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Cytotoxic Properties of Selected *Etlingera* spp. and *Zingiber* spp. (Zingiberaceae) Endemic to Borneo

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ABSTRACT

Zingiberaceae are known as valuable herbs with an important role in the prevention and treatment of various diseases. More than 300 species of Zingiberaceae were documented in Borneo. In this study, methanolic extracts of three species of Zingiberaceae (namely, *Etlingera velutina*, *Etlingera belalongensis* and *Zingiber vinosum*) were analysed for their total phenolic and flavonoid contents and cytotoxic activity *in vitro*. The cytotoxic activities of these extracts were tested against several cancer cell lines, such as hormone dependent breast cancer (MCF-7), non-hormone dependent cancer (MDA-MB-231), ovarian cancer ($CaOV_3$) and cervical cancer (Hela) using MTT assay. Crude extracts from rhizome of *E. belalongensis* and *E. velutina* showed significant cytotoxic activity against MDA-MB-231 cell line proliferation, with IC_{50} values (concentration which inhibit 50% of cell population) of 51.00±4.24 µg/ml and 67.00±9.89 µg/ml, respectively. The methanol extracts were further analysed for the cell cycle analysis using flow cytometry. The results showed that the *Etlingera* species exhibited higher antioxidant activity and stronger cytotoxic activity in selected cancer cell lines, with the highest cell death accumulated in G1 phase as compared to *Zingiber* species. Thus, polyphenol phytochemicals could be the major contributors to the cytotoxic activity of these species. As a conclusion, tropical gingers in Borneo investigated in this study have the potential to be developed as anticancer remedies.

Keywords: Zingiberaceae, *Etlingera* spp., *Zingiber* spp., total phenolic and flavonoid contents, cytotoxic

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INTRODUCTION

Cancer is one of the predominant killers in the world and it represents a real crisis for public health worldwide. According to the statistics by WHO in 2005, out of 7.6 million of deaths worldwide, about thirteen percent were caused by cancer (WHO, 2005) and the number increases by years (WHO, 2008). The use of medicinal plants as an alternative method to cure cancer has been established by WHO since 1978 (WHO, 1978). Several plant-derived compounds are currently successfully employed in cancer treatment with plant products as the main sources of drugs (Hernandez-Ceruelos *et al*., 2002).

Herbs, fruit and vegetables contain a variety of phytochemicals, including flavonoids which have antioxidant and anticancer properties. Of the estimated 250,000 – 500,000 plants species, only a small percentage has been investigated phytochemically and an even smaller percentage has been properly studied in terms of their pharmacological properties (Rates, 2001).

Zingiberaceae consist of 50 genera and 1,500 species worldwide and at least 20 genera and 228 species are found in Malaysia. Generally, Zingiberaceae are valuable herbs with an important role in the prevention and treatment of diseases (Wang & Duan, 1999) and an ingredient in more than half of all traditional Chinese medicines. Besides that, species from the family Zingiberaceae are often used in 'Jamu' (Indonesian traditional herbal medicine). In the present study, the cytotoxic activities of the methanolic extracts of the samples were tested against several cancer cell lines, such as hormone-dependent breast cancer (MCF-7), non-hormone-dependent breast cancer (MDA-MB-231), ovarian cancer ($CaOV_3$), and cervical cancer (Hela) by using MTT assay. In addition, the cell cycle analysis was also conducted to study whether these extracts could affect the cell cycle events. Zingiberaceae have been shown to display anticancer properties. Several Zingiberaceae samples, extracted with different solvents (i.e. petroleum ether, chloroform and ethanol), displayed strong anti-tumour activity (Vimala *et al*., 1999; Murakami *et al*., 1993). Murakami *et al.* (1994) reported that active constituents isolated from *Zingiber cassumunar* Roxd displayed a promising new anti-cancer drug. Besides that, Zerumbone which was extracted from *Zingiber zerumbet* also displayed a significant anticancer activity (Abdul *et al*., 2008). In another study, *Zingiber officinale* varieties (Halia Bara and Halia Bentong) were found contain seven important flavonoids and these compounds have been shown to display dominant anticancer activity (Ghasemzadeh *et al*., 2010).

MATERIAL AND METHODS

Plant Material and Sample Preparation

Fresh samples were collected from Tawau Hills Park and Crocker Range Park in Sabah. The herbarium voucher specimens were identified and deposited by Mr. Januarius Gobilik from Forest Research Centre, Sandakan, Sabah. All the plants were frozen at -20^{0}C and lyophilized for 48 h at 13.3 Pa

in freeze-dryer (Labconco, vacuum pump RV12, Edwards). After drying, the samples were ground and stored in air-tied plastic bags for further use.

Extraction

The freeze-dried samples were ground into fine powder. Fifty gram of the sample was extracted with 100 mL methanol for three days. The resulted slurry was vacuum-filtered through a Whatman No. 3 filter paper and the filtrate was subjected to vacuum rotary evaporation (Rotavapor model R110, Buchi, Flawil, Switzerland) at 40^{o}C. The concentrated methanolic extracts were stored in amber glass vials at 4^{0}C until used.

Determination of Total Phenolic Contents

Total phenolic content was determined using Folin-Ciocelteu, as described by Velioglu *et al*. (1998) with slight modification. Follin-ciocelteu reagent was diluted 10-folds with distilled water. Three hundred microliters of the extract was mixed with 2.25 ml of Folin-Ciocalteu reagent solution. The solution was mixed well using vortex and then allowed to stand for 5 min in the room temperature; 2.25 ml of the sodium bicarbonate (60 g/L) solution was added to the mixture. After 90 min in the room temperature, absorbance was measured at 725 nm using a spectrophotometer. Gallic acid was used as a standard. A standard concentration curve from 1 mg/ml to 5 mg/ml at 1 mg/ml interval was plotted. The total phenolic content of the extracts was determined from the standard graph. The results are expressed as mg gallic acid equivalent.

Determination of the Total Flavonoid Contents

The determination of the total flavonoids content was performed according to the colorimetric assay by Kim *et al*. (2003), with a slight modification. Distilled water (4ml) was added into the extracts (1 ml). Then, 5% sodium nitrite solution (0.3ml) was added, followed by 10% aluminium chloride solution (0.3ml). Test tubes were incubated at ambient temperature for 5 min, and this was followed by additions of 2 ml of 1 M sodium hydroxide and 2.1 ml of distilled water into the mixture after 6 minutes. The mixture was thoroughly vortexed and the absorbance of the pink colour developed was determined at 510 nm. The calibration curve was prepared with catechin and the results were expressed as mg catechin equivalents (CEQ)/100 g sample.

Cell Culture

MCF-7, MDA-MB-231, $CaOV_3$ and Hela cell lines were obtained from American Type Culture Collection (ATCC, USA). The RPMI 1640 Medium (Gibco, USA), supplemented with 10% of foetal calf serum (Gibco, USA) and 1% of penicillin streptomycin (Gibco, USA), was used to culture cell lines in 25 ml flasks (Nunc, Denmark), and incubated in 5% CO_2 incubator (Sanyo, Japan) at 37^{0}C.

MTT Assay for Cell Proliferation

The cytotoxic effects of the plant extracts against previously mentioned human cancer cell lines were determined by a rapid colorometric assay, using MTT bromide and compared with an untreated control (Mosmann, 1983). The concentration of MTT solution used was 5 mg/ml. The MTT solution was prepared by dissolving 0.05 g of the MTT powder in 10 ml PBS (pH 7.2). This solution was filtered through a 0.2 µm filter and covered with aluminum foil to avoid exposure to light. This solution was stored at 4^0C prior to use. Solubilisation buffer was prepared by dissolving 10% SDS (Sodium dodecyl sulphate) in PBS solution. The 96-well microtiter plates, containing cell culture solution, were removed from the incubator after 72 hour of incubation. 10 µL of 5 mg/ml MTT solution was added into each well, including the control wells. After adding the sample extracts, a new medium was added to make up the final volume of 100 µL in each well. The plate was incubated in a 5% CO_2 incubator (Sanyo, Japan) at 37°C for 72 h. Then, 20 µL of MTT reagent was added into each well. This plate was incubated again for 4 h in CO_2 incubator (Sanyo, Japan) at 37^0C. Subsequently, 100 µL of solubilization solution was added into each well to dissolve the remaining purple colour formazon crystals. The cell was then left overnight in 37^0C CO_2 incubator. Finally, the absorbance of the formazan was determined at 550 nm using an ELISA reader (LX-800). Meanwhile, Vincristine (anticancer compound) was used as a positive control.

Cell Cycle Analysis

The cell cycle was analyzed using flow cytometry (FCM) (Model Cyan ADP, Denmark) analysis (Yuan *et al*., 2004). A total of 2 x 10^5 cells were harvested from the control culture and the cells treated with the extracts after 72 hour of the incubation period. The cells were washed twice with PBS and fixed in 70% ethanol for 2 hours. The samples were then concentrated by removing ethanol. Cellular DNA was stained with 500µl of 10µg/ml propidium iodide in 100µg/ml of RNase for 30 minutes in the dark and in room temperature.

Statistical Analysis

All the experiments were carried out in 3 independent experiments and all the data were presented as a mean ± standard deviation of mean using SPSS version 15.0. The data were statistically analysed by one-way ANOVA and Duncan's test. A significant difference was considered at the level of $p < 0.05$.

RESULTS AND DISCUSSION

Many studies have been conducted to determine the contribution of phytochemicals by plants as antioxidants and anti-cancer agents. Plant extracts that are rich in polyphenols and other phytochemicals may contribute to antioxidant and anti-cancer activities. There are many types of phytochemicals including flavonoid, phenolic, steroid, terpenoids and alkaloid. Phenolic and flavonoid, which occur in plants, are very dominant phytochemicals (Manach, 2004) and these compounds

have the potential to benefit human health. In fact, these compounds have been shown as a group of chemicals that may possess antioxidant activity (Shahidi & Wanasundara, 1992) and have physiological functions that include anti-mutagenic and anticancer properties (Kono *et al*., 1995). This effect could be due to their redox properties (Zheng & Wang, 2001), which play important roles in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides.

The total phenolics contents in the methanolic extract were in the range of 41.7 mg GAE/g to 5.3 mg GAE/g dry weight (dw). The rhizomes of *Z. vinosum* and *E. velutina* displayed the highest total phenolic content ($p<0.05$), while the lowest was shown by the stem of *E. velutina*. For all the species tested, the results showed that the phenolic contents were distributed more abundantly in the rhizome as compared to the stem (Table 1). Past studies have also shown that the antioxidant activities of the ginger species were concentrated in the rhizomes (Jitoe *et al*., 1992; Habsah *et al*., 2000). Meanwhile, the rhizome of gingers has been reported to contain antioxidant activity comparable to that of α-tocopherol (Zaeoung *et al*., 2005). The isolated compounds of zerumbone and kaempferol from *Z. aromaticum* showed a potent antioxidant activity (Usia *et al*., 2004). Similarly, Akiyama *et al*. (2006) also reported that diarylheptanoid isolated from *Z. ottensii* displayed a better scavenging activity as compared to L-ascorbic acid or α-tocopherol.

In this experiment, the concentration of flavonoids in the ginger extract was expressed as the mg of catechin equivalents per g of the extract (Table 1). For the total flavonoid content determination, the results showed the same trend with the total phenolic content. The total flavonoid in the

TABLE 1
Total phenolic and total flavonoid contents of methanolic extracts from selected *Etlingera* and *Zingiber* species

Samples	Part	% yield extract	Total phenolic (mg GAE/g)[a]	Total flavonoid (mg CE/g)[b]
E.belalongensis	rhizome	25.76	17.07 ± 0.32[b]	3.77 ± 0.15[a]
	stem	25.53	10.07 ± 0.25[b]	2.57 ± 0.15[a]
E.velutina	rhizome	30.31	25.03 ± 0.46[c]	7.63 ± 0.06[a]
	stem	29.18	5.30 ± 0.10[a]	2.80 ± 0.20[a]
Z.vinosum	rhizome	35.63	41.70 ± 1.11[d]	8.50 ± 0.20[a]
	stem	27.97	27.97 ± 0.93[c]	3.37 ± 0.06[a]

Total phenolic content was expressed as mg gallic acid equivalent in 1 g of dry sample. Values are presented in mean ± S.D (n=3); those with different letters are significantly different at $p < 0.05$, as measured by Tukey HSD test. ANOVA compares the values between rhizomes and stems of each species.
[a] Total phenolic was expressed as gallic acid equivalent (GAE) in 1 g of dry sample.
[b] Total flavonoid was expressed as catechin equivalent (CE) in 1 g of dry sample.
E = Etlingera; Z = Zingiber.

methanolic extracts was in the range of 8.5 mg GAE/g to 1.97 mg GAE/g dry weight (dw). The rhizome of *Z. vinosum* displayed the highest total flavonoid content ($p<0.05$), while the stem of *E. velutina* showed the lowest. From the previous study, the flavonoid compounds in Zingiberaceae have been reported to possess strong antioxidant properties (Cai *et al*., 2006), while the major component of the essential oil extracted from *Z. zerumbet* showed a more promising use as anti-inflammatory and chemotherapeutic agents (Tanaka *et al*., 2001). Masuda *et al.* (1991) reported the occurrence of several sesquiterpenoid and flavonoid in the rhizome of *Z. zerumbet*.

As for the cytotoxicity study, each sample was screened for cytotoxicity against several cancer cell lines, such as MDA-MB-231, MCF-7, CaOV3 and Hela using MTT assays. The cell killing and inhibition of proliferation could be explained by the reduction in the number of cells by particular agent (extract). The results showed that the sample extracts displayed a cytotoxic activity against MDA-MB-231 cancer cell line, with IC_{50} (i.e. the concentration that inhibits 50% of cell lines) ranging from 51 µg/ml to 96 µg/ml after 72 hours of treatment (Table 2). The possible mechanism of the cytotoxic activity of the plant extracts was further investigated using cell cycle analysis through flow cytometry. The results showed that all the extracts arrested cancer cells in sub-G1 phase (Table 3). The results for the samples showed the same trend with a positive control (Vincristine) and displayed significant differences as compared to the control ($p<0.05$).

Numerous cell cycle analyses have proven that good anti-cancer drugs arrested the cell in sub-G1 phase. In agreement to this, Kim (2005) reported that [6]-gingerol isolated from *Z.officinale* inhibited an angiogenesis of human endothelial cells and caused the cell to arrest in the sub-G1 phase. This result was also supported by Choi and Kim (2008) who had shown that daidzein (flavonoid) caused cells to arrest

TABLE 2
IC_{50} values of the methanol extracts of selected *Etlingera* and *Zingiber* species against MDA-MB-231, MCF-7 and CaOV3 cell line

Samples	Plant Part	MDA-MB-231	MCF-7	CaOV3	Hela
E. belalongensis	Rhizome	51.00±4.24^{c}	>100	>100	>100
	Stem	74.00±2.83^{c}	>100	>100	>100
E. velutina	Rhizome	67.00±9.89^{c}	>100	>100	>100
	Stem	89.50±14.85^{d}	>100	>100	>100
Z. vinosum	Rhizome	89.00±7.78^{d}	>100	>100	>100
	Stem	96.00±2.83^{d}	>100	>100	>100
Vincristine		13.00±3.14^{b}	8.50±3.41^{a}	17.50 ±0.82^{b}	3.00±0.73^{a}

E = Etlingera; Z = Zingiber
Values are expressed as mean ± standard deviation (n=3), in which those with different letters are significantly different at $p<0.05$

TABLE 3
Cell cycle analysis of MDA-MB-231 treated with methanol extracts from selected *Etlingera* and *Zingiber* species for 72 hours

Samples	Phase	Control	Treatment
**E. belalongensis* (stem)	sub-G1	5.72±0.18[a]	**68.72±2.03[c]**
	G0/G1	80.22±0.21[d]	25.67±1.75[b]
	S	2.75±0.30[a]	2.46±0.33[a]
	G2/M	11.61±0.16[b]	3.46±0.16[a]
**E.velutina* (rhizome)	sub-G1	5.45±0.07[a]	**83.44±0.63[d]**
	G0/G1	80.3±0.33[d]	13.57±0.48[b]
	S	2.68±0.46[a]	1.86±0.23[a]
	G2/M	11.57±0.16[b]	1.36±0.06[a]
**Z.vinosum* (rhizome)	sub-G1	5.72±0.61[a]	**56.86±1.11[c]**
	G0/G1	76.49±0.58[d]	38.39±0.59[b]
	S	2.75±0.42[a]	1.71±0.02[a]
	G2/M	11.61±0.52[b]	3.15±0.33[a]
Vincristine	sub-G1	5.79±0.23[a]	**90.07±0.43[d]**
	G0/G1	79.79±0.07[d]	5.74±0.26[a]
	S	2.73±0.31[a]	2.07±0.18[a]
	G2/M	11.69±0.19[b]	2.12±0.24[a]

Values are presented in mean ± S.D (n=3); those with different letters are significantly different at $p < 0.05$, as measured by Tukey HSD test.
*Concentration of sample = 80 µg/ml.
Concentration of positive control = 5µg/ml.

in G1 phase in the human breast cancer cells. In addition, green tea polyphenol has been shown to suppress the proliferation of MDA-MB-231 and accumulated the cell at G1 phase (Thangapazham *et al*., 2006). Polyphenol that presents in the species may contribute to the cytotoxicity activities in the cancer cell lines. In conclusion, the methanolic extracts of selected *Etlingera* and *Zingiber* species endemic to Borneo have a great potential to be developed as an anti-cancer agent and are applicable to food and herbal products.

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