# INDUCTION OF APOPTOSIS BY *Elephantopus scaber* L. ETHANOLIC EXTRACT AND EFFECTS OF ITS INTERACTION WITH TAMOXIFEN ON HUMAN BREAST CANCER MCF-7 SPHEROIDS

**By**

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**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

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## DEDICATIONS

I would like to dedicate this thesis to my beloved parents who have raised me up with unlimited love and patience and given me the courage to embrace the challenges in my life. I would also like to extend this dedication to my grandparents, siblings and other members in my family who have also given me their unconditional support and encouragement all these years. Without the inspiration and motivation from all of you, I would not have been able to complete my studies. Thanks to all of you.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Breast cancer has become a major threat to health due to its high incidence among Malaysian women. Although various treatment options are available, resistance to treatment and the risk for health setbacks remain as the main concern of most patients. Frequently, traditional and alternative medicines such as herbs are employed by cancer patients to enhance the effect of the existing therapeutic measures. However, the ultimate efficacy of such medication would remain as a skeptical "myth" until in depth studies are carried out. Therefore, the present study was carried out to determine the cytotoxicity of *Elephantopus scaber* on the human estrogen-dependent breast cancer MCF-7 cell line and to study the potential of combining this herb with tamoxifen to treat MCF-7 cells. In this study, cytotoxicity screening using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay showed that the ethanol extract of *E. scaber* was cytotoxic towards the monolayer culture of MCF-7 and other cancer cell lines. Various cell based assays were then performed to study the mechanism of cell death caused by *E. scaber* on

MCF-7 cells. With evident apoptotic activities shown by the plant, further assessment of the cytotoxic efficacy of combining *E. scaber* with tamoxifen was initiated. In addition, multicellular tumor spheroids (MCTS) that simulate tumor *in vivo* were developed from MCF-7 to bridge the gap between monolayer culture and tumor for precise determination of cytotoxicity. Cytotoxic assays were performed to confirm the elevated resistance of MCTS cultures to treatment as compared to their monolayer counterparts. Scanning electron microscopy and acridine orange/ propidium iodide (AO/PI) staining on the other hand were used to observe the morphological changes of MCTS after treatment using *E. scaber* and tamoxifen. Then, studies on the synergistic effect of *E. scaber* and tamoxifen were carried out using the spheroidal systems. Apart from the cell-based assays, elucidation of the molecular pathways that were targeted by the herb-drug interaction was also studied using real time polymerase chain reaction (PCR) and flow cytometric protein expression studies. The results showed that a combination of *E. scaber* and tamoxifen can stimulate the estrogen dependent pathway of apoptosis that involved the activation of p-53 and caspase cascades in the MCTS cultures. As found with the other herbs with anti-cancer effect, the cytotoxic potential of *E. scaber* might also be related to its potent antioxidant activity and richness in total flavonoid and phenolic content as shown in this study. Apart from this, the hepatoprotective activity of the plant extract on alcohol-induced liver damage in mice could also be attributed to the antioxidant capacity of this extract. Most importantly, *E. scaber* ethanol extract was non-toxic as shown from various toxicity assessments. In conclusion, *E. scaber* is a non-toxic and potent anti-cancer agent, which can be used individually or in combination with tamoxifen, to treat estrogen-dependent human breast cancer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

**INDUKSI APOPTOSIS OLEH EKSTRAK ETANOL *Elephantopus scaber* L. DAN INTERAKSINYA DENGAN TAMOXIFEN TERHADAP KULTUR SFERA BAGI BARAH PAYU DARAH MANUSIA MCF-7**

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Kanser payu dara merupakan ancaman utama kepada kesihatan kerana insidennya yang tinggi di kalangan wanita Malaysia. Walaupun pelbagai jenis rawatan boleh didapati, rintangan terhadap pengubatan dan ancaman terhadap kesihatan masih menjadi kekhuatiran utama bagi kebanyakan pesakit. Lazimnya, perubatan tradisional dan alternatif seperti herba telah diguna oleh pesakit kanser untuk meningkatkan kesan terapi yang sedia ada. Walau bagaimanapun, keberkesanan mutlak bagi rawatan ini akan kekal sebagai 'mitos' melainkan penyelidikan yang lebih mendalam dilaksanakan. Oleh itu, kajian ini telah dijalankan untuk menentukan kesan sitotosik *Elephantopus scaber* ke atas sel kanser payudara MCF-7 yang bergantung kepada estrogen serta mengkaji potensi bagi penggabungan herba ini dengan tamoxifen dalam perawatan MCF-7. Dalam kajian ini, pemeriksaan sitotosik dengan kaedah MTT menunjukkan bahawa ekstrak etanol *E. scaber* mempunyai kesan sitotosik terhadap kultura mono-lapis MCF-7 dan kanser-kanser yang lain. Berikutannya, pelbagai teknik berasaskan sel telah dijalankan untuk menyiratkan mekanisme kematian sel MCF-7 setelah dirangsang oleh *E. scaber*. Berdasarkan

aktiviti apoptosis yang jelas keterampilan, penilaian terhadap keberkesanan gabungan *E. scaber* dan tamoxifen juga telah dijalankan. Sehubungan itu, sfera tumor multisel (MCTS) yang menyerupai tumor *in* vivo telah dihasilkan daripada MCF-7 untuk merapatkan jurang antara kultura mono-lapis dan tumor untuk menilai kesan sitotosik dengan lebih tepat. Ujian sitotosik telah mengesahkan peningkatan rintangan terhadap rawatan oleh kultura MCTS berbanding dengan kultura mono-lapis. Pengimbasan mikroskop elektron dan pewarnaan AO/PI pula dipakai untuk memerhati perubahan morfologi MCTS setelah didedahkan kepada *E. scaber* dan tamoxifen. Seterusnya, kajian ke atas kesan sinergistik *E. scaber* dan tamoxifen telah dijalankan dengan menggunakan sistem sfera tersebut. Di samping teknik berasaskan sel, penguraian jalur molekul yang dirangsang oleh interaksi antara herba-dadah juga telah diselidik dengan menggunakan teknik "real time PCR" dan kaedah aliran sitometri bagi ungkapan protein. Keputusan kajian-kajian tersebut menunjukkan bahawa gabungan *E. scaber* dan tamoxifen dapat merangsang apoptosis oleh pengaruh estrogen yang melibatkan pengaktifan p53 dan rangkain reaksi kaspase. Lantaran pengenalpastian aktiviti antioksidan sebagai faktor kepada kesan anti-kanser sesuatu herba, kesan sitotosik *E. scaber* terhadap sel-sel kanser juga mungkin berpunca daripada aktiviti antioksidannya yang baik serta kandungan flavonoid dan fenoliknya yang tinggi. Selain itu, aktiviti perlindungan hati tikus daripada ancaman alkohol juga boleh dikaitkan dengan kandungan antioksidan ekstrak tumbuhan tersebut. Yang penting sekali, ekstrak alkohol *E. scaber* adalah tidak toksik sepertimana yang dibuktikan oleh pelbagai penilaian kesan tosik ke atas ekstrak tersebut. Kesimpulannya, *E. scaber* adalah tidak tosik dan berpotensi sebagai ejen antikanser yang boleh diambil secara individu atau digabungkan dengan tamoxifen sebagai rawatan kepada kanser payudara yang bergantung kepada estrogen.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements for the Degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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## DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree in Universiti Putra Malaysia or other institution.

**HO WAN YONG**

Date: 18 May 2012

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| | |
|---|---|
| $AlCl_3$ | aluminium chloride |
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| AO | Acridine Orange |
| AST | Aspartate aminotransferase |
| Bax | Bcl-2-associated X protein |
| Bcl-2 | B-cell lymphoma 2 |
| Bcl-XL | B-cell lymphoma-extra large |
| BrdU | Bromodeoxyuridine |
| BSA | Bovine serum albumin |
| CAM | Complementary and alternative medicine |
| CDK | Cyclin dependent kinases |
| CIP | Cyclin-dependent kinase inhibitor |
| Ct | Threshold cycle |
| CYP | Cytochrome P450 |
| DI | Denaturation index |
| DMEM | Dulbecco's modified eagle media |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic acid |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme Link Immunosorbent assay |

| | |
|---|---|
| ER | Estrogen receptor |
| FACS | Fluorescence-activated cell sorter |
| FBS | Foetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| FRAP | Ferric Reducing/Antioxidant Power |
| FRIM | Forest Research Institute Malaysia |
| HBSS | Hanks balance salt solution |
| HC | Concentration that result in 50% of haemolysis |
| HSP | Heat shock protein |
| HT | Hormonal therapy |
| $IC_{50}$ | Concentration that causes 50% of inhibition to cell growth |
| ICAM-1 | Intercellular adhesion molecule-1 |
| ICR mouse | "Imprinting Control Region" mouse |
| JC-1 | 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide |
| JNK | c-Jun NH2-terminal kinases |
| KIP | Kinase inhibitor protein |
| $LD_{50}$ | Median lethal dose |
| LDH | Lactate dehydrogenase |
| MCTS | Multicellular tumor spheroid |
| MDA | Malonyldialdehyde |
| mg/kg BW | mg. per kg. of body weight |
| MOH | Ministry of Health |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| NaOH | Sodium hydroxide |
| $NaNO_2$ | sodium nitrite |

| | |
|---|---|
| NBT | Nitro-blue tetrazolium |
| NCI | National Cancer Institute |
| OECD | Organisation for Economic Co-operation and Development |
| p-Akt | Phosphorylated Akt |
| PBMC | peripheral blood mononuclear cell |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| PE | Phycoerythrin |
| PI | Propidium Iodide |
| PR | Progesterone receptor |
| PS | Phosphatidylserine |
| RBC | Red blood cell |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| S.E.M. | Standard error of the mean |
| SDS | Sodium dodecyl sulfate |
| SEM | Scanning electron microscope |
| SERM | Selective Estrogen Receptor Modulator |
| SOD | Superoxide dismutase |
| Tm | Melting temperature |
| TUNEL | Terminal deoxynucleotidyl transferase (TdT)–mediated biotinylated dUTP nick end-labeling |
| VEGF | Vascular endothelial growth factor |
| WHO | World Health Organization |
| XIAP | X-linked inhibitor of apoptosis protein |

# CHAPTER I

# INTRODUCTION

Cancer is a life threatening disease that is affecting the health of millions each year (World Health Organization, 2011). Breast cancer is not only the most common type of cancer among Malaysian women but is also a leading cause of mortality worldwide (NCR, 2011). Among all the medications that are currently available, tamoxifen is the oldest and most prescribed drug for invasive and metastatic breast cancer. However, the use of tamoxifen had been associated with a number of undesirable side effects and the risk of drug resistance. In spite of this, this drug remains as the main choice for hormonal therapy for breast cancer as no other alternative can demonstrate greater benefit than tamoxifen in the chemoprevention of this cancer (Fisher *et al.*, 2005c), which could prevent or impede the cancer cells from developing invasiveness (Sporn). Thus, the discovery of a safer, more reliable and effective therapeutic option for breast cancer is gaining an increasing interest worldwide.

Much attention has been given to the discovery of a novel therapeutic drug from natural products due to their wealth of bioactive ingredients (da Rocha *et al.*, 2001). Traditional herbs that exemplify powerful source of natural antioxidants are good candidates for anti-cancer activities (Cai *et al.*, 2004). Either alone or as a complementary medicine to the core therapeutics, natural products such as herbal remedies have long been adopted for cancer treatment (NCCAM, 2011; Yamahara *et al.*, 1989). Constituents in herb-drug combination may not only potentiate context-specific mechanism against multiple targets but may also compensate biologically for each other's limitation, thereby allow

for reduced treatment dosage and hence the toxicity of both substances (Lehar *et al.*, 2009). For instance, herbs may enhance cell-killing effect on cancer cell lines when combined in therapy with drugs such as tamoxifen (Izevbigie *et al.*, 2005; Chen *et al.*, 2007).

*Elephantopus scaber,* a local herb that had been recognized by the United Nations Development Programme as one of the good sources for pharmaceutical studies, was selected for this study (Hammer & Johns, 1993). Besides ethnopharmacological records of the use of this plant for treating neoplasm (Balachandran & Govindarajan, 2005), the cytotoxicity of this herb against various types of cancers has also been reported (Ichikawa *et al.*, 2006; Liang *et al.*, 2008; Than *et al.*, 2005; Xu *et al.*, 2006). Therefore, *E. scaber* may possess potent cytotoxic effect against the estrogen dependent human breast cancer MCF-7 cell line. Nonetheless, the interaction between *E. scaber* and tamoxifen against MCF-7 cell line was also of great interest. Therefore, the present study has been undertaken to determine the cytotoxicity of *E. scaber* against MCF-7 cell line, both individually and in combination with tamoxifen. In this study, *E. scaber* was hypothesized to induce cell death in MCF-7 and react synergistically with tamoxifen to trigger apoptosis in the cells. As reduced risk for cancer and anti-neoplastic activities are also related to the cellular redox balance and antioxidant status of the body system (Mena *et al.*, 2009; Valko *et al.*, 2007), it might be relevant to determine the antioxidant potential of *E. scaber* to evaluate the functional role of this bioactivity in promoting the cytotoxic potential of the plant.

In cancer research, monolayer cell culture is most widely used for *in vitro* cytotoxic studies. However, the two-dimensional organization of cells in monolayer culture lack some of the characteristics of the microenvironment in solid tumors (Khaitan *et al.*, 2006). For this reason, the three-dimensional multicellular tumor spheroid cultures (MCTS) that mimic real tumors *in vivo* had been suggested as a more suitable tool for the prediction of cytotoxic potential and the underlying mechanism of a cytotoxic agent against tumor (Fracasso & Colombatti, 2000a; Hamilton, 1998b; Madsen *et al.*, 2006). In this study, spheroid was adopted for cytotoxic studies of the combination of *E. scaber* and tamoxifen on MCF-7 cells. The generated MCTS cultures were hypothesized to provide a more thorough evaluation of cell death mechanism by generating penetration barrier to the herb-drug treatment.

However, combination therapy is meaningful only when the adverse effects are minimized upon treatment, or at least one substance does not poses additive side effects towards the other in a specific regimen (Clark, 2009). Thus, it would be essential to ensure that the toxicity of *E. scaber* is specific towards breast cancer but not to the normal cells. A range of toxicity assays involving both *in vitro* and *in vivo* systems would be crucial to ensure safety of the plant. This includes evaluation of the protective ability of *E. scaber* towards liver, which is the major organ that is responsible for detoxification of foreign compounds that enter the body (Dancygier & Strassburg, 2010).

Objectives:

1. To determine the cytotoxicity of *E. scaber* and to explore its underlying cell death mechanism on human breast cancer MCF-7 cell line.
2. To develop a stable MCF-7 multicellular tumor spheroid (MCTS) culture for cultivation and cytotoxic assays *in vitro*.
3. To study the cytotoxic effect of a combinatorial treatment by *E. scaber* and tamoxifen on multicellular spheroid culture of MCF-7.
4. To evaluate the toxicity and liver protective potential of *E. scaber* in relation to its antioxidant activity.

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