ANTIOXIDANT AND ANTI-PROLIFERATIVE PROPERTIES OF SELECTED ZINGIBERACEAE (*ETLINGERA* SPP. AND *ZINGIBER* SPP.) ENDEMIC TO BORNEO

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries and references, which have been acknowledged.

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ABSTRACT

ANTIOXIDANT AND ANTI-PROLIFERATIVE PROPERTIES OF SELECTED ZINGIBERACEAE: *ETLINGERA* SPP. AND *ZINGIBER* SPP. ENDEMIC TO BORNEO

Zingiberaceae species have been used in traditional medicine since many years. First, ethnobotany of Zingiberaceae was studied in the past literature. The secondary data reviewed showed that about 54 species of Zingiberaceae from 12 genera were used as traditional medicine. *E. belalongensis*, *E. velutina*, *Z. pseudopungens* and *Z. vinosum* were selected for further investigation. Secondly, preliminary phytochemical screening tests using test tube and thin layer chromatography (TLC) were performed. The results showed that flavanoid, alkaloid and saponin were present in the sample extracts. The total phenolic and flavonoid content of sample extracts were evaluated by using folin-ciocalteu and aluminium chloride colorometric method, respectively. The total phenolic and flavonoid content for methanolic extract were in the range of 5.3-41.7 mg GAE/g and 1.9-8.5 mg CE/g, respectively. Rhizome of *Z. vinosum* displayed the highest value of total phenolic and flavonoid content. Evaluation of antioxidant activity was conducted using free radical-scavenging assay (DPPH), radical scavenging assay (ABTS) and ferric reducing power (FRAP assay). Rhizome of *E. belalongensis* had the highest DPPH with 49.5 ± 3.1 mg AA/g in terms of AEAC (acid ascorbic equivalent antioxidant activity). However, rhizome of *E. velutina* showed the highest for ABTS with 3.2 ± 0.18 mM trolox equivalent/mg samples. FRAP value of *E. velutina* (stem) displayed high ferric reducing power with a value of 2.5 ± 0.16 mmol/g. Antioxidant activity of methanolic extracts was higher than aqueous extracts except for the ABTS assay. The third part of this study was to determine the cytotoxicity effects of the extracts by using MTT assays against several cancer cell lines such as MCF-7 (hormone dependent breast cancer), MDA-MB-231 (non-hormone dependent breast cancer), Hela (cervical cancer) and $CaOV_3$ (ovarian cancer). The cancer cell lines were treated by sample extracts with concentration of 60-140 µg/ml at 48 and 72 hours. The sample extract only showed positive effect to the MDA-MB-231 cell lines with IC_{50} in between 51-96 µg/ml except for *Z. pseudopungens*. The sample extracts that were effective to MDA-MB-231 were further analysed by cell cycle analysis for 48h and 72h by flow cytometry. Analysis of cell cycle showed cells were arrested in the sub-G1 phase. *Etlingera* species exhibited the highest antioxidant activity and anti-proliferative activity as compared to *Zingiber* species. The correlations indicated that the total amount of polyphenols is the major contributor to antioxidant and anti-cancer activity. With promising antioxidant and anti-proliferative properties, methanolic extracts of *E. belalongensis*, *E. velutina* and *Z. vinosum* have great potential to be developed into natural remedies based on herbal products, applicable to the food and nutraceutical industries.

ABSTRAK

Spesies Zingiberaceae telah digunakan dalam bidang perubatan tradisional sejak dulu lagi. Bahagian pertama kajian adalah mengenai etnobotani Zingiberaceae melalui kajian-kajian yang terdahulu. Terdapat 54 spesies Zingiberaceae yang terdiri daripada 12 genus telah digunakan dalam perubatan tradisional. Etlingera belalongensis, Etlingera velutina, Zingiber pseudopungens dan Zingiber vinosum adalah spesies Zingiberaceae yang terpilih untuk dikaji. Bahagian kedua adalah ujian awal saringan fitokimia telah dilakukan dengan menggunakan tabung uji dan kromatogram lapis tipis (TLC). Alkaloid, flavanoid dan saponin didapati wujud di dalam sampel tersebut. Jumlah kandungan fenolik dan flavonoid dinilai menggunakan kaedah folin-ciocalteu dan aluminium klorida kolorometrik. Jumlah kandungan fenolik dan flavanoid bagi ekstrak methanol adalah masing-masing diantara 5.3-41.7 mgGAE/g dan 1.9-8.5 mg CE/g. Rizom Z.vinosum menunjukkan nilai tertinggi bagi jumlah kandungan fenolik dan flavonoid. Tiga kaedah digunakan untuk menentukan sifat antioksida bagi sampel ekstrak iaitu penghapusan radikal bebas (DPPH), penghapusan radikal (ABTS) dan penurunan ferik (FRAP). Rizom E. belalongensis menunjukkan nilai tertinggi bagi DPPH iaitu 49.5 $\pm$ 3.1 mg AA/g iaitu mewakili AEAC (asid askorbik bersamaan dengan aktiviti antioksida). Manakala, rizom E. velutina menunjukkan nilai tertinggi bagi ABTS dengan nilai 3.2$\pm$0.18 mM bersamaan dengan trolox/mg sampel. Nilai FRAP iaitu kuasa penurunan yang tertinggi adalah 2.5 $\pm$ 0.16 mmol pada batang E.velutina. Keputusan aktiviti antioksida bagi ekstrak metanol adalah lebih tinggi berbanding ekstrak air kecuali bagi kaedah ABTS. Bahagian ketiga kajian ini adalah penentuan kesan sitotoksik menggunakan kaedah penurunan MTT terhadap beberapa sel kanser seperti MCF-7 (sel kanser payudara yang bergantung pada hormon), MDA-MB-231 (sel kanser payudara yang tidak bergantung pada hormon), Hela (sel kanser servik) dan $CaOV_3$ (sel kanser ovari) pada kepekatan 60-140 µg/ml sampel ekstrak methanol selama 48 dan 72 jam. Bagaimanapun, aras ketoksikan kajian in vitro ini hanya berkesan pada sel MDA-MB-231 dengan julat IC_{50} diantara 51-96 µg/ml bagi semua sampel ekstrak kecuali Z.pseudopungens. Seterusnya, analisis kitar sel telah dilakukan terhadap sel MDA-MB-231 oleh ekstrak yang berkesan melalui 'flow cytometry' pada masa 48 dan 72 jam. Hasilnya, sel mati didapati banyak berkumpul di fasa G1. Spesies Etlingera menunjukkan aktiviti antioksida dan anti-kanser yang tinggi berbanding spesies Zingiber. Jumlah polifenol yang ada di dalam sampel merupakan penyumbang terbesar bagi kedua-dua aktiviti ini. Oleh yang demikian, ekstrak metanol bagi spesis seperti E.belalongensis, E.velutina dan Z.vinosum mempunyai potensi yang tinggi untuk dijadikan ubat semulajadi berasaskan herba serta boleh diaplikasikan di dalam makanan dan industri farmaseutikal.

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DPPH = 1,1-diphenyl-2-picrylhydrazyl

ABTS = 2-2′-azinobis-3-ethylbenzothioazaline-6-sulphonate

FRAP = ferric reducing ability of plasma or ferric reducing / antioxidant power

TPTZ = 2,4,6-tripyridyl-s-triazine

GAE = gallic acid equivalent

CE = catechin equivalent

IC_{50} = half maximal inhibitory concentration

MTT = (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Rf = retention factor

CHAPTER 1

INTRODUCTION

1.1 Background

Borneo is the third largest island in the world with an area of 740,000 km^2. The island is comprised of three neighboring countries: Malaysia (Sabah and Sarawak), Brunei Darussalam and Indonesia (Kalimantan). Being one of the world's hotspots for biodiversity and home to several World Heritage Sites, it is one of the most exciting places on earth to study the richness of plants life. One of the most interesting plant families in this region is the ginger family, Zingiberaceae. Borneo is home to more than 300 ginger species (Poulsen, 2006). Members of Zingiberaceae are great economic and cultural importance as ornamental plants, vegetables and ingredient in traditional medicine (Ibrahim, 1999). Since the Zingiberaceae distribution is focused mainly in Borneo, this geographic region is the target of this study. In Sabah, the diversity of Zingiberaceae remains largely unknown (Mood, 1997), although 156 species have already been described (Gobilik and Yusoff, 2005). On the other hand, more than 160 species are found in Peninsular Malaysia, and no fewer than 155 species in Sarawak, with more waiting to be discovered and described. Besides *Etlingera* and *Zingiber*, other major genera of gingers found in Borneo are *Amomum*, *Boesenbergia*, *Alpinia*, *Hornstedtia*, *Globba* and *Plagiostachys* (Gobilik and Yusoff, 2005).

1.2 Zingiberaceae

Zingiberaceae is a family of herbs consisting of 50 genera and 1,500 species of gingers known worldwide. At least 20 genera and 228 species are found in Malaysia (Ibrahim, 1999). Zingiberaceae is characterized by its aromatic parts, and the plants are used for spices, made into condiments, essential oils and medicine, and grown as ornamentals. The Indo-Malayan region is the centre of diversity for the Zingiberaceae. The majority of the species prefer shaded and moist habitats in the wild. Generally, Zingiberaceae is medicinally valuable and has been used for thousands of years in the prevention and treatment of diseases (Wang and Duan, 1999), and as ingredients in most traditional Chinese medicines (Awang, 1992). Some species from the Zingiberaceae family are often used as ingredients in '*Jamu*', a type of Indonesian traditional herbal medicine. Researches over the years have revealed that gingers from the family Zingiberaceae are rich in antioxidative properties and is believed to have anti-cancer potential. To date, studies on the antioxidant properties of ginger species were confined to rhizomes (Jitoe *et al*., 1992, Habsah *et al*., 2000). The rhizomes have been reported to contain antioxidants comparable to α-tocopherol (Zaeoung *et al*., 2005). For over 15 years the occurrence and chemical classes of anticancer agents from plants have been developed. Species of Zingiberaceae from Borneo, especially in Sabah, are unexplored, and have good potential as anticancer agents.

1.3 Antioxidants

Antioxidants are chemical compounds that bind to free radicals, preventing these radicals from damaging healthy cells. It is important to prevent damage caused by free radicals. Recently, much attention has been placed on the presence of natural antioxidative compounds in plants. It also possessed radical scavenging activity and reducing power would improve human health. As mentioned earlier, the antioxidative potential of a substance is measured by its scavenging activity and its reducing power. Scavenging activity refers to the trapping of free radical by the antioxidants in extracts. The reducing power refers to the ability of the antioxidants in extracts to reduce Fe^{3+} to Fe^{2+}. Natural antioxidants have been reported to occur in many parts of plants such as the leaves, bark, stems and roots (Cai, *et al*., 2006). Various phytochemicals, especially the natural antioxidants from tea, spices and herbs, have been intensively studied for their effects on health. In addition, it has been determined that the antioxidant effect of plant products is mainly due to the occurrence of phenolic compounds, such as flavonoids, phenolic acids, tannins and phenolic diterpenes (Rao *et al*, 2003). Polyphenol had been shown to exhibit antioxidant properties, which is related to their potential for anti-cancer properties (Birt *et al*, 2001).

1.4 Anticancer

Cancer is one of the most predominant causes of death in the world today. According to the 2005 statistics from WHO, about 7.6 million which represents 13 % of world death number is caused by cancer. In 2008, over twelve million people were diagnosed with this disease. Cancer incidense shows a rapid increase annually and the number is estimated to double between the years 2000 and 2020 and triple by 2030 (WHO, 2005 and 2008). Thus, initiatives are needed to decrease the high number of reported deaths caused by cancer each year. The importance of investigating the methods uses of medicinal plants as alternative to cure cancer was established by WHO in 1978 (WHO, 1978). In-depth research has led to the discovery of several potent plant-derived compounds that are currently successfully implemented in cancer treatment in the form of medicinal drugs (Hernandez-Ceruelos *et al.*, 2002). An anticancer compound is defined by the effectiveness of the compound in treating cancer. Additionally, plant constituents that have the potential to kill cancerous cells are describes as cytotoxic. Herbs, fruits and vegetables contain a variety of phytochemicals, including phenolic compounds and flavonoids that have been reported to display potentially strong anticancer activities. However, 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). This study aims to screen the effects of these extracts in cytotoxicity using several cell line cancer and by observing the cell death after treatment.

1.5 Problem Statement

Despite much advancement in cancer therapy, some cancer patients are still ineffectively treated and they become resistant and recurring. There are no treatments that can completely cure cancer. Synthetic drugs have been used as first line therapy for cancer, but they cause side effects. Therefore, alternative ways to cure cancer without any side effects can be found from natural resources. Plant-based medicines have definitely been found to play an important role in this type of treatment as the interaction between phytochemicals and cancer cells has been studied extensively.

Two parts of gingers have always been consumed by local people: the rhizome and the stem. The potential of Borneo's Zingiberaceae, especially in Sabah, with plants rich in phytochemical constituents and biological activities, has been studied. Thus, this research investigates the probability that phytochemicals might be accumulate in gingers and contribute to antioxidant and anti-proliferative activity. Much research has been done on Zingiberaceae, but further studies of the chemistry in terms of biological activity of Zingiberaceae endemic to Sabah are still scarce. Thus, the findings can add new knowledge about potential health benefits of these particular species of Zingiberaceae. Moreover, this study will be great interest to researchers' worldwide who are studying the advantages of medicinal plants for cancer treatment.

1.6 Objectives

In this study, the antioxidant and anti-proliferative potential of four endemic Zingiberaceae (*Etlingera belalongensis*, *Etlingera velutina*, *Zingiber pseudopungens* and *Zingiber vinosum)* were evaluated. The purpose of this study is to determine which plants have high potential to be anticancer drug in the future. To fulfill this purpose, several aspects such as ethnobotany, phytochemical constituents and antioxidant and antiproliferative potential were taken into account. This study is based on the following objectives;

1. To document and review indigenous traditional knowledge of Zingiberaceae through secondary sources and literature regarding the usage as traditional medicine.
2. To evaluate phytochemical contents of the Zingiberaceae extracts by phytochemical screening and thin layer chromatography (qualitative analysis), spectrophotometric methods and HPLC methods (quantitative analysis).
3. To determine the antioxidant activities (DPPH-free radical scavenging activity, ABTS- free radical scavenging activity and FRAP-ferric reducing assay) of the methanolic and aqueous extracts
4. To determine the cytotoxic effects of methanolic plant extracts on several cancer cell lines (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 and cell cycle analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Product and Cancer Discovery

Natural products contribute considerably to the profitability of many pharmaceutical and biotechnology companies. According to the World Health Organization, more than 90% of current therapeutic classes are derived from natural products and interestingly, roughly two-thirds to three quarters of the world's inhabitants rely upon medicinal plants for primary health care (WHO, 2002).

Cancer has emerged as a major public health problem in developing countries, matching its effect in industrialized nations (Parkin *et al.*, 2001). People in South East Asian countries have a much lower risk of colon, gastrointestinal, prostate, breast and other cancers than their Western counterparts, and it is thought that components of their diet may play significant role in protection (Dorai and Aggarwal, 2004). There is considerable interest in identifying potential chemoprotective agents present in foods consumed by the human population. In prior *in vitro* studies, the water or organic solvent extract of ginger was shown to process antioxidative and anti-inflammatory properties. Doxorubicin (Figure 2.1), which was originally derived from *Streptomyces peucetius,* is one of the natural products that was launched in United States in 2002. Its mechanism of action is inhibition of topoisomerase II (Butler, 2004).

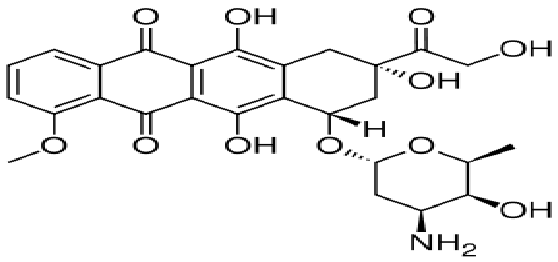

Figure 2.1: Chemical structure of Doxorubicin

One of the most significant examples is the vinca alkaloid family isolated from the periwinkle or *Catharanthus roseus,* which is found in the rain forests of Madagascar (Himes, 1991). The introduction of the vinca alkaloid viscristine (Figure 2.2) was responsible for an increase in the rate of recovery from Hodgkin's disease and some forms of leukemia (DeVita *et al.*, 1970). One active agent known as epipodophyllotoxin (Figure 2.3), which was isolated from the genus of *Podophyllum* ,is known as the anticancer agent (Cragg and Newmann, 2007). Homoharringtonine (Figure 2.4), from the Chinese tree *Cephalotaxus harringtonia* var. *drupacea,* is reported as an anticancer agent in China (Lee *et al.*, 2003). Scientists have defined elements that impact the responsiveness of cancer cells to drugs commonly used as anticancer therapeutics. New studies show plant-based antioxidants: green tea extracts and black raspberry have powerful antioxidants that could prevent or slow the growth of cancer. However, many of the early plant-derived antitumor agents were fortuitous discoveries resulting from the investigation of plants for compounds with other medicinal uses.

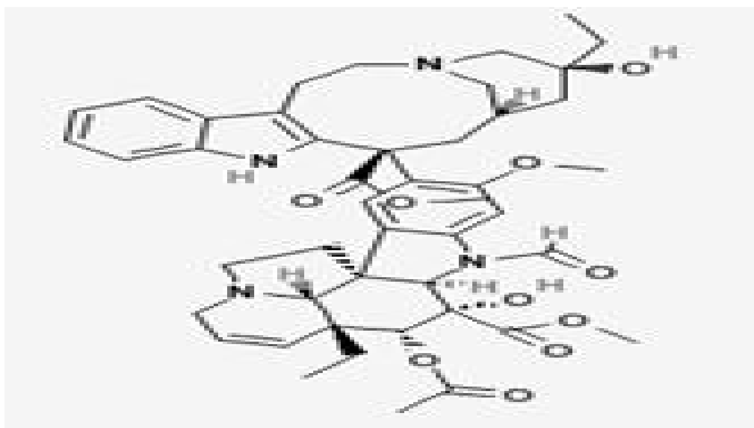

Figure 2.2: Chemical structure of vincristine