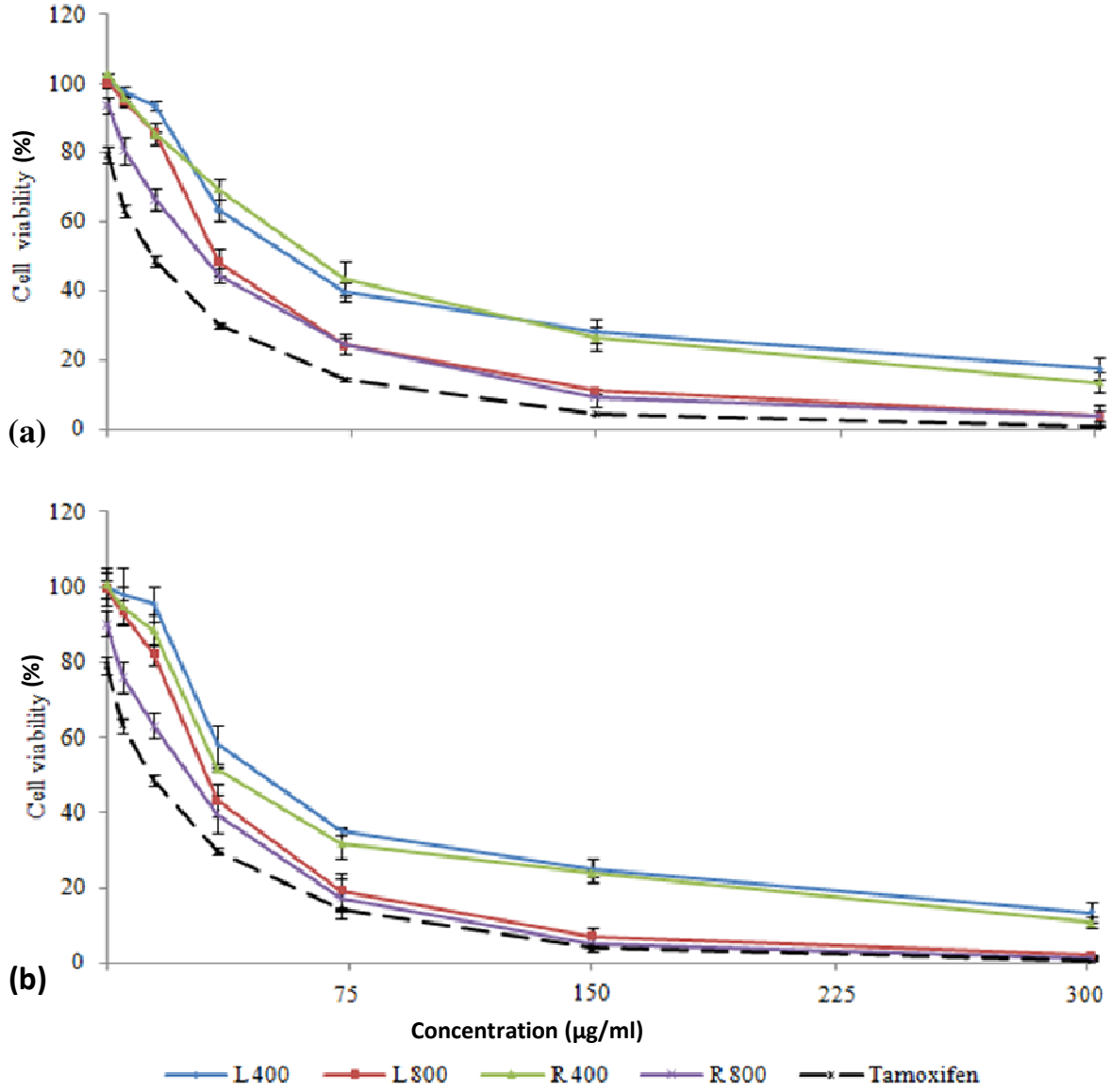

Figure 3. Dose-dependent anticancer of ginger extracts (a: Halia Bentong; b: Halia Bara) towards MDA–MB–231 cell line as determined by the MTT assay. Values are expressed in the mean of triplicate measurements ± SEM. L and R respectively are leaves and rhizomes. 400 and 800 µmol/mol represent ambient and elevated CO_2 concentration.

increasing the CO_2 concentration from 400 to 800 µmol/mol resulted in enhanced quercetin, catechin, kaempferol and fisetin levels in the leaves and rhizomes of Halia Bentong and Halia Bara and following that, the antioxidant activity in young ginger extracts could also be improved (Ghasemzadeh et al., 2010c). This finding in our previous study can support this current finding that showed the increase of anticancer effect of ginger extracts with increasing CO_2 concentration (Table 2). Leaves and rhizomes extract of both varieties did not show inhibition effect on normal cell viability. However, normal cell treated with extracts of enriched ginger (elevated CO_2) showed lower viability compared to non enriched ginger (ambient CO_2).

Conclusion

Cancer is one of the extensive diseases in humans and there is substantial scientific and commercial attention in continuing discovery of new anticancer agents from natural product sources. Currently, about 50% of drugs used in clinical trials for anticancer activity were isolated from natural sources such as herbs and spices or are

Table 3. IC_{50} values of ginger extracts towards MCF–7 and MDA–MB–231 cancer cell lines as determined by the MTT assay.

CO_2 (µmol/mol)	Varieties	Plant parts	MCF–7	MDA–MB–231
400	H. Bentong	Leaves	51.4±1.3[a]	56.1±2.05[b]
		Rhizomes	52±2.1[a]	62.61±1.6[a]
	H. Bara	Leaves	36.8±1.32[c]	46.87±0.45[c]
		Rhizomes	46.8±1.17[b]	39.2±1.8[d]
800	H. Bentong	Leaves	29.75±1.37[d]	34.6±2.16[e]
		Rhizomes	35.2±1.86[c]	32.81±1.05[e]
	H. Bara	Leaves	27.31±2.01[e]	32.85±0.89[e]
		Rhizomes	25.3±0.54[f]	30±0.8[f]
Positive control	Tamoxifen		19.1±0.74	22.61±0.86

All analyses are the mean of triplicate measurements ± standard deviation; Results expressed in µg/ml; Means not sharing a common letter were significantly different at $P \leq 0.05$.

related to them (Newman and Cragg, 2007). A number of active compounds such as flavonoids, diterpenoids, triterpenoids and alkaloids have been shown to possess anticancer activity. The results showed strong inhibitory activity of Malaysian young ginger varieties on human breast cancer cells (MCF–7 and MDA–MB–231). Our results in this study indicate that some compounds in Malaysian young ginger varieties posses anticancer activities and may contribute to the therapeutic effect of this medicinal herb. According to the report of the American National Cancer Institute (NCI), the criteria of anticancer activity for the crude extracts of herbs is an $IC_{50} \leq 30$ µg/ml (Itharat et al., 2004). Thus, according to the results from current study seems that enriched ginger varieties by elevated CO_2 concentration could be employed in ethno-medicine for the management of breast cancerous diseases. Therefore, more focused clinical studies are necessary to establish whether these varieties can be exploited to reach cancer blocking or remedial effects in human body.

ACKNOWLEDGEMENTS

The authors are grateful to the Ministry of Higher Education Malaysia and the Research Management Centre of UPM for financing this work under the Fundamental Research Grant Scheme FRGS/PHASE1-2009/FUNDAMENTAL SCIENCE/UPM/ (01-11-08-646FR).

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