Plants have not only been consumed as food but have also been adopted as folk medicine for centuries. *Elephantopus scaber* Linn, a herb from the Asteraceae family, has traditionally been taken as decoction or tea to cure various ailments and diseases throughout the world. Recent studies had also suggested that this plant posses various bioactivities such as anti-bacterial, anti-inflammatory, hepatoprotective as well as anti cancer properties. In this study, the cytotoxic effect of an ethanolic extract of *E. scaber* on a breast cancer cell line, MCF-7 and the underlying cell death mechanism was examined. *E. scaber* showed cytotoxic effect towards MCF-7 cells with an IC50 value of 15 µg/mL. In comparison to the untreated control, the extract triggered cell death with increased phosphatidylserine externalization, DNA breaks and significant morphological apoptotic characteristics in the MCF-cells. Furthermore, we also found that expression of the tumor suppressor p53 protein was up-regulated in response to the treatment. In conclusion, these results suggested that the ethanolic extract of *E. scaber* may be a potential anti cancer agent for human breast cancer cells by the induction of p53-dependent apoptosis.

**Key words:** Ethanol extract, MCF-7, tumor suppressor protein, DNA fragmentation, phosphatidylserine externalization.

**INTRODUCTION**

Breast cancer is one of the leading causes of cancer mortality worldwide every year. Despite the availability of treatments in the form of surgery, radiation therapy, chemotherapy, hormonal therapy and biologic therapy for breast cancers (American Cancer Society, 2007; World Health Organization, 2011), most of the therapeutic means are associated with some drawbacks such as high cost of treatment and adverse side effects after prolonged exposure. For instance, tamoxifen, the oldest and most-prescribed selective estrogen receptor modulator (SERM) for treating hormone-receptor-positive breast cancer, had been proven to reduce the risk of developing invasive and non-invasive breast cancer among women (Fisher et al., 2005). However, clinical cases had also witnessed the susceptibility of women who underwent tamoxifen therapy for more than five years to have relatively higher risks for stroke, cataracts, cardiac arrhythmia or atrial fibrillation, hypertriglyceridemia, deep-vein thromboembolic events and even death (Fisher et al., 2005; Veronesi et al., 2007). There is indeed a need to search for more easily available and much more reliable therapeutic sources into overcoming the problems associated with current breast cancer treatments.

Natural products have been used for a long time in folk medicine to treat a great variety of diseases. In ethnopharmacology, plant-derived products are not only useful as medications to cure common injuries and ailments but are also well known for preventing and treating dreadful diseases such as cancers (Mann, 2000; Yeap et al., 2010). The ethnopharmacological evidence of natural products usage has therefore become an important lead for the discovery of anticancer drugs.
In modern medicine, herbal products are used widely but not limited to in many developed countries as a major complementary/alternative medicine (CAM) (Ernst, 2000; Molassiotis et al., 2005). The wide acceptance of traditional Chinese medicine in the Western countries has also popularized herbs as an alternative for cancer therapy (Chiaramonte and Lao, 2010).

Elephantopus scaber Linn, or more commonly known as Elephant’s foot, is a perennial herb from the Asteraceae family that can be easily found in tropical countries. The whole plant of this herb can be consumed for the treatment of various ailments and diseases (Gurib-Fakim et al., 1993; Inta et al., 2008; Ho et al., 2009). Traditionally, E. scaber is used for treating diabetes, enteritis, flatulence, diuresis and is also adopted as an analgesic, astringent and antiemetic agent (Ong and Nordiana, 1999; Daisy et al., 2007; Lee et al., 2008). In India’s Ayurvedic medicine, this herb, along with a few others is also used to treat neoplasm of different stages (Balachandran and Govindarajan, 2005). Nevertheless, there is indeed scientific evidence that has proved the antineoplastic activity of E. scaber. This plant showed anti proliferative activities towards carcinoma cell lines from the renal, liver, cervix and colon in vitro and inhibited the growth of a human cervical cancer tumor in vivo (Than et al., 2005; Ichikawa et al., 2006; Xu et al., 2006; Liang et al., 2008; Liang and Min, 2002). However, the lack of in depth study has limited our understanding of the cell death mechanism induced by E. scaber in human cancers. Therefore, in this study, the potential cytotoxic effect of E. scaber on the human breast cancer MCF-7 cell line was evaluated by elucidation of the cell death mechanisms and regulation of tumor suppressor protein underlying the cytotoxicity of this herb.

MATERIALS AND METHODS

Preparation of E. scaber ethanol extract

E. scaber used in this work was collected from Georgetown, Penang, and was identified by science officer Mr. Lim Chung Lu from the Forestry Division of Forest Research Institute Malaysia (FRIM), Kepong, Selangor. The voucher number deposited in FRIM for E. scaber is FR165693. The leaves of E. scaber were thoroughly washed, shade dried and ground into fine powder. The leaf powder was then subjected to extraction for three times with absolute ethanol at room temperature. All the extracts were then mixed and filtered through grade 1 Whatman filter paper with subsequent evaporation to dry under reduced pressure using Aspirator A-3S (EYELA, Japan) at < 40°C. The final yield was 8% of the initial dried leaves’ weight. A stock of the extract was prepared by dissolving the powder in DMSO at a concentration of 10 mg/ml and it was stored at 4°C.

Cell lines and cell culture

Human breast carcinoma MCF-7 and human breast cell control MCF-10A was purchased from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7 was routinely cultured in RPMI-1640 (Sigma) while MCF-10A was maintained in a 1:1 mixture of DMEM and F12 medium (DMEM-F12) supplemented with 5% (v/v) horse serum, hydrocortisone (0.5 µg/ml), insulin (10 µg/ml) and epidermal growth factor (20 ng/ml). All media were supplemented with 10% (v/v) heat-inactivated foetal bovine serum, penicillin (100 U/ml) and streptomycin (50 U/ml). The cells were cultured at 37°C in a humidified incubator with 5% CO₂.

MTT cytotoxicity assay

The cytotoxic potential of ESEE against MCF-7 and MCF-10A cell lines was determined via the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described (Mosmann, 1983). In brief, the cells that were cultured overnight in 96-well plates were treated with fresh media containing different concentrations of ESEE ranging from 0.47 to 100 µg/mL (Tamoxifen with concentrations from 0.47 to 100 µg/mL was used as positive control for MCF-7 cell line). After further incubation of 24, 48 and 72 h, the absorbance was recorded at 570nm in a µ Quant ELISA Reader (Bio-tek Instruments, USA). Triplicates were carried out for all cell lines. Percentage of proliferation was calculated using the following formula:

\[
\text{Percentage of cell viability} = \left( \frac{\text{OD sample}}{\text{OD control}} \right) \times 100\%
\]

Cells seeding and treatment

MCF-7 cells were used for the acridine orange and propidium iodide dual staining, terminal deoxyxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (Tunel) assay, phosphatidylserine externalization analysis and protein phenotyping. In all these assays, the cells were seeded at a concentration of 2 X 10⁵ cells/well in 6-well plates for 24 h prior to treatment. On the following day, RPMI medium was discarded and replaced with 2ml of fresh medium in each well. The cells were subjected to treatment by 15.0 µg/ml of E. scaber ethanol extract (ESEE). After incubation for 24, 48 and 72 h, the control and treated cells were detached using recombinant trypsin (TrypLE™ Express, Gibco) and spun down by centrifugation (1000 rpm for 10 min) before proceeding to the corresponding assays.

Acridine orange and propidium iodide double staining

Apoptosis was determined by staining MCF-7 cells with acridine orange (AO) and propidium iodide (PI). The harvested cells were dissolved in 100 µl of PBS. Then, staining dyes (10 µg/ml of AO and PI each) were added into the cell suspension and 10 µl of the stained cells were immediately observed under an inverted fluorescent microscope (Nikon, Japan).

Tunel assay

Tunel assay was carried out according to the protocol of the APO-DIRECT™ kit (Becton Dickinson, USA). In brief, the harvested cells were suspended in 1% (w/v) paraformaldehyde in PBS (pH 7.4). The cell suspension was placed on ice for 60 min followed by centrifugation at 300 x g. Pellet was then washed twice with PBS. Cell concentration was adjusted to 1 x 10⁶ cells per ml of 70% (v/v) ice cold ethanol and let to stand for 1 h on ice before storing at -20°C. After 24 h of incubation, the cells were stained according to
the protocol for measurement of apoptosis and then subjected to analysis using the FACS Calibur flow cytometer.

**Phosphatidylserine (PS) externalization**

The annexin V method was used to investigate PS externalization. Cells were washed twice with cold PBS followed by double staining with Annexin V-FITC and propidium iodide (PI) using the FITC Annexin V Apoptosis Detection Kit according to the manufacturer’s protocol (Becton Dickinson, USA). Data acquisition and analysis were performed in FACS Calibur flow cytometer (Becton Dickinson, USA) using CellQuest 3.3 software.

**p53 monoclonal antibody staining**

The fixation and staining of cells with p53 monoclonal antibody was carried out according to the protocol of Cytofix/Cytoperm™ Fixation/Permeabilization Kit (Becton Dickinson, USA). First, 250 µl of fixation/permeabilization solution was added into each of the harvested cell pellet and incubated at 4°C for 20 min. Then, the cells were washed twice in BD Perm/Wash™ buffer. For staining of cells, the cells were resuspended in 50 µl of BD Perm/Wash™ buffer containing the p53 monoclonal antibodies followed by 30 min of incubation at 4°C in the dark. The cells were then washed twice and further conjugated with FITC Goat Anti-Mouse IgG and incubated for another 30 min at 4°C in the dark. The cells were washed twice and resuspended in staining buffer prior to flow cytometric analysis.

**Statistical analysis**

Results were expressed as mean ± standard error (S.E.M.). Three separate experiments were performed for each measurement. Differences between the groups were evaluated by using ANOVA test (one way) followed by Duncan test and p ≤ 0.05 was taken as being statistically significant.

**RESULTS AND DISCUSSION**

Despite the increasing amount of marketed antineoplastic drugs, a huge population of the world is still using herbs as a mean for cancer therapy. Previous researches had shown that *E. scaber* exerted differential cytotoxic effects towards a few cancer cell lines (Than et al., 2005; Ichikawa et al., 2006; Xu et al., 2006; Liang et al., 2008; Liang and Min, 2002). However, most studies involving cytotoxicity of its compounds were limited to screening of their anti proliferative activities with no further evaluation on the cell death mechanism. We were thus attempted to examine further the cytotoxic and apoptotic properties of ESEE in MCF-7 cells. The MTT assay was carried out to assess the cytotoxicity of ESEE on MCF-7 and a normal human breast cell line MCF-10A. The IC_{50} value, concentration of the extract that causes 50% growth inhibition or 50% cell kill of the cell lines was determined from this assay. Table 1 shows the mean average IC_{50} values after 48 and 72 h of incubation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cancer cell type</th>
<th>Cell line</th>
<th>Average mean values of IC_{50} (µg/mL)</th>
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</thead>
<tbody>
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<td>ESEE</td>
<td>Human breast carcinoma</td>
<td>MCF-7</td>
<td>14.69 ± 0.29 7.33 ± 1.20 7.17 ± 1.09</td>
</tr>
<tr>
<td></td>
<td>Human normal breast cell</td>
<td>MCF-10A</td>
<td>&gt; 80  &gt; 80  &gt; 80</td>
</tr>
<tr>
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<td>Human breast carcinoma</td>
<td>MCF-7</td>
<td>11.6 ± 0.3 9.6 ± 0.65 10.2 ± 0.45</td>
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**Table 1. IC_{50} values of MCF-7 and MCF-10A upon treatment with ESEE and tamoxifen.**

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become a favorable therapeutic strategy for designing treatments against cancerous diseases (Alitheen et al., 2010, 2011). The occurrence of apoptosis is described as a cascade of programmed processes involving a series of characterized morphological and biochemical criteria (Majno and Joris, 1995; Sgonc and Gruber, 1998; Fadeel and Orrenius, 2005). Direct examination of the ESEE treated cells in culture plates (Figure 1) revealed characteristics such as intense surface ziotic blebbing, protrusion of thin surface microspikes and loss of substratum adhesion from the cell culture plate, that were described by Collins et al. (1997) as signs of apoptosis. An anti-inflammatory study carried out by Ichikawa et al. (2006) concluded that isodeoxyelephantopin isolated from *E. scaber* is potential therapeutic agent for proinflammatory diseases by blocking of the NF-κB pathway. Also in their study, the compound was also identified to potentiate apoptotic effects through down-regulation of some NF-κB regulated gene products modulating cell proliferation, invasion and antiapoptosis. Therefore, the study was extended to investigate the occurrence of apoptosis in MCF-7 after ESEE treatment.

Cells treated with 15 µg/mL of ESEE after the respective periods were stained with AO and PI dyes to distinguish among viable, apoptotic and necrotic cells. Viable cells, with intact membranes, were stained green with AO and showed well-defined nuclear structures. Apoptotic cells, on the other hand, were also stained green with AO but exhibited chromatin condensation, nuclear genome fragmentation and membrane blebbing, which differentiated them from viable cells. The late apoptotic cells that possessed disintegrated membranes were additionally stained orange by PI dye but were differentiated from the morphological similar necrotic cells by the occurrence of fragmented DNA. As shown in Figure 2, these apoptotic events were increased significantly up to about 8 fold higher than control cells after 24 (52.72%) and 48 h (53.85%) of treatment with ESEE and the percentage was even higher after 72 h whereby 63.55% of apoptotic cells were detected. The percentage of necrotic cells observed was also increased but by a much lower extent from 4.50% at 24 h to 5.45 and 6.20% at 48 and 72 h, respectively.

Next, Tunel assay was carried out to confirm apoptosis in MCF-7 cells upon ESEE treatment. The method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to incorporate biotinylated deoxyuridine to 3'-OH ends at sites of DNA breaks and accurate quantification of the incorporation signals was done using a flow cytometer. ESEE treatment was shown to mediate apoptosis through DNA fragmentation in MCF-7 cells (Figure 3). It is apparent that the untreated cells showed minute tunnel positive signals (less than 0.3%) at all three incubation periods. As opposed to this, treatment with ESEE significantly induced DNA fragmentation in the cells whereby 43.09% of the treated cells were TUNEL positive at 24 h and the rate increased to 95.23 and 96.94% after 48 and 72 h, respectively. A high percentage of DNA fragmentation at the late time points indicated that these cells were in the late stage of apoptosis after long exposure to ESEE and early apoptosis had take place at an earlier time point. However, DNA fragmentation was shown to occur at a late stage during apoptosis (Collins et al., 1997). Hence, the cytotoxicity effect of ESEE on MCF cell line was also examined at earlier time frames (3, 6 and 12 h) to investigate the initiation of apoptotic event by using Annexin V/PI analysis, which serves as a useful specific marker for early phase apoptosis when the cell membrane is still intact (Koopman et al., 1994; Yeap et al., 2011). Assessment from the flow cytometric analysis showed increase of phosphatidylserine externalization in treated cells with time. As shown in Figure 4, early apoptotic signals could be traced in the cells as early as 3 h after incubation with 15 µg/mL of ESEE. Early apoptotic as well as late apoptotic and necrotic events were significantly increased when compared to untreated cells in the early time frames (3, 6 and 12 h). However, as the incubation period was increased, a shift from early apoptosis to late apoptosis was observable in the cells. In
Figure 2. Percentage of viable (V), apoptotic (A) and necrotic (N) cells in control and ESEE treated cells as determined by dual staining with acridine orange and propidium iodide. The cells were incubated for 24, 48 and 72h, respectively and the results are presented as means ± SEM of three independent experiments (*P < 0.05 for comparison of control cells to cells treated with ESEE).

Figure 3. Effects of ESEE on DNA fragmentation. MCF-7 cells were cultured for 24, 48 and 72 h with or without the presence of 15 µg/mL of ESEE. Cells were then stained by TUNEL and analyzed by flow cytometry. Results are expressed as percentage of cells in the T (Tunel positive) region (*P < 0.05 for comparison of control cells to ESEE treated cells).
Figure 4. Determination of apoptotic events in MCF-7 cells treated with ESEE based on recognition of phosphatidylserine at the outer leaflet of cell membrane. (a) control group of untreated cells and (b) cells incubated with 15 µg/mL of ESEE for respective periods as indicated on the right. The proportion of cells in each quadrant is represented as mean percentage in replicates of 3 independent experiments (*P < 0.05 for comparison of control cells to cells treated with ESEE).
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Figure 5. Relative expression of p53 protein in ESEE treated MCF-7 cells. The values are expressed as percentages of the untreated control group (expression of untreated control = 100%). Results are presented as means ± SEM of three independent experiments (*P < 0.05 for comparison of control cells to cells treated with ESEE).

In conclusion, this study has proven that the ethanolic extract of *E. scaber* could inhibit growth and triggered time dependent and dosage dependent cell death in the MCF-7 breast cancer cell line via a p53 dependent apoptotic pathway. p53 appears to be a central protein modulating many critical pathways leading to development and design for anti-cancer agents. Hence, investigation on the downstream proteins associated with p53 would provide important clues for evaluating ESEE as a potential treatment for human breast cancer. In future studies, the effect of ESEE on estrogen receptor status and cell cycle regulation may be investigated to explore the potency of ESEE in targeting breast cancers.
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