SCURRULA FERRUGINEA METHANOL EXTRACT INDUCES REACTIVE OXYGEN SPECIES-MEDIATED AND MITOCHONDRIAL-DEPENDENT APOPTOSIS IN BREAST CANCER CELLS

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I would like to dedicate this thesis to my beloved wife, my lovely unborn child and my lovely father and mother for their endless support and encouragement.
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ABSTRACT

The purpose of this study is to investigate antioxidant and anticancer activities of *Scurrula ferruginea* extracts. The antioxidant activities of the extracts were evaluated using various assays. The extracts were further investigated to examine their cytotoxic activity on human breast cancer cell lines; MDA-MB-231, MDA-MB-468 and MCF-7 using MTT assay. Microscopic examinations of cells were carried out to elucidate the modes of cell death. The effect of the extracts on cancer cells colony formation and migration were determined. Changes in mitochondrial membrane potential and level of reactive oxygen species (ROS) were measured. Western blot and cell cycle analysis were performed to unravel the mechanism of action of extracts against the breast cancer cells. Using GC-MS analysis, chemical composition of extracts were characterized to reveal the presence of anti-cancerous compounds. Our study on stem methanol extract has shown the highest amount of phenolic, flavonoid contents, strong DPPH radical scavenging and metal chelation activity in comparison to other extracts. The stem aqueous and methanol extracts have shown higher cytotoxic effect towards MDA-MB-231 cells compared to other cell lines with IC$_{50}$ value of 50.35 and 19.27 µg/mL, after 72 h of treatment, respectively. Morphological observations revealed properties of apoptosis in the treated cells. The results displayed that the extracts have the ability to stop migration of cancer cells and also inhibit the colony formation of cancer cells. Moreover, the results have shown that the extracts induced apoptosis in breast cancer cells by ROS generation and mitochondrial depolarization. Furthermore, this study demonstrated that methanol extract inhibited the proliferation of breast cancer cells via induction of cell cycle arrest at G0/G1 phase and apoptosis through a mitochondria-dependent apoptosis pathway. The findings of present study revealed the potential antioxidant and anticancer activities of *S. ferruginea* stem methanol extract which may serve as a promising candidate in the search of a new anti-cancer drug.
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4.39 Fluorescence imaging for detection of apoptosis in MCF-7 cells. Cells were treated with tamoxifen (positive control) for 24 hours at concentrations of 31.25 and 250 μg/mL. Left panel displays Hoechst 33342 staining while right panel displays PI staining of the same field. The morphological alterations in the cells were visualized under fluorescence microscope (20×).

4.40 Effect of *S. ferruginea* extracts on colony-forming abilities of MDA-MB-231 cells. Methanol (a) and aqueous (b) extracts suppressed colony formation in a dose dependent manner. The methanol extract inhibited the clonogenicity of MDA-MB-231 cells more effectively than aqueous extract. The images were taken using an inverted phase contrast microscope (Zeiss Axiovert 100) at 4× magnification.

4.41 Quantitative measurement of colony formation of selected extracts on MDA-MB-231 cells at different concentrations (31.25-1000 μg/mL). The numbers of the colonies were estimated under dissection (stereo) microscope (Wild Heerburgg M3). The colony forming ability of the cells at each dose of extracts is expressed in terms of percent of control and represented as means ± SD of three replicates in three independent experiments. * indicates statistically significant different from their respective control (one way ANOVA, P < 0.05).

4.42 Effect of *S. ferruginea* extracts on colony-forming abilities of MDA-MB-468 cells. Methanol (a) and aqueous (b) extracts suppressed colony formation in a dose dependent manner. The methanol extract inhibited the clonogenicity of MDA-MB-468 cells more effectively than aqueous extract. The images were taken using an inverted phase contrast microscope (Zeiss Axiovert 100) at 4× magnification.
Quantitative measurement of colony formation of selected extracts on MDA-MB-468 cells at different concentrations (31.25–1000 µg/mL). The numbers of the colonies were measured under dissection (stereo) microscope (Wild Heerburgg M3). The colony forming ability of the cells at each dose of extracts is expressed in terms of percent of control and represented as means ± SD of three replicates in three independent experiments. * indicates statistically significant different from their respective control (one way ANOVA, P < 0.05).

Effect of *S. ferruginea* extracts on colony-forming abilities of MCF-7 cells. A. Methanol (a) and aqueous (b) extracts suppressed colony formation in a dose dependent manner. The methanol extract inhibited the clonogenicity of MCF-7 cells more effectively than aqueous extract. The images were taken using an inverted phase contrast microscope (Zeiss Axiovert 100) at 4x magnification.

Quantitative measurement of colony formation of selected extracts on MCF-7 cells at different concentrations (31.25–1000 µg/mL). The numbers of the colonies were estimated under dissection (stereo) microscope (Wild Heerburgg M3). The colony forming ability of the cells at each dose of extracts is expressed in terms of percent of control and represented as means ± SD of three replicates in three independent experiments. * indicates statistically significant different from their respective control (one way ANOVA, P < 0.05).

Effect of *S. ferruginea* methanol and aqueous extracts on the cell migration of MDA-MB-231 cells. Scratch closure activity of treated MDA-MB-231 cells upon creation of scratch using a scratcher in control and treated well. The images of scratched MDA-MB-231 cell monolayer treated with extracts captured under an inverted phase-contrast microscope at different time intervals (0, 6, 12 & 24 h).

Quantitative measurement of cell migration of methanol (a) and aqueous (b) extracts on MDA-MB-231 cells at different concentrations (31.25–1000 µg/mL). Scratch closure rates were analyzed quantitatively as the difference between scratch width at 0, 6 and 12 or 24 h and results are expressed as percentage of cell migration. Results showed that in presence of selected extracts the migration of the MDA-MB-231 cells was dose- and time-dependently inhibited. Data are represented as means ± SD of three replicates in three independent experiments. * indicates statistically significant different from their respective control (one way ANOVA, P < 0.05).
4.48 Effect of *S. ferruginea* methanol and aqueous extracts on the cell migration of MDA-MB-468 cells. Scratch closure activity of treated MDA-MB-468 cells upon creation of scratch using a scratcher in control and treated well. The images of scratched MDA-MB-468 cell monolayer treated with extracts captured under an inverted phase-contrast microscope at different time intervals (0, 6, 12 & 24 h).

4.49 Quantitative measurement of cell migration of methanol (a) and aqueous (b) extracts on MDA-MB-468 cells at different concentrations (31.25-1000 µg/mL). Scratch closure rates were analyzed quantitatively as the difference between scratched width at 0, 6 and 12 or 24 h and results are expressed as percentage of cell migration. Results showed that in presence of selected extracts the migration of the MDA-MB-468 cells was dose- and time-dependently inhibited. Data are represented as means ± SD of three replicates in three independent experiments. * indicates statistically significant different from their respective control (one way ANOVA, P < 0.05).

4.50 Effect of *S. ferruginea* methanol and aqueous extracts on the cell migration of MCF-7 cells. Scratch closure activity of cells upon creation of scratch using a scratcher in control and treated well. The images of scratched MCF-7 cell monolayer treated with extracts captured under an inverted phase-contrast microscope at different time intervals (0, 6, 12 & 24 h).

4.51 Quantitative measurement of cell migration of methanol (a) and aqueous (b) extracts on MCF-7 cells at different concentrations (31.25-1000 µg/mL). Scratch closure rates were analyzed quantitatively as the difference between scratch width at 0, 6 and 12 or 24 h and results are expressed as percentage of cell migration. Results showed that in presence of selected extracts the migration of the MCF-7 cells was dose- and time-dependently inhibited. Data are represented as means ± SD of three replicates in three independent experiments. * indicates statistically significant different from their respective control (one way ANOVA, P < 0.05).

4.52 Qualitative evaluation of ROS generation in MDA-MB-231 cells using the fluorescent probe DCF-DA. MDA-MB-231 cells were treated with 31.25 µg/mL, 250 µg/mL and positive control (50 µM H2O2) for 12 h. Fluorescence microscopic images (10×) indicated that methanol extract induced intracellular ROS formation in MDA-MB-231 cells.
4.53 Qualitative evaluation of ROS generation in MDA-MB-468 cells using the fluorescent probe DCF-DA. MDA-MB-468 cells were treated with 31.25 μg/mL, 250 μg/mL and positive control (50 μM H₂O₂) for 12 h. Fluorescence microscopic images (10×) indicated that methanol extract induced intracellular ROS formation in MDA-MB-468 cells.

4.54 Qualitative measurement of ROS generation in MCF-7 cells using the fluorescent probe DCF-DA. MCF-7 cells were treated with 31.25 μg/mL, 250 μg/mL and positive control (50 μM H₂O₂) for 12 h. Fluorescence microscopic images (10×) indicated that methanol extract induced intracellular ROS formation in MCF-7 cells.

4.55 Effects of *S. ferruginea* methanol extract on ROS generation in different breast cancer cell lines. Cells were treated with different concentrations of methanol extract and positive control (50 μM H₂O₂) for 12 h. Data are represented as means ± SD of three replicates in three independent experiments. * indicates statistically significant different from corresponding controls (one way ANOVA, P < 0.05).

4.56 Effect of methanol extract on mitochondrial membrane potential (MMP) in MDA-MB-231 cells using JC-1 fluorescence dye. Methanol extract induced MMP depolarization in MDA-MB-231 cells. The cells were treated with 31.25 μg/mL, 250 μg/mL and positive control (50 μM CCCP) for 12 h. Images were obtained with an inverted fluorescent microscope (Zeiss Axiovert A) (40×). The emitted green fluorescence indicates MMP depolarization which is an early event in apoptosis.

4.57 Effect of methanol extract on mitochondrial membrane potential (MMP) in MDA-MB-468 cells using JC-1 fluorescence dye. Methanol extract induced MMP depolarization in MDA-MB-468 cells. The cells were treated with 31.25 μg/mL, 250 μg/mL and positive control (50 μM CCCP) for 12 h. Images were obtained with an inverted fluorescent microscope (Zeiss Axiovert A) (40×). The emitted green fluorescence indicates MMP depolarization which is an early event in apoptosis.

4.58 Effect of methanol extract on mitochondrial membrane potential (MMP) in MCF-7 cells using JC-1 fluorescence dye. Methanol extract induced MMP depolarization in MCF-7 cells. The cells were treated with 31.25 μg/mL, 250 μg/mL and positive control (50 μM CCCP) for 12 h. Images were obtained with an inverted fluorescent microscope (Zeiss Axiovert A) (40×). The emitted green fluorescence indicates MMP depolarization which is an early event in apoptosis.
4.59 Relative quantity of mitochondrial membrane potential ($\Delta \Psi_m$) in different breast cancer cell lines. Cells were treated with different concentrations of methanol extract and positive control (50 μM CCCP) for 12 h. Methanol extract disrupts mitochondrial transmembrane potential ($\Delta \Psi_m$). Data are represented as means ± SD of three replicates in three independent experiments. * indicates statistically significant different from corresponding controls (one way ANOVA, P < 0.05).

4.60 Western blot analysis of pro-apoptotic Bax protein in MDA-MB-231 cells. MDA-MB-231 cells were treated with IC$_{50}$ concentration of S. ferruginea methanol extract and control cells (0.1% DMSO) for indicated times. β-actin was used as loading control. Densitometry analysis showed time-dependent up-regulation of Bax protein. The expression of Bax protein increased as early as 2 hour. The densitometer-intensity data are represented as means ± SEM of three replicates in three independent experiments. * indicates statistically significant different from control (one way ANOVA, P < 0.05).

4.61 Western blot analysis of anti-apoptotic Bcl-2 protein in MDA-MB-231 cells. MDA-MB-231 cells were treated with IC$_{50}$ concentration of S. ferruginea methanol extract and control cells (0.1% DMSO) for indicated times. β-actin was used as loading control. Densitometry analysis showed time-dependent down-regulation of Bcl-2 protein. The densitometer-intensity data are represented as means ± SEM of three replicates in three independent experiments. * indicates statistically significant different from control (one way ANOVA, P < 0.05).

4.62 Western blot analysis of caspase-3 protein in MDA-MB-231 cells. MDA-MB-231 cells were treated with IC$_{50}$ concentration of S. ferruginea methanol extract and control cells (0.1% DMSO) for indicated times. β-actin was used as loading control. Densitometry analysis demonstrated that procaspase-3 (32-kDa) was cleaved to yield a catalytically active 17-kDa fragment after treatment with methanol extract. The densitometer-intensity data are represented as means ± SEM of three replicates in three independent experiments. * indicates statistically significant different from control (one way ANOVA, P < 0.05).
Western blot analysis of caspase-7 protein in MDA-MB-231 cells. MDA-MB-231 cells were treated with IC_{50} concentration of *S. ferruginea* methanol extract and control cells (0.1% DMSO) for indicated times. β-actin was used as loading control. Densitometry analysis demonstrated that procaspase-7 (35-kDa) was cleaved to yield a catalytically active 17-kDa fragment after treatment with methanol extract. The densitometer-intensity data are represented as means ± SEM of three replicates in three independent experiments. * indicates statistically significant different from control (one way ANOVA, P < 0.05).

Western blot analysis of PARP protein in MDA-MB-231 cells. MDA-MB-231 cells were treated with IC_{50} concentration of *S. ferruginea* methanol extract and control cells (0.1% DMSO) for indicated times. β-actin was used as loading control. The PARP protein (116-kDa) was cleaved into its signature 85-kDa fragment, a marker of apoptosis, after treatment with methanol extract. The densitometer-intensity data are represented as means ± SEM of three replicates in three independent experiments. * indicates statistically significant different from control (one way ANOVA, P < 0.05).

Effect of *S. ferruginea* methanol extract on the cell cycle progression in MDA-MB-231 cell. MDA-MB-231 cells were treated with IC_{50} concentration of methanol extract for 24 and 48 h, stained with PI and its content was analyzed by flow cytometry. The data are represented as means ± SEM of three replicates in three independent experiments. * indicates statistically significant different from control (one way ANOVA, P < 0.05).

Effect of *S. ferruginea* methanol extract on the cell cycle progression in MCF-7 cell. MCF-7 cells were treated with IC_{50} concentration of methanol extract for 24 and 48 h, stained with PI and its content was analyzed by flow cytometry. The data are represented as means ± SEM of three replicates in three independent experiments. * indicates statistically significant different from control (one way ANOVA, P < 0.05).

Proposed schematic diagram of *S. ferruginea* methanol extract-induced apoptosis in human breast cancer cells MDA-MB-231. Treatment of MDA-MB-231 cells with *S. ferruginea* methanol extract induced high level of ROS generation and subsequently reduced ΔΨm levels which leading to changes in the expression levels of Bax/Bcl-2. This results in mitochondrial dysfunction and caspase-3 and caspase-7 activation. These events all contribute to the subsequent degradation of PARP in MDA-MB-231 cells via G0/G1 cell cycle arrest, which mediates apoptosis.
### LIST OF ABBREVIATION

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NCR</td>
<td>The National Cancer Registry</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal Carcinoma In Situ</td>
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<tr>
<td>LCIS</td>
<td>Lobular Carcinoma In Situ</td>
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<tr>
<td>HER</td>
<td>Human Epidermal Growth Factor Receptor</td>
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<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
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<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>CAM</td>
<td>Complementary and Alternative Medicine</td>
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<tr>
<td>DPPH</td>
<td>Diphenyl-2-picryl hydrazine</td>
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<tr>
<td>ABTS</td>
<td>2, 2'-azino bis-(3-ethyl benzo thiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>TPC</td>
<td>Total Phenolic Content</td>
</tr>
<tr>
<td>TFC</td>
<td>Total Flavonoid Content</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectroscopy</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PDT</td>
<td>Population Doubling Time</td>
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<tr>
<td>MTT</td>
<td>Thiazolyl Blue Tetrazolium Bromide</td>
</tr>
<tr>
<td>AO/EB</td>
<td>Acridine orange/Ethidium bromide</td>
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<tr>
<td>MMP</td>
<td>Mitochondrial Membrane Potential</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial Outer Membrane Permeabilization</td>
</tr>
<tr>
<td>DISK</td>
<td>Death-Inducing Signaling Complex</td>
</tr>
<tr>
<td>ML-I</td>
<td>Mistletoe Lectin I</td>
</tr>
<tr>
<td>HR-QOL</td>
<td>Health-Related Quality Of Life</td>
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<tr>
<td>VA</td>
<td><em>Viscum album</em></td>
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<tr>
<td>ADCC</td>
<td>Antibody-Dependent Cell-mediated Cytotoxicity</td>
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<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor alfa</td>
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<tr>
<td>CRF</td>
<td>Cancer Related Fatigue</td>
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<tr>
<td>TCM</td>
<td>Traditional Chinese Medicine</td>
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<tr>
<td>LS</td>
<td>Life Satisfaction</td>
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<tr>
<td>TEAC</td>
<td>Trolox Equivalent Antioxidant Capacity</td>
</tr>
<tr>
<td>RT</td>
<td>Retention Time</td>
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CHAPTER 1

INTRODUCTION

1.1 Research Background

1.1.1 Breast Cancer

Cancer of breast formed due to formation of malignant tumor in the cells of breast. Initially the growth of breast cancer is local which is followed by extension within lymph vessels into regional lymph nodes and invasion of small vein which results in systematic metastatic spread (Spratt & Tobin, 1995). Breast cancer is the most common type of non-skin malignancy among women worldwide. It has been reported that the incidence and mortality of breast cancer have increased during the last two decades (American Cancer Society Global Cancer Facts & Figures 2nd Edition, 2011; Jemal et al., 2011; Ferlay et al., 2013). Based on 2006-2010 statistics, the number of deaths in the United States was 22.6 per 100,000 women per year. It is predicted that an estimated 231,840 new cases of breast cancer and 40,730 breast cancer-related deaths will occur among women in 2015 worldwide (“American Cancer Society. Cancer Facts & Figures,” 2015)

The incidence rate of breast cancer is highest in North America with the age standardized rates of 99.4 per 100,000 population, followed by countries in the Eastern Europe, South America, Southern Africa, and western Asia with moderate incidence rates, while the lowest incidence rates are reported in most African countries (Yip et al., 2006; Ferlay et al., 2010).
It is reported that approximately one million females are diagnosed with breast malignancy with an estimated 410,000 deaths every year, worldwide (Coughlin & Ekwueme, 2009). The incidence and mortality of breast cancer were reported lower in low-resource countries compared to high-resource countries (Smith, 2006). In most of the Asian countries, the incidence rate of breast cancer is increasing (Abdullah et al., 2013). An increasing in the prevalence of breast cancer was reported in Malaysia as well (Abdullah et al., 2013). The highest incidence rate for breast cancer in Malaysia was observed at women between 50-60 years old (Dahlui et al., 2011). It is estimated that one out of twenty Malaysian women have a chance to get breast cancer at some point of their lives (Dahlui et al., 2011).

Breast cancer is the most common cancer among Malaysian women (Lim et al., 2008). The National Cancer Registry (NCR) 2003-2005 reported an age-standardized rate (ASR) of 47.3 per 100 000. The incidence is highest in Chinese (59.9 per 100 000) followed by Indians (54.2 per 100 000) and Malays (34.9 per 100 000) (Lim et al., 2008). The International Agency for Research in Cancer (GLOBOCAN) 2012 estimated the ASR of breast cancer in Malaysia as 38.7 per 100,000 with 5410 new cases in 2012 (“http://globocan.iarc.fr,”).

### 1.1.2 Breast Cancer Treatment

Different treatment options are currently available including local therapy and systemic therapy. Local therapy includes surgery, radiotherapy or a combination of the two, applied to kill cancer cells from a limited (local) area such as lymph nodes, breast and chest wall. Systemic therapy includes endocrine or hormone therapy and chemotherapy which administered following primary surgery or radiotherapy to kill or inhibit metastases and to improve survival. Table 1.1 represents various methods of breast cancer treatment and their common side effects. Selection of treatment strategies depend on tumor size, metastatic potential, axillary lymph node status and molecular and patient profile (Liao et al., 2013). Systemic therapy with cytotoxic chemotherapy and endocrine therapy were found to be effective in prolonging disease-free and survival time (Peto et al., 2000).
Table 1.1: Summary of various methods of breast cancer treatment and their common side effects.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Mechanism of action</th>
<th>Side effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>Conservative and mastectomy</td>
<td>Lymphedema, chronic nerve damage, infection at the incision site, armpit discomfort</td>
<td>(Karen <em>et al.</em>, 2002; Ridner <em>et al.</em>, 2011)</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>Using high dose of radiation</td>
<td>Skin reactions of the area being radiated</td>
<td>(Sjövall <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>Biological targeted therapy</td>
<td>Using monoclonal antibody and medicine Herceptin (Trastuzumab) Tykerb (lapatinib)</td>
<td>Weakness, diarrhea, Pain, fever Itchy and dry skin, diarrhea</td>
<td>(Nahta <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>Endocrine or hormone therapy</td>
<td>Using aromatase inhibitors and tamoxifen by blocking the action of estrogen Tamoxifen:</td>
<td>Vaginal discharge, an increase in thromboembolic events and uterine sarcoma Musculoskeletal adverse effect, hot flashes, increased LDL, loss of libido, vaginal dryness</td>
<td>(Kalidas &amp; Brown, 2005; Connor &amp; Attai, 2013)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>The most commonly type of treatment using anti-breast cancer drugs Carboplatin, Cisplatin: Cyclophosphamide:</td>
<td>Nephrotoxicity Pulmonary toxicity</td>
<td>(Yao <em>et al.</em>, 2007; Chandwani <em>et al.</em>, 2012; Gianni <em>et al.</em>, 2008)</td>
</tr>
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</table>
Despite of varied side effects, using chemotherapy either as a single compound or combination therapy with multiple-agents is still the most commonly used treatment option by breast cancer patients (Ozer et al., 2000). Chemotherapy uses anti-breast cancer drugs and cytotoxic agents for treatment of metastatic breast cancer (ER-negative tumors). Tumor cell response to chemotherapy and cytotoxic agents through an active form of cell death is known as apoptosis or programmed cell death. It is now well established that other modes of cell death such as necrosis and autophagy also take place following chemotherapy in tumor cells (Brown & Attardi, 2005).

1.2 Problem Statement

Although many treatment methods are currently established including surgery, radiotherapy, biological therapy, hormone therapy and chemotherapy, these therapies are less effective and recurrence is still occurring in breast cancer patients due to side effects and toxicity of drugs in normal cell and aggressive behaviour of the tumours (Table 1.3). In spite of many improvement in the use of hormonal and adjuvant cytotoxic therapies in breast cancer patients, there is no considerable reduction in mortality of breast cancer today (Eggenschwiler et al., 2007). Costly treatment methods and serious side effects associated with available therapies may cause greater tendencies among people to use herbal medicines for health care.

Complementary and alternative medicine (CAM) as one of the major aspect of cancer therapy has been developed in last few years in order to alleviate drug side effects and relief pain in breast cancer patients (Ostermann et al., 2009). A large proportion of cancer patients (up to 80%) use complementary and alternative medicine (CAM) (Vardy et al., 2013). Breast cancer patients are among the most likely users of CAM (Bennett et al., 2009). Among CAM, herbal supplements (antioxidants) is the most commonly used group of cancer treatment. Cancer treatment using herbal medicine has a history of more than 2000 years (Craig, 1999).
Harmful effects of conventional treatment as well as toxicity of chemotherapy create a significant problem in breast cancer therapy. The alternate solution to decrease side effects of chemotherapeutic drugs is the use of medicinal plants. Use of medicinal plants which have fewer side effects as compared to synthetic drugs can provide an alternative to the use of conventional allopathic medicine for treatment of breast cancer. In addition, any practical solution to manage cancer progression is of paramount importance. Therefore, there is a need to evaluate whether medicinal plant extracts are able to act as potent anticancer agent by controlling the cancer progression or arresting the carcinogenic process.

Previous research findings have shown that various European mistletoe extracts from different host trees are capable of inducing apoptosis and cell death in numerous tumor cells and human cancer cell lines (Ramaekers et al., 2007; Harmsma et al., 2006).

Although various studies investigated the effect of European mistletoe on cancer, not many studies focused on other species of mistletoe from other continents. Malaysia’s rainforest being part of the world’s tropical rainforest is also considered as one of the most evolved and diverse rainforest in the world. *Scurrula ferruginea* is one of the mistletoe species in Malaysia which is used as a folk medicine for treatment of several ailments (Barlow, 1991). It has been reported that a decoction of *S. ferruginea* leaves along with *Millettia sericea* used for bathing malarial patients. In addition, a poultice of the pounded leaves administered as a post-partum protective medicine and also applied for snake bite and wound (Burkill et al., 1966) (Perry, 1978). Moreover, this plant are traditionally employed in the treatment of many diseases including gastrointestinal malfunction, high blood pressure and hypertension (Ameer et al., 2009).

Ethno-medical knowledge plays an important role in selection of plants for discovery of novel drugs. Therefore, *S. ferruginea* was selected for the present study based on its reputation in folk medicine. There is no report on antioxidant capacity, anticancer activity and mechanism of action of *S. ferruginea*. The current study
provide the scientific rational for antioxidant and anti-breast cancer activities of *S. ferruginea*.

### 1.3 Objectives of Study

Based on the above-mentioned problem statements, the objectives of the present study are as follow:

1. To evaluate potential of *S. ferruginea* crude extracts based on the antioxidant activity and phytochemical analysis

2. To investigate the selective cytotoxic effects of selected extracts on breast cancer cells and study apoptosis-inducing effects of extracts

3. To study the mechanism of growth arrest and unravel apoptotic pathway involve in breast cancer cell death by selected extract

### 1.4 Scope of Study

Aerial parts of *S. ferruginea* (Jack) Danser including stems, leaves and flowers were used in the present study. Different types of breast cancer cell lines including MCF-7 (luminal A breast carcinoma), MDA-MB-231(Claudin-low breast carcinoma) and MDA-MB-468 (basal-like breast carcinoma) which are differ in molecular markers status and invasiveness have been selected for the present study.

To achieve the listed objectives, the study was confined to the following scopes:

1. Determination of total phenolic and total flavonoid content by Folin-Ciocalteu and aluminum chloride methods, respectively and antioxidant activities of different extracts by assessing DPPH free
radical scavenging activity, ABTS and metal chelation capacity of *S. ferruginea* extracts.

2. Analysis of chemical composition using GC-MS of *S. ferruginea* extracts.

3. Evaluation of selective cytotoxic activities of selected extracts against breast cancer cell lines and non-cancerous cell line using MTT assay and characterization of the cell death using AO/EB and Hoechst/PI staining methods.

4. Determination of cell migration inhibition efficiency and colony forming ability of treated cancer cells using scratch assay and colony forming assay respectively.

5. Measurement of mitochondrial membrane potential by JC-1 assay and investigation on the potential mechanism of apoptosis as the result of oxidative stress by measuring intracellular ROS level using DCF-DA assay.

6. Determination of cell death mechanism pathway of selected extract against breast cancer cell through the regulation of bcl-2, bax, caspase-3, caspase-7 and PARP proteins using western blot analysis and possible cell cycle arrest using flow cytometric analysis.
1.5 **Significant of Study**

i. Growth inhibitory effects on different carcinoma cell types may be crucial for effective control of breast cancer; therefore, the present study is of great importance to introduce a novel candidate in battling breast cancer particularly ER-negative breast carcinoma.

ii. The present study is also paving the way for further research on *S. ferruginea* in the field of pharmaceutical industry and anti-cancer drug discovery for the development of anticancer agents.

iii. This study provides an experimental basis for systematic and clinical research of medicines for treatment of breast cancer in the future.

1.6 **Methodology**

- **Antioxidant activity assays**
  - Extracted with
    - Methanol
    - Water
    - Ethyl acetate
    - Hexane

- **In vitro cell culture assays**
  - 1. LSPH assay
  - 2. ABTS assay
  - 3. Metal chelation activity assay
  - 4. Total phenolic content
  - 5. Total flavonoid content
  - 6. GC-MS analysis

- **Chemical composition of extracts**

- **Assays to determine mechanism of action**
  - 1. JC-1 assay
  - 2. ROS generation assay
  - 3. Cell cycle analysis
  - 4. Western blot analysis

- **Bcl-2, Bax, Caspase, PARP**
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