SAFETY AND EFFICACY EVALUATION OF *Christia vespertilionis* EXTRACTS ON BREAST CANCER CELL LINES

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by

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for the degree of

Master of Science (Biomedicine) Mixmode

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DECLARATION

I hereby declare that this thesis is the result of my own investigation, except what I had duly acknowledge. I also declare that it has not been previously and concurrently submitted as a whole for any other masters at Univesrsiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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(MUHAMMAD ASYAARI BIN ZAKARIA)

Date:…….

CERTIFICATE

This is to certify that the thesis entitles" Safety and Efficacy Evaluation of *Christia vespertilionis* Extracts Against Breast Cancer Cell lines" is a record of research done by Mr. Muhammad Asyaari bin Zakaria during the period February 2018 to December 2018 under my supervision.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS, ABBREVIATIONS AND SYMBOLS

<	Less than
%	Percentage
±	More or less
×	Multiply by
≥	More than or equal to
°C	Degree Celsius
CO_2	Carbon dioxide
µl	Microliter
ml	Mililiter
l	Litre
µg	Microgram
mg	Miligram
min	Minutes
hr	Hour
m/z	Mass to charge ratio
W	Watt
RF	Radio frequency
FBS	Fetal bovine serum
PBS	Phosphate buffer saline
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoilum bromide
et al	et al - 'and others'
i.e.	*id est* - 'that is'
IC_{50}	50% Inhibitory Concentration
CV	*Christia vespertilionis*
CVME	*Christia vespertilionis* methanol extract
CVAE	*Christia vespertilionis* aqueous extract
PS	Phosphatidylserine
AnnV	Annexin V-FITC
PI	Propidium iodide
EGCG	Epigallocatechin
ATCC	American Type Culture Collection
WHO	World Health Organization

ABSTRAK

Kanser payudara adalah salah satu kanser malignan yang biasa berlaku dalam kalangan wanita di seluruh dunia. Terdapat permintaan yang berterusan untuk terapi baru bagi merawat penyakit yang kompleks ini. Dapat diperhatikan, penyelidikan saintifik sedang menumpukan perhatian ke arah tumbuhan berubat yang berpotensi menjadi sumber kepada agen antikanser. *Christia vespertilionis* adalah tumbuhan tempatan yang dikatakan mempunyai sifat antikanser. Ia banyak dipasarkan sebagai makanan tambahan di Malaysia sekali gus membangkitkan persoalaan mengenai keselamatan dan keberkesanan tumbuhan ini. Tambahan lagi, penyelidikan semasa mengenai potensi mekanisma antikanser tumbuhan ini juga adalah terhad. Oleh itu, kajian ini bertujuan untuk menilai keberkesanan ekstrak-ekstrak *C. vespertilionis* terhadap sel-sel kanser payudara (MDA-MB-231 dan MCF-7) dan untuk menyiasat cara kematian sel yang diakibatkan oleh ekstrak tersebut. Di samping itu, sebatian kimia dalam CVME dan unsur-unsur logam tumbuhan ini juga dikaji. Aktiviti ketoksikan terhadap sel oleh CVME, CVAE dan tamoxifen dinilai oleh eksperimen MTT manakala cara kematian sel adalah dinilai oleh penggunaan pewarna Annexin V-FITC dan PI. Analisis sebatian kimia dan unsur-unsur logan disiasat menggunakan GC-MS dan ICP-MS. Hasil eksperimen MTT menunjukkan bahawa CVME mempunyai kesan ketara dalam menghalang pertumbuhan sel-sel MDA-MB-231 ($p<0.05$) dan kesannya adalah melalui ketoksikan sel terpilih iaitu terhadap sel-sel kanser sahaja. Selain itu, kajian ini mendedahkan bahawa CVME menyebabkan sel-sel mati melalui cara apoptosis yang ditunjukkan melalui peningkatan peratusan sel-sel apoptosis ($p<0.05$) jika dibandingkan dengan sel-sel kanser yang tidak dirawat. CVME juga didapati mengandungi beberapa sebatian kimia yang aktif dan memiliki aktviti antikanser

seperti phytol dan asid 10-undecenoic. Analisis unsur logam merbahaya juga mendedahkan bahawa tumbuhan ini mengandungi kandungan kromium yang tinggi yang boleh menyebabkan kesan-kesan sampingan yang berbahaya. Sebagai kesimpulan, kajian ini menunjukkan *C. vespertilionis* mempunyai potensi untuk dijadikan sumber rawatan bagi kanser payudara.

ABSTRACT

Breast cancer is one of the common malignancies among women worldwide. There is a constant demand for new therapies to treat this complex disease. Notably, scientific research is drawing its attention towards medicinal plants as a potential source for anticancer agents. *Christia vespertilionis* is a local herb claimed to have anticancer properties. It is widely marketed as a food supplement in Malaysia thus raise an awareness regarding their efficacy and safety. Moreover, current research about the mechanisms of its anticancer potential is also limited. Herein, this study aimed to evaluate the efficacy of *C. vespertilionis* extracts on breast cancer cell lines (MDA-MB-231 and MCF-7) and to investigate the mode of cell death that underlies its anticancer effects. Besides, the phytochemical in CVME and heavy metals of the plant were also investigated. The cytotoxic activities of CVME, CVAE and tamoxifen was evaluated by MTT assay while the mode of cell death was evaluated by Annexin V-FITC and PI staining. The phytochemical and heavy metal analysis were investigated by GC-MS and ICP-MS, respectively. Results from MTT assay showed that CVME significantly inhibits proliferation of MDA-MB-231 cells line ($p<0.05$) and the effect was selectively cytotoxic towards cancerous cells only. Furthermore, this study showed that CVME induces apoptosis as indicated by a significant increase of apoptotic cell percentage ($p<0.05$) when compared with untreated cells. CVME was also found to contain numbers of pharmacologically bioactive compounds that possess anticancer activities such as phytol and 10-undecenoic acid. Hazardous heavy metal analysis revealed that this plant contains a high concentration of chromium which may cause toxic side effects. Overall, this study demonstrates the potential applications of *C. vespertilionis* as an anticancer drug for breast cancer treatment.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Cancer is a condition of abnormal cell growth in the body that disobeys the normal rules of cell division and cell death. Cancer cells also have an abnormal structure and are unable to differentiate into functioning normal cells. This group of cancer cells will eventually form a tumor that has the potential to become malignant and metastasize to different parts of the body, thus jeopardize the normal physiologic body function (Umar et al., 2012).

Cancer is a second leading cause of death worldwide, estimated to cause 9.6 million of death in 2018 (WHO, 2018). It is also predicted that the cases of cancer diagnosed will increase and reach 24 million by 2035 (GLOBOCAN, 2012). In Malaysia, 12% of mortality in the hospital were caused by cancer, making it as a major cause of death after cardiovascular and blood diseases. The most common type of cancer detected among Malaysians was breast cancer followed by colorectal and respiratory tract cancers (Azizah et al., 2016). Breast cancer was also a common malignancy among women in the United States, estimated to affect 268,670 individuals and cause more than 40,000 of death in 2018 (Siegel et al., 2018).

The increment of breast cancer cases and the toxic side effects of current breast cancer treatments have created a demand for new therapeutic agents to treat the disease. In this regard, the medicinal plant have been identified as having the potential to be develop into staple drugs for cancer. The secondary metabolites produced by some

plants have been scientifically reported by researchers to be effective in treating a number of illnesses and diseases such as bacterial infection, diabetes and physical injuries (Raman et al., 2012; Reghu et al., 2017; Sharma et al., 2013). Moreover, the medicinal plant has been preferred as they have been successfully isolated, modified, synthesized and approved to be used as anticancer drugs for years (Demain and Vaishnav, 2011). To be specific, 108 medicinal plant-based drugs were studied in various testing procedures such as in preclinical, clinical phases I to III, and preregistration in early 2008 (Harvey, 2008).

The search for potential anticancer agents still continues until recent time and one of the plants screened to have anticancer properties is *Christia vespertilionis* (CV) or locally known as 'daun rerama' (Nguyen-Pouplin et al., 2007). This plant has 'butterfly' shaped leaves, widespread in tropical Southeast Asia and native in Malaysia. Recently, this plant was promoted as a 'cancer remedy' among some herb's practitioners in the local market and commercialized in the form of tea. Thus, raises an awareness regarding the safety and efficiency of *C. vespertilionis* in treating cancer. Therefore, the present study was designed to assess the cytotoxicity of *C. vespertilionis* towards breast cancer cell lines. Analysis of the mode of cell death was also conducted to confirm either the cell dead through apoptosis or necrosis mechanism.

1.2 Rationale of study

The risk and detrimental side effects of radiotherapy and chemotherapy for cancer treatment are acknowledged to fast compensating their benefits. Thus, increasing the urged for the development of a new therapeutic agent. Medicinal plants have gained an attention as a source of novel anticancer drugs by the scientific community. They

have been perceived as safe by the public because it is natural and have been traditionally used. Nevertheless, their natural origin is not a guarantee of safety as concerning many toxic cases due to the herbal product consumption had been noted. Recently, *Christia* vespertilionis-based products which have been claimed to have anticancer activity have been distributed and consumed in Malaysia, even though the scientific evidence of anticancer activities of *C. vespertilionis* is still not fully elucidated. Moreover, the toxic profile of *C. vespertilionis* also is not fully understood. Hence, through the cytotoxic and safety evaluation conducted in this study, it could provide knowledge and safeguard to the public regarding the efficacy and safety of *C. vespertilionis*.

1.3 Objectives of the study

1.3.1 General objective

The general objective of this study was to evaluate the cytotoxic activity of *C. vespertilionis* aqueous extract (CVAE) and *C. vespertilionis* methanol extract (CVME) in MDA-MB-231 and MCF-7 breast cancer cell lines.

1.3.2 Specific objectives

1. To evaluate the IC_{50} of CVAE and CVME in MDA-MB-231 and MCF-7 cell lines.
2. To determine the mode of cell death after treatment with CVME or CVAE.
3. To identify the phytochemicals present in CVME or CVAE by using gas chromatography-mass spectrometry (GC-MS).
4. To identify the heavy metals present in CV by using inductively coupled plasma-mass spectrometry (ICP-MS).

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is an abnormal cell which divides uncontrollably beyond their natural boundaries. Accumulating cancer cell can form a mass of tissue or tumor (Aktipis et al., 2015). The type of tumor can be divided into benign or malignant. If the tumor does not occupy the surrounding tissue, it is denoted as benign. In contrast, if the tumor successfully invades to distant part of the tissues through the local spread, blood, and lymphatic vessels, it is said to have metastasized and denoted as malignant (Vogelstein et al., 2013).

Cancer cells act intelligently in many ways to maintain their survival. One important myriad of cancer cells is the capability to avoid apoptosis; a natural programmed cell death (PCD). Besides, cancer cells can sustain proliferative signalling, evading growth suppressors, activating metastasis, enabling replicative immortality and inducing angiogenesis during metastasis. Cancer cells also can avoid immune cell destruction, deregulating cellular energetics, contain mutated genome and capable of promoting inflammation (Hanahan and Weinberg, 2011).

There are over 100 types of cancer recognized and they are classified in two ways; by the location in the body and by the type of cell or tissue that cancer arises (histological type). Based on the histological type, cancer can be classified into carcinoma, sarcoma, myeloma, lymphoma, and leukaemia. Carcinoma is an epithelial tissue malignancies account for 80-90% of cancer cases worldwide (Almeida and Barry, 2011). Breast,

lung, colorectal, skin and prostate cancer are the examples of cancer that can be classified under carcinoma (National Cancer Institute, 2015). The global incidence and death of top five cancers is shown in Table 2.1.

2.1.1 Breast cancer

Breast cancer is characterized by abnormal growth of cells in the mammary epithelial tissue (Hinck and Näthke, 2014). The type of breast cancer depends on the type of cells in the breast that turn into cancer. The most common type of breast cancer is ductal carcinoma; cancer cells that grow from the ducts of the breast, and lobular carcinoma; cancer cells that grow from the lobules of the breast. Both types of breast cancer cells can invade surrounding tissues and metastasize to distant parts of the body through blood and lymphatic vessel (Centre for Disease Control, 2018).

2.1.2 Epidemiology of breast cancer

Breast cancer is the most common cancer worldwide diagnosed with an estimated 2.4 million incidence cases reported in 2015 (Fitzmaurice et al., 2017). In the United States, 63,960 cases of carcinoma in situ and 87,290 cases of melanoma in situ of breast cancer were expected to be diagnosed in 2018 (Siegel et al., 2018). In Malaysia, breast cancer accounted for 32.1% of all cancers among women with estimated number of 18,206 cases from 2007-2011. Based on ethnic group, the incidence of breast cancer was found to be the highest among Chinese women with the ratio of 1 in 22 people followed by Indian with the ratio of 1 in 24 people and the least is among Malay with the ratio of 1 in 35 people (Azizah et al., 2016).

Table 2.1 Global incidence and deaths for top five cancers in 2015. Adapted from Fitzmaurice et al. (2017)

Ranking	Cancer	Incident Cases, Thousands	Deaths, Thousands
1	Breast	2422	534
2	Tracheal, bronchus, and lung	2019	1722
3	Colon and rectum cancer	1653	832
4	Prostate	1618	366
5	Stomach	1313	819

2.1.3 Breast cancer treatment

According to Senkus et al. (2015), the treatment of breast cancer was given according to the cancer stage identified by node-tumor-metastasis staging. For stage I and II, mastectomy or breast-conserving surgery is the preferred treatment. For breast-conserving surgery, radiation therapy is given after the surgery to reduce local recurrence and increase the survival rate (Clarke et al., 2005). Early stage breast cancer patients also receive adjuvant systematic therapies. The choice of adjuvant systematic therapies are endocrine therapy, tissue-targeted therapy, and chemotherapy. For stage III, the standard treatments are induction chemotherapy followed by surgery and radiation therapy. While, for stage IV, the treatments are endocrine therapy or chemotherapy. (Maughan and Lutterbie, 2010). The type of therapy and list of medication commonly used to treat breast cancer is shown in Table 2.2.

2.1.3.1 Tamoxifen

Tamoxifen is a drug that has been used for the treatment of estrogen receptor-positive breast cancer for more than 30 years. It has been used in both adjuvant and metastatic settings (Matteo et al., 2012). Tamoxifen can be used alone or in combination with chemotherapy to treat advanced breast cancer (Fisher et al., 1998). Despite its effectiveness in preventing recurrence and treat breast cancer, tamoxifen has been associated with several side effects such as increased the risk of developing uterine cancer, endometrial cancer, blood clots, stroke and cataracts (Sugerman, 2013). Therefore, the search for a new therapy that has less adverse side effects is in huge demand nowadays to treat breast cancer.

Table 2.2 Medication used in the treatment of breast cancer. Adapted from Maughan and Lutterbie (2010)

Type of therapy	Medication
Chemotherapy	Doxorubicin Epirubicin Docetaxel Paclitaxel
Endocrine	Anastrozole Exemestane Letrozole Goserelin Tamoxifen
Tissue-targeted	Trastuzumab

2.1.3.2 Side effects of cancer treatments

Treatments for cancer such as surgery, radiation, hormonal therapy, and chemotherapy come with a burden of side effects to the cancer patients (Cleeland et al., 2012). The most often side effect experienced by cancer patients is fatigue, estimated to affect between 60% to 90% of patients during their treatment or survivorship (Bower et al., 2000). This cancer-related fatigue is usually accompanied with other symptoms such as reduce cognitive function, insomnia, depression, and anxiety (Valentine and Meyers, 2001). To be worsen, fatigue can effect patients' compliance with medical treatment as reported by Berger et al. (2015).

Radiotherapy is a choice of treatment for cancer patient that has many deleterious side effects. Radiotherapy utilizes radiation that can damage the DNA of both cancerous and normal cells and inhibiting its ability to reproduce (Berkey, 2010). The most often side effects associated with radiotherapy are hair loss, weight changes and anemia. Radiotherapy also can cause radiation dermatitis such as itching, burning, scaling, pigmentation changes, and ulceration (Berger et al., 2011; Berkey, 2010). For breast cancer patients receiving radiotherapy, cardiovascular disease and radiation pneumonitis are well-recognize adverse effect. The risk of these side effects increases with longer follow-up of treatment and increases dose of radiation (Adams et al., 2003; Monsuez et al., 2010). Besides, cancer patients receiving chemotherapy were also reported to develop several side effects such as thrombosis, a condition of blood vessel blockage due to blood clot (Swystun et al., 2011) and early menopause among female young patients survivors (Partridge et al., 2008).

2.2 Mechanism of cell death in cancer

2.2.1 Apoptosis

Cell division is a normal physiologic process that needs to be counterbalanced by cell death. This crucial cell death process is known as apoptosis or PCD. The term "apoptosis" was introduced by Kerr to describe a form of hepatocellular cell death in ischemic liver disease (Kerr et al., 1972). The morphological features of apoptosis are membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation, and loss of adhesion to neighbour cells and extracellular matrix followed by phagocytosis of the fragments by nearby cells (Hotchkiss et al., 2009) (Figure 2.1). Meanwhile, the biochemical changes of apoptosis are activation of proteases (caspases), chromosomal DNA cleavage into internucleosomal fragments, phosphatidylserine externalization and intracellular substrate cleavage by proteolysis (Ouyang et al., 2012).

Apoptosis was reported to be dysregulated and lose its beneficial effect in numerous pathological conditions such as auto-immune disease, Alzheimer's disease, Parkinson's disease as well as cancer (Portt et al., 2011). According to Hanahan and Weinberg (2011), one of the cancer biology features is the imbalance between cell proliferation and apoptotic cell death as cancer cell was known to be able to avoid PCD. Thus, the search for a therapeutic agent that can induce apoptosis mode of cell death to treat cancer has been an indispensable approach by many studies as apoptosis is one of the important markers of potential anticancer drugs. In this regards, researchers have identified that some medicinal plants and their bioactive compounds capable of inducing apoptosis that is blocked in cancer cells (Safarzadeh et al., 2014)

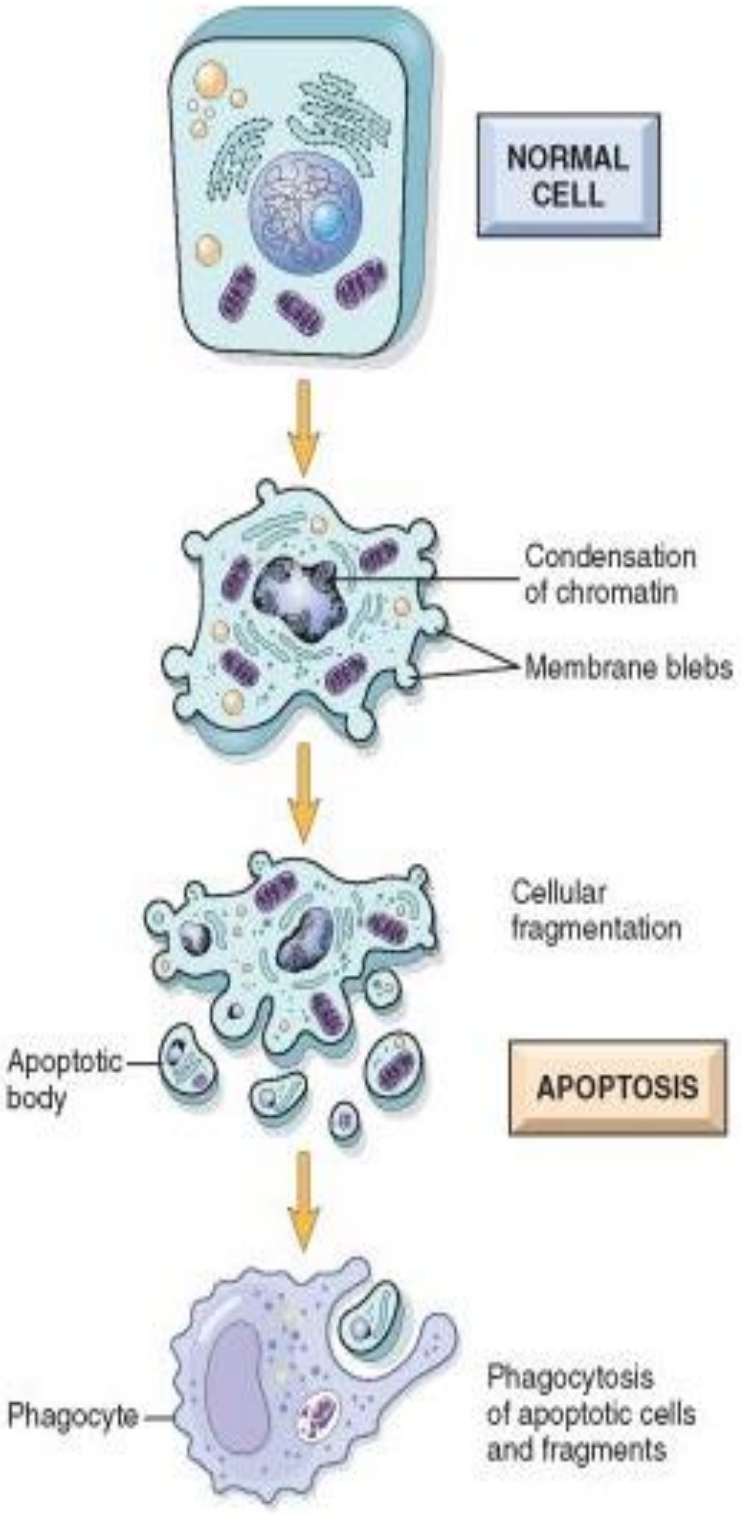

Figure 2.1 Cytology and morphological hallmarks of apoptosis (Nunes et al., 2014)

2.2.2 Necrosis

Necrosis is another form of cell death. Morphologically, necrotic cell death can be characterized by a gain in cell volume, swelling of organelles such as mitochondria and endoplasmic reticulum, plasma membrane rupture and loss of intracellular contents to extracellular matrix (Figure 2.2). The biochemical hallmarks of apoptosis such as activation of proteases (caspases) and fragmentation of oligonucleosomal DNA are usually absent in necrotic cells (Proskuryakov and Gabai, 2010).

Necrosis occurs due to several factors such as physicochemical trauma, and also during viral and bacterial infection (Mohammad et al., 2015). Besides, necrosis is a common feature of human solid tumors in the core region due to oxygen and glucose depletion (Ouyang et al., 2012). Necrosis also associated with activated angiogenesis, reduced vascular maturation and presence of vascular invasion. Consequently, leads to the tumor vascular formation and metastatic spread (Stefansson et al., 2006). In breast cancer, necrosis has been related to high-grade disease, increased tumor size and high micro vessel density. While, in endometrial cancer, necrosis is associated with increase in tumor proliferation rate (Bredholt et al., 2015).

According to Portt et al. (2011), necrosis is an alternative cellular suicide pathway if the normal apoptosis is blocked or defected. However, the inflammatory reaction produced from the necrotic cell was reported to promote cancer cell growth because the immune cells which react to the inflammation produced essential cytokines to nurture the surviving cancer cells (Vakkila and Lotze, 2004). Briefly, these evidences bring into a suggestion that necrosis form of cell death is highly up regulated during cancer progression.

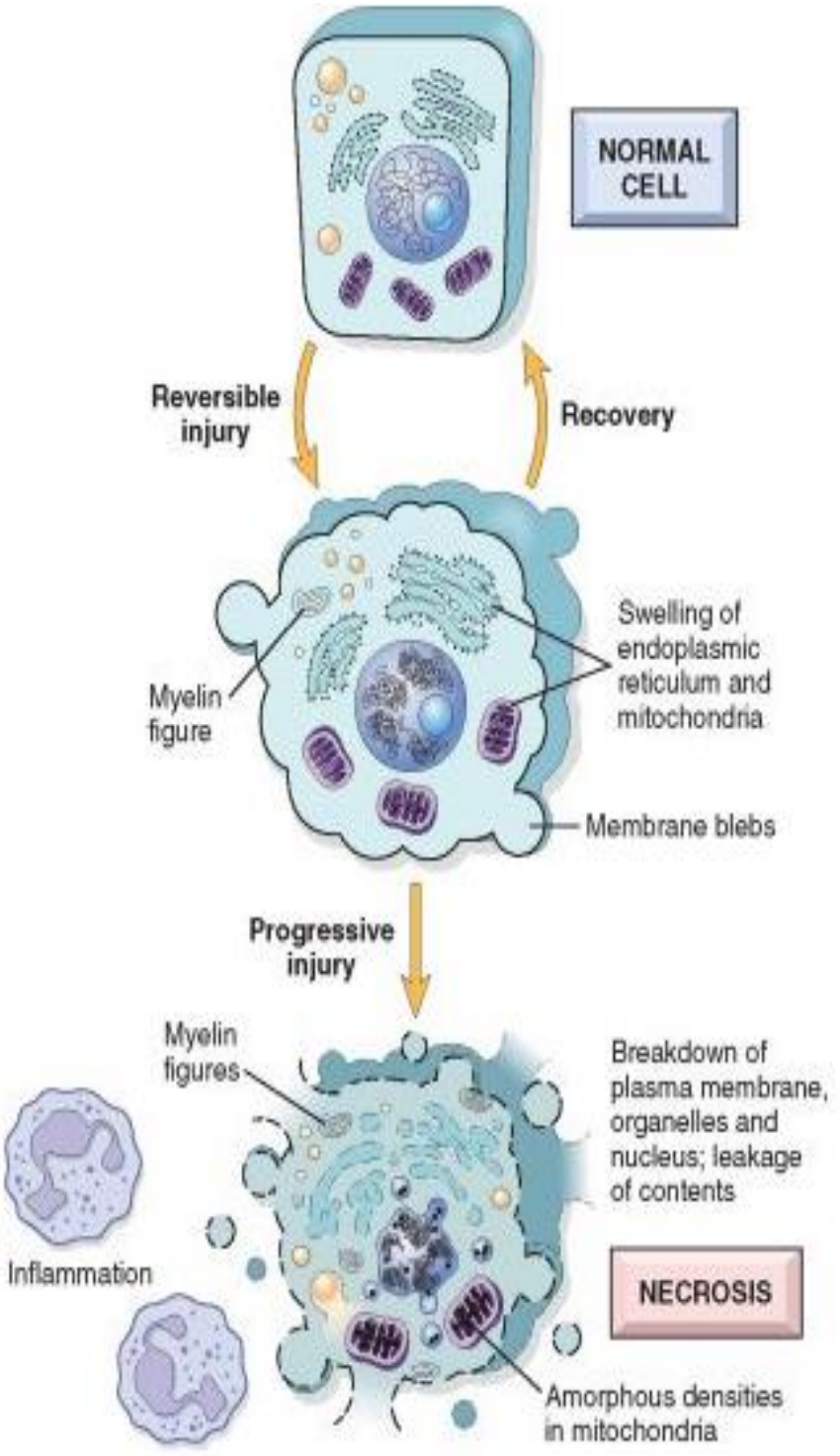

Figure 2.2 Cytology and morphological hallmarks of necrosis (Nunes et al., 2014)

2.3 Medicinal plants

2.3.1 Medicinal plants as an anticancer agent

For decades, secondary metabolites or phytochemical naturally produced by medicinal plants were utilized as a source of drug candidates for various ailments including cancer. In fact, 48 out of 65 new drugs registered from 1981-2002 for cancer treatment were derived from medicinal plants. For example are vincristine (Oncovin®) from *Catharanthus roseus* plant and paclitaxel (Taxol®) from *Taxus brevifolia* plant which have been used to treat several neoplasms including breast cancer (Nobili et al., 2009; Safarzadeh et al., 2014). Plants are also rich with other phytochemicals that are reported to have anticancer activities such as polyphenols, flavonoids and plant hormone; brassinosteroids (M.Greenwell, 2015).

2.3.2 Medicinal plants induce apoptosis

Apoptosis induction is one of the ideal characteristics of the anticancer agent as it does not elicit an inflammatory reaction. Recent findings suggested that natural compounds have the capability to stimulate apoptosis in cancer cells. A few examples of the phytochemicals that are capable of inducing apoptosis in cancer cells are epigallocatechin gallate (EGCG) from green tea, resveratrol from the skin of red grapes, and curcumin from the rhizome of Curcuma species (Mohammad et al., 2015). EGCG was reported to induce apoptosis by inhibiting proteasome activity thus led to the accumulation of IkB-α and p27 protein that eventually cause growth arrest (Kazi et al., 2003). Meanwhile, resveratrol and curcumin induce apoptosis by increasing the sensitivity of resistant cancer cells and elevate pro-apoptotic factor Bax respectively (Hayun et al., 2009; Sprouse and Herbert, 2014).

2.3.3 Safety of medicinal plants

Nowadays, the safety of food including medicinal plants is in increase concern. Many adverse side effects have been reported due to consumption of medicinal plants such as direct toxic effects, allergic reactions, effects from contaminants, and interactions with drugs and other herbs (Bent and Ko, 2004). Some medicinal plants and their constituents has been shown to cause liver injury or carcinogenicity as reported by Moreira et al. (2014). For example are *Aristolochia* sp. that can cause upper tract urothelial carcinoma (Chen et al., 2013) and *Piper methysticum G.* Forst. that can cause hepatocellular and cholestasis pattern of liver injury (Olsen et al., 2011). These reports suggest that even though the plant was perceived as 'natural' and had been used for a long time as a traditional medicine, it does not ensure the safety and effectiveness of the medicinal plants. In this regards, strict evaluation of plant safety is vital before being commercialized as a product.

2.3.4 *Christia vespertilionis* (CV)

2.3.4.1 Ethnobotanical view

Christia vespertilionis (CV) is commonly known as a butterfly wing or 'rerama' (butterfly in Malay). This plant is a non-climbing perennial herb that comes from Fabaceae family and can be found widespread in tropical Southeast Asia. This plant is one of the popular ornamental plants because of its uniquely shaped trifoliate leaves. The juvenile leaves of CV usually have a purple tint and dark green along prominent veins and the plant can grow until 60-120 cm. The picture of CV plants is shown in Figure 2.2.

Figure 2.3 *Christia vespertilionis* plant. Retrieved from https://sv.m.wikipedia.org/wiki/Fil:Christia_vespertilionis_Blanco1.201.jpg

2.3.4.2 Traditional uses

Traditionally, the whole plant of CV has been reported to be used for the relief of tuberculosis, bronchitis, inflamed tonsils, muscle weakness, snake bites, bone fracture, cold and to increase blood circulation (Brach and Song, 2006; Bunawan et al., 2015). Besides, CV was also used for the treatment of scabies by applying the crushed fresh leaves topically (Upadhyay et al., 2013).

2.3.4.3 Phytochemicals

CV was reported to contain various phytochemicals including alkaloids, pheophorbid-a (Chlorophyll derivative), isoquinoline alkaloids, triterpenes, fatty acids, phenols, alkanes, palmitine, corynoxidine, and long chained alcohols (Hofer et al., 2013). Besides, CV also contains other phytochemicals such as christine, christanoate, pentacyclic triterpenes, flavonoid, steroids, and monoterpene as reported by Upadhyay et al., (2013).

2.3.4.4 Anticancer activity of CV

A study conducted by Nguyen-Pouplin et al. (2007) is one of an early study that highlighted the anticancer potential of CV extract. The study reported that the cyclohexane extract of CV showed high cytotoxicity against human cervix carcinoma cells Hela. Furthermore, CV extracts also showed high cytotoxicity against neuroendocrine tumor as studied by Hofer et al. (2013). The study concluded that ethyl acetate fraction of CV yields highest cytotoxic activity against human medullary thyroid carcinoma and human small intestinal neuroendocrine tumor cell lines.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 General instruments and apparatus

All general instruments and apparatus used in this study are listed in Table 3.1

3.1.2 Consumable items

All consumable items used in this study are listed in Table 3.2

3.1.3 Chemicals and reagents

All chemicals and reagents used in this study are listed in Table 3.3

3.1.4 Plant materials

The green butterfly wing of *Christia vespertilionis* plant was bought from Rural Transformation Centre Kelantan, Malaysia. The complete set of the plant was sent to Herbarium Kulliyah of Pharmacy, International Islamic University Malaysia and the taxonomic identity of the plant was authenticated by Dr Shamsul Khamis, a botanist. The voucher specimen is PPIUM 0273-1 and the scientific name of the plant was obtained. The scientific classification of CV was identified belongs to the genus of *Christia* and species of *vespertilionis.*

Table 3.1 General instruments and apparatus used in this study

Instruments and apparatus	**Manufacturer**
Analytical balance	Denver instrument, USA
Biological safety cabinet	Thermo Fisher Scientific, USA
Centrifuge	Hettich, Germany
CO_2 incubator	Heraeus, Japan
Deep freezer -80 ºC	Sanyo, Japan
Drying oven	Binder GmBH, Germany
Flow cytometer (FACS Calibur)	Beckman-Coulter, USA
Fridge (-4 ºC and -20 ºC)	SHARP, Japan
Fume hood	ERLA, Malaysia
GC-MS	Conquer Scientific, USA
Grinder	Buffalo Machinery Co. Ltd., Taiwan
Inverted microscope	Olympus, Japan
Microplate reader	Biorad, USA
MiniSpin Micro centrifuge	Eppendorf, Germany
Orbital shaker	Heidolph, UK
Rotary evaporator	Heidolph, UK
Spectrophotometer	Shimadzu Corp., Japan
Vortex mixer	ERLA, Malaysia
Water bath	Memmert, Germany
Hemacytometer set	LO-laboroptik Ltd, UK

Table 3.2 Consumables used in this study

Consumables	**Manufacturers**
96-wells plates	Costar Cornin, USA
6-wells plate	SPL life science, Korea
0.22 µm syringe filter membrane	TPP, Switzerland
Centrifuge tube (1.5 ml, 15 ml)	Axygen Scientific, USA
Cryogenic vials	Invitrogen, USA
Falcon tube (50 ml)	Thermo Fisher Scientific, USA
Filter paper	Whatman, UK
Flow cytometry tube	BD, USA
Plastic petri dish	Thermo Fisher Scientific, USA
Pipette tips (1-1000 µl)	Axygen Scientific, USA
Pipette tips (1-200 µl)	Axygen Scientific, USA
Pipette tips (0.2-10 µl)	Thermo Fisher Scientific, USA
Serological pipette	TPP, Switzerland
Syringe (10 ml)	BD, USA
T25 tissue culture flask	TPP, Switzerland
T75 tissue culture flask	TPP, Switzerland

Table 3.3 Chemicals and reagents used in this study

Chemical or reagents	**Manufacturer**
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) powder	Amresco, Inc. USA
Absolute ethanol	Merck, Germany
Dimethyl sulfoxide (DMSO)	VWR life science, USA
Dulbecco's Modified Eagle Medium (DMEM)	ATCC, USA
Ethyl acetate	HmbG, Malaysia
Fetal bovine serum	Gibco Laboratories, USA
Methanol	Merck, Germany
Penicillin streptomycin	Gibco Laboratories, USA
Petroleum ether	HmbG, Malaysia
Phosphate buffer saline (PBS)	Invitrogen, USA
Propidium iodide	Merck, Germany
Tamoxifen	Sigma-Aldrich, USA
Trypan blue	Gibco Laboratories, USA
Trypsin-EDTA 0.25%	Gibco Laboratories, USA

3.2 Methods

This study started with the collection and identification of the plant. Then, extraction was performed to obtain the extracts. The extracts obtained were used in MTT assay to evaluate the IC_{50} of the extracts against cancerous and non-cancerous cell lines. Then, the cytotoxicity of the extracts was further evaluated by Annexin V apoptosis assay to determine the mode of cell death. Concurrently, identification of bioactive compounds in the most potent CV extract and heavy metals in the leaves powder of CV were conducted by using GC-MS and ICP-MS, respectively. The flowchart of this study is shown in Figure 3.

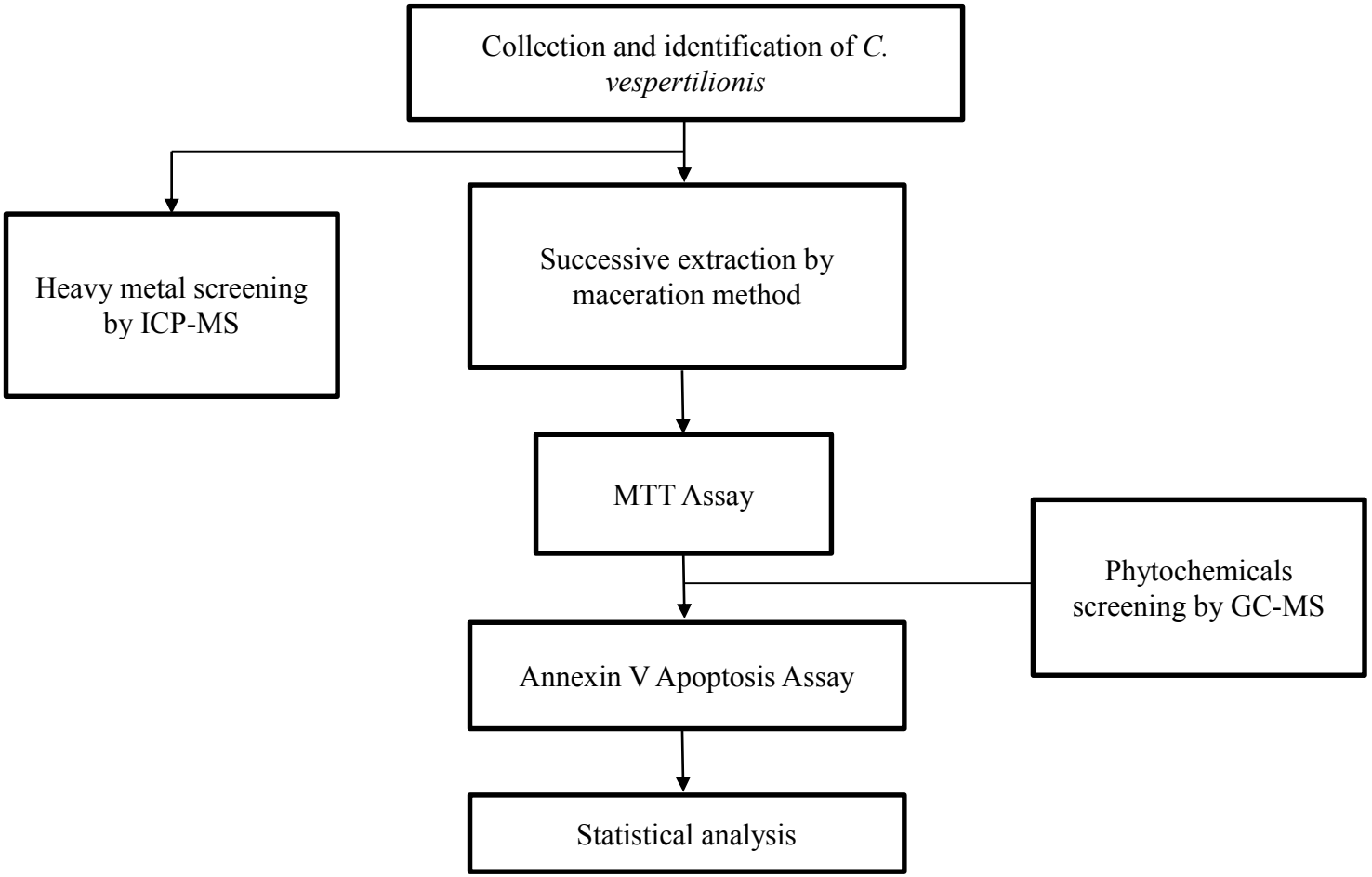

Figure 3.1 Experimental flowchart

3.2.1 Extraction of CV leaves

3.2.1.1 Preparation of CV leave extracts

The fresh leaves of CV were cleaned and rinsed with tap water and distilled water to remove surface pollutants. After that, the leaves were dried in an oven at 40ºC in a ventilated drying oven for four days or until constant weight was measured. Then, the leaves were ground into fine powder by using a grinding mill. Then. The ground sample was stored in the chiller at 4ºC until further use.

The ground CV leaves were extracted successively by maceration method in petroleum ether, ethyl acetate, methanol and water (1:10 plant/solvent ratio) for 24 hours in each solvent as shown in Figure 3.2. In this study, 50 g of the ground CV leave were macerated in 500 ml of solvents. After 24 hours of extraction, the extract solutions were filtered by using Whatman filter paper to remove any left residues. Then, the extracts were concentrated under reduced pressure until complete elimination of organic solvents and subsequently freeze-dried for water solvent providing CV petroleum ether extract (CVPEE), CV ethyl acetate extract (CVEAE), CV methanol extract (CVME) and CV aqueous extract (CVAE). All extracts were stored in the chiller at 4ºC until further use. The yields of the extracts were weighed by electronic balance and calculated by using the following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of extract (g)}}{\text{Weight of sample taken for extraction (g)}}$$