# CYTOTOXIC ANALYSIS OF *Christia vespertilionis* LEAVES EXTRACTS TREATED ON HUMAN CERVICAL CANCER CELL (HeLa)

**MOHAMMAD MIZWARUDDIN BIN SIDEK**

**UNIVERSITI SAINS MALAYSIA**

**2019**

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**by**

**MOHAMMAD MIZWARUDDIN BIN SIDEK**

**Dissertation submitted in fulfilment of the requirements**
**for the degree of**
**Master of Science (Health Toxicology)**

**AUGUST 2019**

## DECLARATION

I hereby declare that this research was sent to Universiti Sains Malaysia (USM) for the degree of Master of Science in Health Toxicology. It has not been sent to other universities. With that, this research can be used for consultation and photocopied as reference.

Sincerely,

…….

MOHAMMAD MIZWARUDDIN BIN SIDEK

(P-IPM0055/18)

## ACKNOWLEDGEMENT

All great abundant thanks to the Allah Almighty, the Omniscient and the sole nourish the sustainer of the universe, who taught me and guided my steps to these humble efforts. My deep gratitude goes to Dr. Hasni Bin Arsad and Dr Nur Nadhirah Mohamad Zain, who expertly guided me through my research project. Other than that, I wish to express my appreciation and thanks to those who provided their time, effort and support for this project. To the members of my dissertation committee, thank you for sticking with me. My profound appreciation is extended to staffs of Animal Research Centre (ARC) and staffs in Advance Medical and Dental Institute (AMDI). Lastly, it is my utmost pleasure to dedicate this work to my dear parents and my family, who granted me the gift of their unwavering belief in my ability to accomplish this goal: thank you for your support and patience. Their continuous support, encouragement and leadership kept me constantly engaged with my research, and for that, I will be forever grateful.

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## LIST OF SYMBOLS

| | |
|---|---|
| µg | Microgram |
| mL | Microliter |
| mg | Milligram |
| nm | nanometer |
| g | Gram |
| cm | Centimeter |
| ± | Plus Minus |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ARC | Animal Research Centre |
| CAOV-3 | Ovarian Cancer Cell Line |
| $CO_2$ | Carbon Dioxide |
| *C. vespertilionis* | *Christia vespertilionis* |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DCM | Dichloromethane |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl Sulfoxide |
| EDTA | Ethylenediaminetetraacetic acid |
| FBS | Fetal Bovine Serum |
| GC-MS | Gas Chromatography-Mass Spectrometry |
| H22 | Hepatocellular carcinoma of the mouse |
| HeLa | Human Cervical Cancer Cell Line |
| HPLC | High Pressure Liquid Chromatography |
| $IC_{50}$ | Half Inhibitory Concentration |
| KRJ-I | Human Small Intestinal Neuroendocrine Tumours |
| LC-MS | Liquid Chromatography-Mass Spectrometry |
| LLE | Liquid-Liquid Extraction |
| MCF-7 | Breast Cancer Cell Line |
| MRC-5 | Normal Lung Fibroblast Cell Line |
| MTC-SK | Medullary Thyroid Carcinoma |
| NIST | National Institute Slandered and Technology |
| NMR | Nuclear Magnetic Resonance |
| PBS | Phosphate Buffered Saline |
| Pen-Strep | Penicillin-Streptomycin |
| RPM | Revolution Per Minutes |
| RT | Retention Time |
| S180 | Murine Sarcoma Cancer Cell Line |
| SD | Standard Deviation |
| SRB | Sulforhodamine B |

| | |
|---|---|
| TMS | Trimethylsilyl |
| USM | Universiti Sains Malaysia |
| WHO | World Health Organization |

**ANALISIS SITOTOKSIK EKSTRAK DAUN *Christia vespertilionis* KE ATAS SEL KANSER SERVIKAL (HeLa)**

## ABSTRAK

Tumbuhan herba telah lama digunakan oleh orang-orang tua pada zaman dahulu kerana mempunyai banyak kesan terapeutik. Pada zaman moden ini, populariti penggunaan tumbuh-tumbuhan ini terutamanya kerana kesan antikanser yang terdapat di dalam tumbuhan semakin meningkat. Banyak kajian telah dilakukan untuk mengekstrak kesemua sebatian aktif yang terdapat di dalam tumbuhan yang dipercayai mempunyai kesan terapeutik teutamanya kesan antikanser. Tambahan pula, banyak testimoni yang menggunakan herba sebagai pengubat kanser tanpa mempunyai bukti saintifik telah digunakan. Di dalam kajian ini, ekstrak daun *Christia vespertilionis* telah digunakan untuk mengkaji kesan-kesan sitotoksik keatas sel-sel kanser servikal. Ekstrak daun *C. vespertilionis* telah melalui proses pecahan menggunakan ekstrak pecahan mentah dari larutan metanol untuk menghasilkan beberapa pecahan ekstrak lagi mengikut turutan kekutuban seperti heksana, diklorometana, kloroform, n-butanol dan akueus. Berdasarkan kajian ini, ekstrak diklorometana (DCM) telah menunjukkan kesan-kesan sitotoksik tertinggi kepada sel-sel kanser berdasarkan kepekatan merencat separuh ($IC_{50}$). Ekstrak DCM menghasilkan kepekatan merencat separuh ($IC_{50}$) pada nilai 63.68, 55.42 and 53.04 µg/mL pada setiap 24, 48 dan 72 jam. Kehadiran badan apoptotik dan ketanggalan sel-sel kanser seawal 24 jam kemungkinan menandakan kesan sitotoksik ekstrak-ekstrak tersebut. Tambahan pula, analisis menggunakan gas kromatografi-jisim spektrometer telah menunjukkan kehadiran pelbagai sebatian aktif di dalam ekstrak DCM seperti 9,12,15-asid oktadecatrienoic, metil ester, (Z, Z, Z)-, asid heksadekanoik, metil ester, fitol, syclododecane, dan 2-asid propenoik. Oleh yang demikian, tumbuhan ini berkemungkinan besar mempunyai kepentingan dari segi farmakologi untuk masa hadapan.

**CYTOTOXIC ANALYSIS OF *Christia vespertilionis* LEAVES EXTRACTS TREATED ON HUMAN CERVICAL CANCER CELL (HeLa)**

## ABSTRACT

Plants have been used by old folk for many therapeutic effect long time ago. In modern age, the popularity of using plants especially for anticancer activities has kept increasing. Many studies have been conducted to extracted out all phytochemical compound contained in the plant who might be responsible for therapeutic effects such as anticancer activities. Plus, many testimony of herbal supplements as cancer treatment has been conducted without any scientific evidence. In this study, *Christia vespertilionis* leaves extracts have been used to investigate the cytotoxic activities of the plants towards cancer cell line. *C. vespertilionis* leaves have been fraction using partition extraction from crude (methanol) extract followed by partitioning extract with increasing polarity namely hexane, dichloromethane, chloroform, n-butanol and aqueous fractions. From the findings, dichloromethane (DCM) extract of *C. vespertilionis* leaves has shown the highest cytotoxicity properties in human cervical cancer cell line (HeLa) indicated by half inhibitory concentration ($IC_{50}$). DCM fraction exhibited significant cytotoxicity with $IC_{50}$ value of 63.68, 55.42 and 53.04 µg/mL at 24, 48 and 72 hours respectively. The present of apoptotic bodies and considerable percentage of cell to detach in 24 hours exposure might suggest cytotoxicity effect of the extract. Plus, the presence of various phytochemical compounds in the extract using gas chromatography-mass spectrometry such as 9,12,15-octadecatrienoic acid, methyl ester, (Z, Z, Z)-, hexadecanoic acid, methyl ester, phytol, cyclododecane, and 2-propenoic acid might be the reason for the cytotoxic and anti-proliferative activity exhibited by extracts onto the HeLa cell lines. It was suggested that this plant might has pharmacological importance in the future.

# CHAPTER 1
# INTRODUCTION

## 1.1 General Background of Research

*Christia vespertilionis* leaves have been used as a Malay traditional medicine to treat various diseases (Whiting, 2007; Nguyen-Pouplin et al., 2007). A few studies proved that the plant extract can be used for treatment in cancer prevention as it exhibits anticancer properties (Hofer et al., 2013; Wu et al., 2012). However, the extractions of phytochemical contents from plant that have anticancer properties depend on many factors such as extraction solvent and method used. Referring to the study conducted by Nguyen-Pouplin et al., 2007, the cyclohexane extraction of *C. vespertilionis* showed cytotoxicity activity. However, in the same study, no cytotoxic activity of *C. vespertilionis* was observed when difference solvent extraction such as methanol, crude extract, methylene chloride solvent extractions were used. Hence, the study aim is to determine *in vitro* effects of *C. vespertilionis* leaves using difference types of extraction solvent on the human cervical cancer cell (HeLa cell line). The cytotoxicity effects towards HeLa cell line indicated by half inhibitory concentration ($IC_{50}$) and morphological changes will be observed. The study also aims to show that natural product has potential as cancer preventive agent by looking to the major phytochemical compound present in the extracts.

## 1.2 Problem Statement

The use of natural product especially from plants among Malay folk for therapeutic effect has increased years by years. Plus, many research has been conducted to investigate the therapeutic effect that might present in these plants. Anticancer activities of natural product

were a great interest as it was inexpensive, applicable and accessible for cancer prevention. The anticancer properties exhibited by plant extracts is believed caused by the present of phytochemical or bioactive compound in the extract. The method that mostly used by researcher to extracted out all phytochemical compound was by conducting extraction process using solvent such as methanol or ethanol. Even though this conventional method can be used to access anticancer properties of plant extract, it might also cause various interaction among phytochemical compound itself due to abundant of compound within one single crude extract hence produce less cytotoxic effect. So, in order to overcome this problem, the phytochemical compound from the crude methanol or ethanol extract need to be separated based on solubility and polarity of phytochemical compound in certain solvent to produce several partition extracts. In addition, little information exists on the cytotoxic activity of the crude extract and partition extracts of this plant. Due to this fact, the study was conducted to undertake a detailed evaluation of the *in vitro* cytotoxicity activity of a *C. vespertilionis* leaves extracts using partition extraction method.

### 1.3 Objectives

Main Objective:

To determine the *in vitro* effects of crude methanol extract and its solvent partitioning extracts from *C. vespertilionis* leaves on the human cervical cancer cell (HeLa cell line)

The following were the specific objectives of this study:

1) To determine the cytotoxic effect of crude methanol extract and its solvent partitions extracts of *C. vespertilionis* leaves on HeLa cell line indicated by half inhibitory concentration ($IC_{50}$)

2) To determine the morphological changes of HeLa cell line after being exposed to the selected extract of *C. vespertilionis* leaves using inverted microscope
3) To determine the major phytochemical contents in the selected extract of *C. vespertilionis* using gas chromatogram-mass spectrometry (GC-MS)

### 1.4 Research Question

What are the half inhibitory concentration ($IC_{50}$) of differences solvent extractions of *C. vespertilionis* leaves on HeLa cell line?

What are the morphological changes that can be observed in HeLa cell line after being exposed with *C. vespertilionis* leaves extract that produce lowest half inhibitory concentration ($IC_{50}$)?

What are the main phytochemical compounds that can be detected by GC-MS for the selected extract of *C. vespertilionis* leaves?

### 1.5 Research Hypothesis

The differences solvent extractions of *C. vespertilionis* leaves on human cervical cancer cell line (HeLa) will be expected to produce difference results in term of half inhibitory concentration ($IC_{50}$). Some extraction may produce lower half inhibitory concentration than other extraction types. Low $IC_{50}$ value indicate that the extraction has higher cytotoxicity effect than the extraction that produce high $IC_{50}$ value. Moreover, the leaves extraction of *C. vespertilionis* may exhibits anticancer properties and potentially can treat human cervical cancer cell.

## 1.6 Significance of the Study

The findings of this study are expected to increase the database regarding the cytotoxicity of difference solvent partitioning extractions of *C. vespertilionis* leaves on human cervical cancer cell line (HeLa) indicated by half inhibitory concentration. Therefore, for the in vivo study, the researchers may only use the selected extraction that produced the effect on vertebrae model.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 *Christia vespertilionis*

*Christia vespertilionis* or commonly known as butterfly wing or Mariposa that belonging to Fabaceae (Leguminosae) family is one of the most common herb plant found in Malaysia. Other synonym scientific names used for this plant include *Vespertilionis*, *Hedysarum vespertilionis* and *Lourea vespertilionis*. The plant is mainly distributed around China and South East China which include Thailand, Vietnam, Cambodia, Indonesia, Malaysia as well as Myanmar. This plant can grow up to 60 - 120 cm tall. According to (Brach & Song, 2006), the plant has 3 leaflets with few species that have the purplish red stripes on the leaves, red colour leaves and green colour leaves. Due to the butterfly shape shown on the leaves, the plant is well known as butterfly wing plant (Figure 2.1). In the Malay communities, this plant is used in the traditional medicine to treat various diseases such as tuberculosis, snake bite, hypertension, dengue, diabetes and asthma (Whiting, 2007). Normally in the old Malay folk medicine practice, the leaves of the plant are boiled in the hot water for about 10 minutes before the water can be drunk as medicinal tea. In this modern year, the leaves of the plants were dried and packed in teabag by the producer to be commercialised in the market (Nurul et al., 2018).

Nowadays, the benefits of this *C. vespertilionis* plant has been widely known as it can treat various diseases, this plant has been commercialized as supplements, even the seed and the plant were also sold throughout the Malaysia. Apart from that, *C. vespertilionis* can be used to treat various diseases. This was proved by another study done by Nguyen-Pouplin et al. (2007) on the cyclohexane extraction from *C. vespertilionis* showed this plant have good

antiplasmodial activity in malaria diseases. This plant exhibited antiplasmodial activity with $IC_{50}$ value of 10.8±1.3 µg/mL. However, they concluded that the plant extraction has high cytotoxicity toward the mammalian cell with selective index less than 2. Other than that, this plant also has anticancer properties and anti-inflammatory characteristics that can treat cancer. The same study showed the toxicity of *C. vespertilionis* (whole plant) extraction towards HeLa cell line by 9.9 µg/mL of half inhibition concentration ($IC_{50}$). This study however showed that the HeLa cell line was affected by cyclohexane extraction of *C. vespertilionis* only compared to other extraction solvents performed in the study such as methylene chloride, crude extract, and methanol. This study has suggested that the difference extraction method might produce difference cytotoxic effects on cancer cell line.

Based on the study done by Hofer et al. (2013), their findings showed that *C. vespertilionis* plant extract at the concentration of 10 µg/mL can inhibit the proliferation of neuroendocrine tumours cells after 72 hours. However, the plant extraction showed weak inhibition activity as compared to DMSO treated control cells. Nevertheless, the 10 µg/mL of ethyl acetate fraction (CV-45) of the plant revealed that the plant extraction contains anti-proliferative and pro-apoptotic effect in human medullary thyroid carcinoma (MTC-SK) and human small intestinal neuroendocrine tumours (KRJ-I). In another study, in vivo study was conducted whereby mice were used to study the toxicity of *C. vespertilionis* on S180 and H22 tumor cells (Wu, Tang, and Lu, 2012). The cancer was induced to the mice and the intervention (*C. vespertilionis*) was given after the induction process. Significant inhibition of the tumour was observed from the in vivo study.

Figure 2.1: *Christia vespertilionis* plants can be found abundantly in Malaysia and has been used widely as medical plants among Malay people

## 2.2 Extraction Method

Extraction method is a process to separate the desired natural products from the raw materials according to their solubility and polarities. Extraction processes are divided into 2 categories, liquid-liquid extraction and solid phase extraction. The process is commonly used in natural products to extract their active ingredient or phytochemical contents. The same concept was applied on tea production to produce sweet, aromatic tea taste from dried leaves of tea. There are several factors that influence the yield of extraction process such as temperature, pH, and solvents types.

Before the extraction process was conducted, pre-extraction of plants must be conducted to ensure the entire active compound or phytochemical compound in the plants can be extracted during extraction process. The sample preparation of plants extraction can be divided into 2 groups, dry and wet sample. Dry sample referred to the sample that has been dried first using air dried or assisted dryer while wet sample referring to fresh sample without undergoes dried process. According to Sulaiman et al. (2011), wet sample's active compound is fragile and tends to deteriorate faster than dried samples. Thus, extraction process needs to be conducted as soon as possible after pre-extraction process. Another factor that influences the extraction process later is rupturing the cell plants wall by lowering the particle either through grinding or powdering process. As the particle of plants became lower, more surface contact will be exposed to the solvents later and more active compound can be extracted out (Azwanida, 2015).

In order to prepare the crude extract, commonly methanol or ethanol solvent is used to dissolve the compound. The mixture of 80% methanol and 20% water has produce variant active ingredients or phytochemical content in extraction process compared to methanol or ethanol alone (Aktumsek et al., 2013). The initial crude extract of the plants commonly contains complex various active compounds. In order to extract out the entire compound,

methanol solvent or mixture of methanol and water was used as initial solvent to produce crude extract. Methanol solvent can extract complex various type of active component from the plants compared to other solvents. This was due to the fact that the methanol-water mixture has high polarity and thus greater efficacy towards the extraction of polar phytochemicals such as flavonoids and phenolics (Nordin et al., 2017). Another factor that influences extraction process is temperature. Increasing temperature may accelerate the extraction process. However, too high temperature might degrade the active compound in the plant (Mustafa and Turner, 2011).

For the extraction process, one of the major factors that influence the extraction process is solvents. Solvents are the liquid that dissolve certain solute from the mixture based on polarities. In extraction process, solvents can be categorized into 2 major groups, polar and non-polar (Das, Tiwari, and Shrivastava, 2010). The examples of polar solvents are ethanol, butanol and water while the less polar solvents are chloroform, dichloromethane, diethyl ether or ethyl acetate. Polar solvents can extract higher polarity compound such as glycosides, sugars, amino acids, proteins and polysaccharides while less or non-polar solvent can extract less polar compound such as isoflavones, flavanones, methylated flavones and flavonols. Polarity index is a relative measure of the degree of interaction of the solvent with various polar solutes. For extracting non-polar compounds like fats, oils and lipids, non-polar solvents are used.

The determinants of optimal solvent used during extraction process will result in the cytotoxic effect after exposing the extraction solvent to the cell line culture. Common cell line culture that have been used to investigate the cytotoxicity effects of the plant extract is cancer cell line such as human cervical cancer cell (HeLa), breast cancer cell (MCF-7), ovarian cancer cell (CAOV 3) and many more. According to the study conducted by Nguyen -Pouplin et al. (2007), cyclohexane extraction of whole *C. vespertilionis* plant has produce

half inhibitory concentration ($IC_{50}$) by 9.9 µg/mL on human cervical cancer (HeLa) and 12.9 µg/mL on normal lung fibroblast (MRC-5) cell line respectively. However, in the same study, no half inhibitory concentration has been identified in other solvent extraction such as methanol, crude extract, and methylene chloride on both cell lines (Table 2.1). In another study conducted by Ganesan, Kumar, and Bhaskar (2008), difference solvent fractions of Indian red sea weeds obtained from total (methanol) extract exhibit higher antioxidant activities as compared to the total extract. This was suggested that crude extract of methanol tend to have more interfering substances as compared to fractions. However, some interfering substances in methanol extract might also cause antagonist interaction and thus decreased the effect of antioxidant and anti-proliferative properties (Silva, Lee, and Kinghorn, 1998).

Table 2.1: Extraction and *in vitro* bioassays on *Plasmodium falciparum* and human cells of the selected plants (Nguyen-Pouplin et al., 2007)

| **Selection** | | | **Extraction** | | **Antiplasmodial activity[a], *Plasmodium falciparum* FcB1** | | **Cytotoxicity, $IC_{50}$ (µg/ml)** | | **Selectivity index (SI)[b]** | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Scientific name** | **V.N.[c]** | **Part[d]** | **Extraction[e]** | **Yield (%)[f]** | **Inhibition (%) 10µg/ml** | **$IC_{50}$ (µg/ml)** | **Hela cells** | **MRC5 cells** | **Hela cells** | **MRC5 cells** |
| *Christia vespertilionis* | JN63 | w | E | 11.8 | 1 | NT | - | - | - | - |
| | | | C | 2.2 | 69 | 10.8±1.1 | 9.9 | 12.9 | 0.9 | 1.2 |
| | | | D | 0.3 | 0 | NT | - | - | - | - |
| | | | M | 77.6 | 0 | NT | - | - | - | - |

[a] NT, not tested.

[b] Selectivity index (SI), ratio of cytotoxicity on Hela or MRC5 cells to antiplasmodial activity against FcB1 strain of *Plasmodium falciparum*.

[c] V.N., voucher number; n.d., voucher not done.

[d] B, bark; L, leaves; R, roots; S, stem; Se, seeds; W, whole plant.

[e] C, cyclohexane extract; D, methylene chloride extract; E, crude extract; M, methanol extract; T, methanol extract after tannins removal.

[f] Extract yield %: E extract yield percentage is regarding the dry powdered plant; C, D and M extract percentages are regarding E extract.

## 2.3 Cervical Cancer

Cervical cancer is a cancer that occurred in cervical region in female. The cancer is due to abnormal growth of cell around cervical region. In severe condition, the abnormal cells may move to another organ or cells in the body. The condition is known as metastasis process. There are four stages of the cervical cancer, stage I, II, III and IV (Detterbeck, 2017). As the stage increased, the survival rates of the cancer patients start to decrease. The risk factors include smoking, a weak immune system, birth control pills, starting sex at a young age, and having many sexual partners.

In 2018, cervical cancer was listed as the fourth most cancer occurred in women globally (Vu et al., 2018) while in Malaysia, it was placed as third most cancer in women between 20 - 44 years old after breast cancer and colorectal cancer for 2007 - 2011 (Azizah et al., 2016). According to the study by Masood (1999), cervical cancer mostly occurred in developing countries where 70% was recorded due to multifactorial such as lack of political commitment to maintain healthcare infrastructures and facilities. Plus, more than 90% cases of cervical cancer were believed due to infection of human papilloma virus (HPV). The history has showed that women who did not take human papilloma virus vaccine prone to get cervical cancer. So, human papilloma virus vaccine was encouraged to be taken by women as early as 13 years old according to Malaysia vaccination programme (Wong, 2008). The vaccine need to be taken 3 doses within 6 months for life time. The screening of cervical cancer is Pap smear which needs to be done at least once in every 3 years for women between 21 to 65 years old. The early screening of cervical cancer can prevent the occurring of the cancer by 80% (Arbyn et al., 2010). The symptoms of cervical cancer in women are abnormal vaginal bleeding, pelvic pain, or pain during sexual intercourse.

For the treatments of cervical cancer, there were two common treatments currently, radiation and chemotherapy (Thomas, 1999). Surgical or removal of certain part in cervix (hysterectomy or trachelectomy) can be another option for the treatment. This surgical treatment however did not promise recurrences of the cancer did not occur again in the future. Some abnormal cell might be escaped from the process and might proliferate again. Meanwhile, for the radiation treatment, the radiation might reduce the possibility of recurrence from occurring again in the future since the radiation ray can kill cancer cell. However, healthy cell nearby will also be affected and die due to radiation. For the chemotherapy treatment, cisplatin or tamoxifen might be used intravenously to the patients. Similar to the radiation, this chemotherapy treatment can also kill the healthy cell (Peer et al., 2007). Plus, the adverse effect of the treatment such as bone marrow suppression, hearing problems, kidney problems, and vomiting might be occurred to the patients (Eifel et al., 2004). The needs to further investigate the safety of the current treatment must be conducted to prevent toxicity effect of the treatment to the patients.

### 2.4 Natural Product in Cervical Cancer Treatment

Natural products have been used as medicine in folklore for treatment of diseases and illnesses over thousand years in all over the world. For instance, the oil extraction from *Cupressus sempervirens* (Cypress) and *Commiphora* species (myrrh) that were used to treat coughs, colds and inflammation were the earliest records of natural product recorded from Mesopotamia since 2600 B.C (Dias, Urban, & Roessner, 2012). Moreover, there are many natural products used in traditional medicine have become potential drug candidates for the treatment of diseases because of their wide range of biological activity and diversity. The advanced developments of technology help researchers to identify and discover new potential drugs that can be applied in medical practices.

Although conventional treatment of human cervical cancer such as radiotherapy and chemotherapy can kill cancer cell but some healthy cell nearby will also be affected. Plus, low success rate such as to reduce mortality, prevent recurrences of cancer and prevent adverse effect using conventional treatment in cervical cancer treatment indicated that new treatment derived from natural plants can be an ideal treatment candidate.

Some promising anticancer activity by apoptosis process to the human cervical cancer cell mode (HeLa) has been exhibited by the *Boerhaavia diffusa* (punarnava) roots extraction (Hsu et al., 2013). The extraction product can reduce the proliferation rate of the HeLa cell. Plus, the morphology changes of the cell have been detected. The other promising candidate against cervical cancer cell was *Morinda citrifolia*. It showed synergism and enhanced therapeutic effects in combination with chemotherapy drug (cisplatin) in two human cervical carcinoma cells model, HeLa and SiHa cell lines (Gupta, 2013). The apoptotic events produced by the plant suggested that intrinsic mitochondrial pathway via up regulation of p53 and pro-apoptotic Bax protein, Bcl-XL protein and up regulation of caspase 9 and 3 activities. However, the promising of these candidates in anticancer treatment must also be evaluated to ensure the toxicity of the compound reach the target cell only and do not give any toxic effect to healthy cell.

# CHAPTER 3

# RESEARCH METHODOLOGY

## 3.1 Materials

### 3.1.1. Chemicals & Reagents

Table 3.1 List of chemicals and reagents.

| Chemicals & Reagent | Brand/Company |
|---|---|
| Dulbecco's Modified Eagle Medium (DMEM) | Gibco®, USA |
| Fetal Bovine Serum (FBS) | Gibco®, USA |
| Penicillin-Streptomycin | Gibco®, USA |
| Phosphate Buffered Saline | Gibco®, USA |
| Dimethyl Sulphoxide (DMSO) | Gibco®, USA |
| Paclitaxel | Santa Cruz Biotechnology, USA |
| Trypan Blue Dye | Gibco®, USA |
| Sulforhodamine B (SRB) reagents | Merck, USA |
| Trichloroacetic acid (TCA) | Sigma Aldrich, USA |
| Tris Buffer | Invitrogen, USA |
| Acetic acid | Sigma Aldrich, USA |
| Absolute methanol | HmbG® Chemicals, Germany |
| Hexane | Fisher Scientific, UK |
| Dichloromethane | Fisher Scientific, UK |
| Chloroform | Fisher Scientific, UK |
| n-Butanol | Fisher Scientific, UK |

3.1.2. Disposable Consumable Items

Table 3.2 List of disposable consumable items.

| **Disposable Consumable Items** | **Brand/Company** |
|---|---|
| Disposable gloves | LabServ, Ireland |
| Face mask | Medicos, Malaysia |
| Tissues Culture Flask (T25, T75) | Falcon, USA |
| Serological Pipette (5 mL, 10 mL, 25 mL) | Greiner Bio-One, Austria |
| Micropipette tips (10 µL, 100 µL, 1000 µL) | Eppendorf ,Germany |
| Microcentrifuge tube (2 mL) | Eppendorf, Germany |
| Falcon tubes (10 mL, 15 mL, 50 mL) | Thermo Scientific, USA |
| 96-well plates | Falcon, USA |
| Membrane filters (Pore size: 0.22 µm) | HmbG, Malaysia |
| Syringe | Terumo, Japan |
| Cryogenic vials (2 mL) | Corning Incorporated, USA |
| Parafilm | Bemis Company, USA |
| Eppendorf tubes (2.0 mL) | Eppendorf, Germany |
| Aluminum foils | Diamond, China |
| GC-MS vial | Agilent, USA |

### 3.1.3 Laboratory Apparatus and Instruments

Table 3.3 List of laboratory apparatus and instruments

| **Laboratory Apparatus & Instruments** | **Brand/Company** |
|---|---|
| Schott Duran Bottles (200 mL, 500 mL, 1000 mL) | Duran, Germany |
| Glass Beakers (50 mL, 100 mL, 500 mL, 1000 mL) | Pyrex, USA |
| Separatory Funnel | Pyrex, USA |
| Rotary Evaporator | Thermo Fisher Scientific, USA |
| Cell Counter | Tamaco, Taiwan |
| Hemocytometer | Hirschmann Instruments, Germany |
| Pipette tips box | Eppendorf, Germany |
| Micropipette(10 µL, 100 µL, 1000 µL) | Eppendorf, Germany |
| Inverted Microscope | Olympus, USA |
| Class II Biohazard Safety Cabinet | Labconco Corporation, USA |
| $CO_2$ Incubator | Thermo Fisher Scientific, USA |
| Chiller (4 °C) | Samsung, Korea |
| Freezer (-20 °C) | Haier, China |
| Freezer (-80 °C) | Sanyo, Japan |
| Water Bath | Memmert, USA |
| Vortex Mixer | DLAB Scientific Co., Ltd., China |
| Centrifuge | Thermo Fisher Scientific, USA |
| Analytical Balance | Sartorius, Germany |
| Plate Reader Infinite® 200 Pro | Life Sciences, Tecan, Switzerland |
| Blender | Panasonic, Japan |
| Gas Chromatography-Mass Spectrometry | Agilent, USA |
| Autoclave | Hirayama, Japan |
| Freeze Dryer | Christ, Germany |

### 3.2 Collection of *C. vespertilionis* Leaves

The *C. vespertilionis* plants were purchased from local nursery store, Guar Perahu Herbal Valley at Kubang Semang, Pulau Pinang. Later, the leaves were registered to Herbarium centre under School of Biological Sciences, Universiti Sains Malaysia for vouchering process. The plant was identified by Dr Rahmad Zakaria and registered with the voucher specimen number, 11777 (Appendix A).

### 3.3 Preparation of Crude Extract of Green *C. vespertilionis* Leaves

The leaves parts of *C. vespertilionis* plant were air dried and grinded to the powder using electronic blender. The dried leaves were weighed using the analytical balance before proceed to the extraction process. The dried powder was extracted using 80 % methanol solvent using shaker at room temperature for 72 hours with 3 repeated times each 24 hours of the methanol. In order to produce 80 % methanol solvent, the ratio of methanol to water was set up 4:1. The extraction process was carried out until a dark greenish colour of crude was obtained. Then, the solvent containing crude extract was filtered using filtered paper into a 1 L beaker. The extraction was evaporated using rotary evaporator at 40 $^{0}$C to remove the solvent. After removal of solvent under reduced pressure followed by freeze dried using freeze dryer to remove all remaining water, the remaining extract was weighed again and stored in 15 mL falcon tube and sealed with aluminium foil. The crude extract was kept in a refrigerator at 4 $^{0}$C until use. The percentage yield of methanol extract and percentage yield of partition extracts was obtained using the equation 1 and 2 respectively.

$$Percentage\ Yield\ (\%) = \frac{Weight\ of\ crude\ extract(methanolic\ extract)\ (g)}{Weight\ of\ dry\ leaves\ powder\ (g)} \times 100\% \quad (1)$$

$$Percentage\ Yield\ (\%) = \frac{Weight\ of\ each\ partition\ extract(partition\ extracts)\ (g)}{Weight\ of\ methanol\ extract\ (g)} \times 100\% \quad (2)$$

### 3.4 Preparation of Solvent Extracts using Partition Extraction Method

Fraction of every solvent extract was conducted using kupchan method with slight modification (Chaity et al., 2016). The crude extract was partitioned with the solvents with increasing polarity namely hexane, dichloromethane, chloroform and n-butanol fractions followed by solvent evaporation using rotary evaporator respectively. The ratio of aqueous fraction to each solvent fraction was 1:3. The separation of each fraction with aqueous fraction was based on density of these two solvent.

After obtaining dried crude methanol extract, the extract was rinsed with water and mixed with hexane with ratio 1:3 respectively. The two solvents were shaken in the separatory funnel and left until two immiscible phase formed. Since the density of water was higher than hexane, water was extracted out first from separatory funnel and the hexane extract later was evaporated using rotary evaporator to remove all the solvent before being stored in refrigerator until use. The crude extract rinse with water was known as aqueous fraction now. The aqueous fraction was continuously partitioned using other solvents with increasing polarity. The Figure 3.1 showed the process of partitioning the extract using modified Kupchan method extraction.

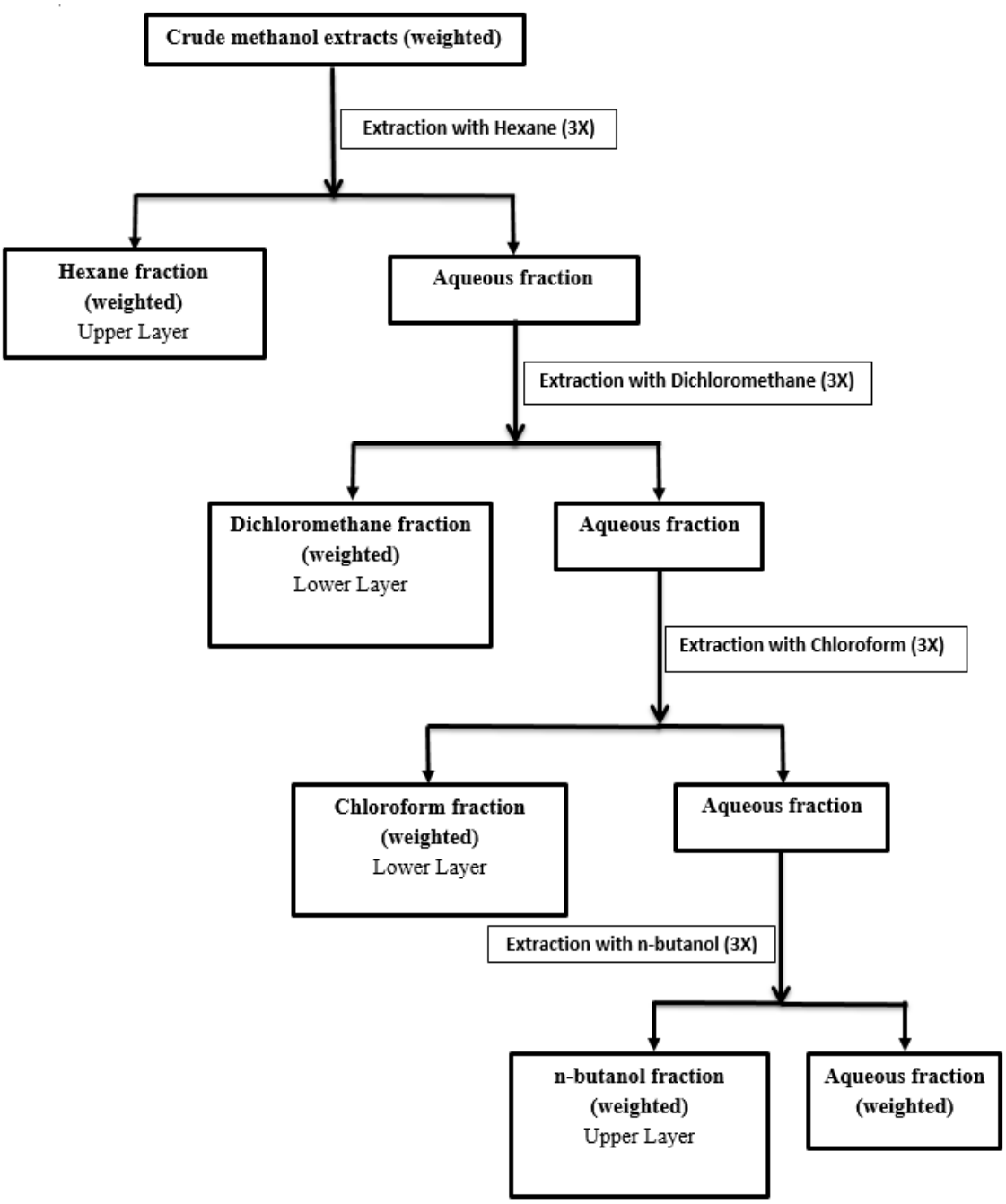


Figure 3.1: Partition extraction method adapted from Kupchan method of partitioning with slight modification

### 3.5 Preparation of *C. vespertilionis* Stock Solution

Stock solutions of *C. vespertilionis* extracts such as hexane, dichloromethane and chloroform were diluted with 100 % DMSO solvent so that the final concentration of the extracts did not exceed 0.1 % DMSO concentration where it can affect the cell growth of human cervical cancer cell (HeLa). Thus, a 200 mg/mL stock solution of hexane, dichloromethane, and chloroform extract were prepared by diluting 1000 mg of crude extracts with 5 mL of 100 % DMSO respectively followed by sterilisation using a 0.22-micron Whatman nylon syringe filters. Meanwhile, for stock solution extracts such as methanol, n-butanol and aqueous, they were directly diluted with complete growth media followed by filtering the extract solution with the 0.22-micron Whatman nylon syringe filter. All the preparation of extracts stock solution was prepared freshly. The stock solution later was stored at 4 $^{0}$C.

### 3.6 Preparation of Paclitaxel Drug Stock Solution

Paclitaxel (Molecular weight: 853.906 g/mol) is an anticancer chemotherapy drug that was used in this study as the positive control. The preparation of the stock solution at the concentration 1 mg/mL was formulated by diluting 1 mg of drug with 1 mL of DMSO. The solution was filtered with 0.22-micron syringe filters and kept in the refrigerator at 4 $^{0}$C. The preparation of positive control was conducted in biosafety cabinet fume chamber.

### 3.7 Preparation of Complete Growth Medium

The complete growth medium used in this experiment consists of Dulbecco's Modified Eagle Medium (DMEM) with 10 % (v/v) Fetal Bovine Serum (FBS) and 1 % (v/v) penicillin-streptomycin solution (PenStrep). The solution was prepared by mixing 50 mL of FBS with 5 mL of penicillin-streptomycin solution into 500 mL DMEM solution. The

mixture was gently swirled and 50 mL of complete growth medium was aliquot into few 50 mL falcon tubes. The solutions then were stored at 4 $^{0}$C.

### 3.8 Preparation of Sulforhodamine B (SRB) Reagent

The SRB cell proliferation assay was used in this experiment to measure the cell proliferation and cell viability after treated with the plant extracts and chemotherapeutic drug (positive control) at every time point, 24, 48 and 72 hours (Orellana, and Kasinski, 2016; Bhagat et al., 2014). The SRB reagent was prepared at 0.004 g/mL by adding 100 mL of 1 % acetic acid to 0.4 g of SRB powder and the mixture were vortex for few seconds until it dissolved. The solution was kept at 4 $^{0}$C.

### 3.9 Cell Lines Preparation

Cell culture preparation was performed under sterilised condition in biosafety cabinet in Animal Research Centre (ARC), Universiti Sains Malaysia. All of the materials, apparatus and other equipment were sterilized with 70 % alcohol before conducting any cell culture works. The human cervical cancer cell line (HeLa) used in this experiment was originally procured from American Type Culture Collection (ATCC), USA and the cells were stored in -80 $^{0}$C nitrogen tank at Animal Research Centre (ARC), Institut Perubatan dan Pergigian Termaju.

### 3.10 Cell Thawing

The complete growth medium container was placed in a water bath set at 37 $^{0}$C. Cryovial tube containing the frozen human cervical cancer cell (HeLa) was removed from the -80 $^{0}$C nitrogen tank. The cells were quickly thawed by gentle agitation in a 37 $^{0}$C water bath

until half of the ice was melt. In order to reduce the possibility of contamination, the vial cap was not completely thawed in the water bath. Before the vial was transferred into the biosafety cabinet, the vial was wiped with the 70 % ethanol to prevent any contamination to the cells. The thawed cells were transferred to 15 mL falcon tube containing 9 mL pre-warmed complete growth medium. The cell suspension was centrifuged at 1000 rpm for 5 minutes. The supernatant was removed from the tube and the cell was re-suspended with fresh complete growth medium before transferring into T-75 flasks. The cells were plated in T-75 flask at seeding density of 3 x $10^4$ viable cells/$cm^2$ and the flask was transferred to a 37 $^0$C in 5 % $CO_2$ incubator. The flask was labelled with the cell line, passage number, date and owner initials.

### 3.11 Cell Maintenance

The human cervical cancer cell lines (HeLa) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10 % Fetal Bovine Serum (FBS) and 1 % Penicillin-Streptomycin (Pen-Strep) in T-75 flasks. The cells were maintained at 37 $^0$C in an incubator with humidified atmosphere of 5 % $CO_2$. The culture was observed daily under the inverted microscope to check for cell growth and any bacteria contamination. The culture media was changed every 2 or 3 days.

### 3.12 Sub Culturing and Cell Counting

The human cervical cancer cells (HeLa) were cultured for about 48 hours. After 48 hours incubation, the flasks were observed under inverted microscope to check the confluency of cells on the surface of the flasks. The confluency of the cell needed to reach 70 % to 80 % confluence on the surface of the flasks. If the confluency of the cells in the flask did not reach 70 % to 80 % confluence, the culture media solution was replaced and incubate

for another 24 hours. If the confluency of cells reached 70 % to 80 %, the media solution was removed and the cell was washed with PBS solution. Next, 2 mL to 3 mL of trypsin-EDTA solution was added into the flasks and incubated it in $CO_2$ incubator at 37 $^0$C within 5 to 15 minutes. After the short incubation, the flasks were observed under the inverted microscope to ensure the detachment of cell from surface of flasks. Afterwards, 6 to 8 mL of media solution was added and transferred into 15 mL falcon tube. The 15 mL falcon tube was centrifuged at 1000 rpm speed for 5 minute. Later, the supernatant was removed and sediment was suspended with 1 mL of media solution. Afterwards, 10 µL of re-suspend solution was pipetted into micro centrifuge tube follow by addition of 10 µL of trypan blue into same micro centrifuge. Subsequently, 10 µL of mixture was pipetted out onto hemocytometer and count the number of cells. The cells were counted using the following equation:

$$Cell\ Concentration\ (cells/mL) = \frac{Total\ cells}{Number\ of\ Grids\ Counted} \times Dilution\ Factor \times 10^4\ cells/mL \quad (3)$$

**3.13 Cell Cryopreservation**

The cryovials tube were labelled with the name of cell line, passage number and date. The cryopreservation medium was prepared by adding 10 % of dimethyl sulfoxide (DMSO) into cold complete growth medium. 1 mL of DMSO was mixed with 9 mL of cold complete growth medium and the medium was placed in 4 $^0$C until it was ready to be used. The T-75 flask containing cells were washed with phosphate buffered saline (PBS) before adding trypsin into the flask for cell detachments. The cells containing trypsin were neutralized by adding equal volume of complete growth medium. The cell suspension was re-suspended and transfer into falcon tube for centrifugation at 1000 rpm for 5 minutes. After centrifugation,

the supernatant was removed and the pellet was re-suspended in the cold cryopreservation medium at a viable cell density of 3 x $10^4$ viable cells/mL by gentle pipetting up and down. The cryovials were then placed back into the -80 $^0$C nitrogen tank.

### 3.14 Cytotoxicity of HeLa Cell Line using SRB Assay ($IC_{50}$)

The half inhibitory concentration ($IC_{50}$) of *C. vespertilionis* leaves extract on the viability of HeLa was assessed using SRB assay (Orellana, and Kasinski, 2016; Bhagat et al., 2014). The cancer cell line of HeLa was seeded at density of 3 x $10^4$ per mL on the 96-well plates (Wang et al., 2009) and left for 24 hours to allow the cells to attach on the surface of the well. After 24 hours, HeLa cell line was treated with different concentrations of the extracts that diluted by serial dilution method. There were five different concentrations of *C. vespertilionis* extracts (200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, and 12.5 µg/mL) that were used to determine $IC_{50}$ in 96-well plates for every time point incubation (24, 48 and 72 hours) in the early screening of cytotoxicity effect of the extracts. The concentrations of each extract were adjusted later based on dose response relationship until optimum $IC_{50}$ can be obtained. As for untreated group (negative control), the cells were seeded at the same seeding density and were incubated with media without any treatment. Meanwhile, for positive control, Paclitaxel drug that act as anticancer drug or chemotherapy drug was used to verify the result of $IC_{50}$ of the plant extract. The determination half inhibitory concentration, $IC_{50}$ of the positive control was conducted using five different concentrations, 0.17 µg/mL, 0.085 µg/mL, 0.0425 µg/mL, 0.02125 µg/mL, and 0.010625 µg/mL (Wang et al., 2000). The cells were exposed to the positive control after 24 hours cell attachment in 96 well plates and cytotoxicity effects were recorded for every time point (24, 48 and 72 hours). Each sample was assayed for three independent experiments in four replicates. The viability of the cells was measured using SRB assay.