



**EFFECTS OF *CLINACANTHUS NUTANS* ON DRUG RESISTANT
CANCER CELL PROLIFERATION AND MIGRATION**

BY

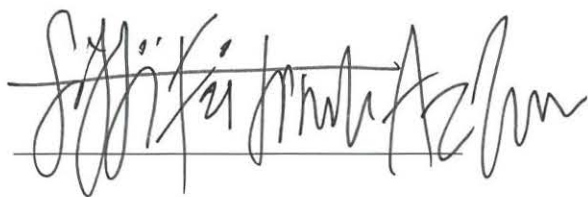
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**DISSERTATION SUBMITTED IN PARTIAL FULLFILMENT OF
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MEDICINE (MEDICAL RESEACRH)**

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DECLARATION

I hereby declare that this research has been sent to Universiti Sains Malaysia for the degree of Master of Medicine in Medical Research. It is not to be sent to any other universities. With that, this research might be used for consultation and can be photocopied as references.

A handwritten signature in black ink, appearing to read 'Sitti Fatimah Az Zahra BT MD Arzali', written over a horizontal line.

SITTI FATIMAH AZ ZAHRA BT MD ARZALI

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LIST OF ABBREVIATIONS

EDTA	Ethylene Diamine Tetra-acitic Acid
PLC- γ 1	Phospholipase C- γ 1
DMSO	Dimethyl sulfoxide
CO ₂	Carbon dioxide
FBS	Foetal bovine serum
MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
rpm	Revolutions per minute
HER2	Human epidermal g
T25	Cell culture flask (25cm ²)
Tam-R	Tamoxifen-resistant cell line derived from UACC 732 cell

LIST OF SYMBOLS

g	Gram
h	Hour
kg	Kilogram
L	Litre
M	Molar
m	metre
mg	Milligram
Min	Minute
w/v	Weight for volume
w/w	Weight for weight
°C	Degree celcius
µg	Microgram
µL	Microlitre
µm	Micrometre
mL	Millilitre
mm	Millimetre
mM	Millimolar

EFFECTS OF *CLINACANTHUS NUTANS* ON DRUG RESISTANT CANCER CELL PROLIFERATION AND MIGRATION

ABSTRAK

Clinacanthus nutans adalah tumbuhan terapeutik yang terkenal di negara Asia. Baru-baru ini ia menjadi tumpuan penyelidikan untuk aplikasi bagi rawatan dan pencegahan penyakit kanser. Oleh itu, matlamat penyelidikan ini ialah terhadap kesan ekstrak *Clinacanthus nutans* terhadap perencatan pertumbuhan dan penghijrahan sel kanser HER2 (UACC 732) yang tahan terhadap ubat. Kaedah: Daun *Clinacanthus nutans* dibeli dalam bentuk serbuk dan pengestrakan dilakukan menggunakan pelarut akueus dan pelarut etanol. Kesan ekstrak ini terhadap ujian proliferasi kanser tahan dadah ditentukan menggunakan kit ujian MTT. Seterusnya konsentrasi penghalang separuh maksimum (IC_{50}) bagi kedua-dua ekstrak yang dikaji mengenai migrasi sel kanser tahan dadah. Keputusan: Peratus kuantiti ekstrak bagi pelarut akueus ialah 2.7% dan 2.8% bagi pelarut etanol. Nilai IC_{50} bagi sel utama pelarut etanol ialah 837.98 $\mu\text{g/mL}$ dan nilai IC_{50} bagi sel tahan dadah pelarut etanol ialah 923.98 $\mu\text{g/mL}$ bagi tempoh 24 jam rawatan. Manakala, tiada nilai IC_{50} bagi sel utama dan sel tahan dadah untuk pelarut akueus. Sel-sel migrasi bagi pelarut akueus dan pelarut etanol menunjukkan penghijrahan sel-sel dari ekstrak *Clinacanthus nutans* menunjukkan peningkatan penghijrahan sel sehingga penutupan kawasan luka ketika dirawat dengan 100 mg/mL. Perbincangan: Kaedah pengestrakan akueus dan etanol menghasilkan kuantiti ekstrak yang hampir sama bagi kedua-dua kaedah. Kesan sitotoksik kedua-dua ekstrak UACC 732 sel menunjukkan perbezaan yang ketara. Telah dinyatakan bahawa ekstrak etanol menyebabkan penghambatan ibu bapa dan tahan UACC 732 sel berbanding ekstrak akueus.

Penyiasatan lanjut menggunakan ujian penyembuhan luka menunjukkan bahawa kedua-dua ekstrak menghalang penghijrahan sel pada 24 jam. Kesan yang sama tidak berada di dalam sel-sel UACC 732 . Ini menunjukkan bahawa sebatian yang ada dalam ekstrak air dan etanol dapat mengurangkan penghijrahan sel. Kesimpulan: Kajian ini menunjukkan bahawa ekstrak *Clinacanthus nutans* mempamerkan kegiatan antiproliferatif dan anti-penghijrahan. Walau bagaimanapun, siasatan lanjut perlu dilakukan untuk mengesahkan penemuan ini.

EFFECTS OF *CLINACANTHUS NUTANS* ON DRUG RESISTANT CANCER CELL PROLIFERATION AND MIGRATION

ABSTRACT

Clinacanthus nutans is a renowned therapeutic plant in Asian countries. Recently is has gained research interest in the applications for treatment and prevention of cancer diseases. Therefore, the aim of this research will focus on effects of *Clinacanthus Nutans* extracts towards drug resistant HER2 overexpression (UACC 732) cancer cell proliferation and migration. Method: *Clinathanus nutans* leaves was purchased in powder form and extraction was done using aqueous and ethanol solvents. Effects of these extracts on drug resistant cancer proliferation assay was determined using MTT assay kit. Next, half-maximal inhibitory concentration (IC_{50}) for both extracts were studied on drug resistant cancer cell migration. Result: Cytotoxic effects of ethanol *Clinacanthus nutans* extract on parent and resistant UACC 732 cells viability at various concentrations, respectively showed the IC_{50} value for 24 hours treatment were 923.98 $\mu\text{g/mL}$ and 837.98 $\mu\text{g/mL}$. Meanwhile, no cytotoxic effects shown in aqueous *Clinacanthus nutans* extract on parent and resistant UACC 732 cells. The cell migration of *Clinacanthus nutans* extracts indicated an increase in cell migration until the closure of the wound area when treated with 100 mg/mL. Discussion: Extraction method produced almost equal yields with aqueous and ethanol extracts. Cytotoxic effects of both extracts on parent and resistant UACC 732 cells showed significant difference. It was noted that ethanol extracts caused inhibition of parent and resistant UACC732 cells compared to aquoeus extract. Further investigation using wound healing assay showed that both extracts inhibited cell migration at 24 hours. Similar effects were not observed in parent and resistant UACC

732 cells. This indicated that compounds present in aqueous and ethanol extracts were able to inhibit cell migration. Conclusion: This study shows that *Clinacanthus nutans* extracts exhibits antiproliferative and anti-migration activity. However, further investigation need to be done to confirm these findings.

CHAPTER 1

INTRODUCTION

1.1 CANCER

Cancer is a wide term for a class of diseases regarded as the uncharacteristic cells that grow uncontrollably and attack the healthy cells in the body. Cancer has been existing in human nature since the older times. It is the second major cause of death in developed and developing countries. Diverse aspects such as pollution, sedentary lifestyle, smoking and imbalanced diet together with population aged have been pronounced as the core factors for the advancement of this disease. Moreover, lung, prostate, colorectal, stomach and liver malignancy are the most likely types of cancer occurred in men. In contrast, breast, colorectal, lung, cervix and stomach cancer are the utmost common types of cancer that affected women worldwide. In 2015, it was expected there were approximately 8.8 million death caused by cancer according to the World Health Organisation (WHO, 2015).

The Malaysia National Cancer Registry (MNCR, 2016) report based on data from 2007-2011 showed there was nearly a huge number of 103,507 new cases. The data presented just about 46,794 cancer cases were reported in males with calculated percentage of 45.2%. While, there were 56,713 cases of cancer identified involving females with 54.8%. Besides, the ratio of threat of men contracting cancer was 1 in 10 meanwhile for females was 1 in 9 with the slightly differences compared to the males. The age standardised incidence rates (ASR) is 86.9 for male and 89.0 for female per 100,000 population correspondingly. The ASR in Chinese was the highest which for males were 112.9 per 100,000 population whereas in females were 126.2 per 100,000

population. Among the Malay men it was 70.7 per 100,000 population and women was 81.9 per 100,000 population. Lastly, Indian ethnic group showed for men was 70.4 per 100,000 population and 110.1 for women per 100,000 population.

Worldwide breast malignancy is the second prominent cause of mortality among women. It is indicated as the most common malignancy affecting women (Siegel et al., 2015). Even though there are significant improvements in survival outcome in patients over the years. This disease is not a single entity but it is a heterogeneous group with its subtypes revealing distinct variances in biological and clinical performance (Dawson et al., 2013).

In 2006, almost 3500 breast cancer cases registered in NCR represented 16.5% of all cancer cases listed. The general ASR was 39.3 per 100,000 population. For age outline in 2006, data revealed a peak incidence rate at the age of between 50-59 years. Among Chinese ethnic group, the incidence was maximum where the ASR was 46.4 per 100,000 population. This was followed by Indian with the ASR of 38.1 per 100,000 population and finally Malay with 30.4 per 100,000 correspondingly. In addition, the Penang Cancer Registry for 2004-2008 reported an incidence of 48 per 100,000 of population.

1.2 BREAST CANCER SUBTYPES

1.2.1 HER2 overexpression subtypes

The human epidermal growth factor receptor-2 (HER2) protein is highly expressed about 20% of all breast cancer cases. The HER2 gene is also called the *EEBB2* (*erb-B2 receptor tyrosine kinase 2*) gene. Its activation stimulates pathways involving the cell proliferation and development. HER2 expression in breast cancer is related with a development of the disease, greater mortality rate and an increase level of metastasis. In

addition, HER2 status is a key player in determining the appropriate routine of treatment to use for the patient. Mostly, a breast cancer tumour with HER2 protein converses a measure of resistance to treatment based chemotherapy.

HER2 expression is frequently associated to development of resistance towards tamoxifen treatment due to the oestrogen receptor in breast cancer. At this time, there are at least two kinds of treatment for patients with HER2 breast cancer. They are Herceptin whose active constituent is Trastuzumab. Lapatinib is also given to the patients as primary care for breast cancer patients.

Even with the administration of Trastuzumab and Lapatinib for patients with HER2 overexpression or gene amplification of breast cancer, there are quite a lot of patients will either not respond to the treatment given or eventually progress regardless of the treatment. Currently, development of resistance to HER2 therapies becomes a serious concern and in the long run results in shorter time to tumour progression and limited survival. Furthermore, there are a number of mechanism by which HER2 overexpressing tumour cells may evolve resistance to HER2 healings.

1.3 DISCOVERY OF MEDICINAL PLANT

Cancer treatment involved chemotherapy and radiotherapy. Although numerous advancement, the control of cancer progression still have their own drawback. It is important to address this challenge. Thus, natural therapies derived from plants could be added in existing therapies to reverse adverse effects of the cancer treatments. Medicinal plants have been used widely for centuries and recently some of these plants have shown promising findings in cancer therapies (Desai et al., 2008).

Numerous therapeutic drugs and new compounds have been found in plants and they have huge healthcare benefits. Furthermore, medicinal plants are foundation of new therapeutics (Siew et al., 2014; Abdi et al., 2014). Increasing public awareness for safe and healthier therapies caused wide use of natural products and drugs derived from plants. These plants contain bioactive complexes that show therapeutic effects such as antioxidant, anti-viral and anti-inflammatory effects.

1.3.1 *Clinacanthus nutans*

Clinacanthus nutans originates from the South East Asian countries predominantly in Thailand, Malaysia, Indonesia and China. The taxonomic classification and nomenclature of *Clinacanthus nutans* is as follow:

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Lamiales

Family: Acanthaceae

Genus: *Clinacanthus*

Species: *nutans*

Scientific name: *Clinacanthus nutans*

Most of the species from this family are used in traditional medicine to cure several diseases mainly those involving analgesic processes (Matheus et al., 2005). It has been verified that *Clinacanthus nutans* can be used as anti-inflammatory drug to treat insect bites, herpes infection and allergic responses (Wanikiat et al., 2008). Besides, a clinical trial was performed to compare *Clinacanthus nutans* with benzydamine in the prevention and to ease off radiation-induced oral mucositis to the neck and head of cancer

patients. The results showed that the average time required for the onset of oral mucositis for patients treated with *Clinacanthus nutans* is significantly later compared to the patients treated with benzydamine (Putwatana et al., 2009). In Thai traditional medicine, *Clinacanthus nutans* has been long used for the treatment of skin rashes, snake bites and varicella-zoster virus (VZV) lesions.



Figure 1.1: *Clinacanthus nutans* plant

1.4 RESEARCH PROBLEM STATEMENT

Prevalence of breast cancer is reported to increase over the last decade. Although numerous new therapies, drug resistance remains as a challenge in cancer treatment. The anti-HER2 monoclonal antibody trastuzumab and the tyrosine kinase inhibitor lapatinib have complementary mechanisms of action and synergistic antitumour activity in models of HER2-positive breast cancer. However, the efficacy of trastuzumab in combination with lapatinib remains controversial.(von Minckwitz et al., 2018) Potentials of *Clinacanthus nutans* have been extensively investigated in various types of cancers. However, there is lack of information with regard to its effect on HER2 overexpressed

and resistant cancer cell. Therefore, there is a need to explore potentials of this plant to overcome HER2 overexpressed drug resistant breast cancer.

1.5 OBJECTIVES

1.5.1 General objectives

To study the effects of *Clinacanthus nutans* towards HER2 overexpressed drug resistant cancer cell proliferation and migration.

1.5.2 Specific objectives

1. To optimise aqueous and ethanol extraction methods of *Clinacanthus nutans* leaves.
2. To compare the effects of *Clinacanthus nutans* extracts on HER2 overexpressed drug resistant cancer cell proliferation using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay.
3. To compare the effects of *Clinacanthus nutans* extracts on HER2 overexpressed drug resistant cancer cell migration with wound healing assay.

1.6 HYPOTHESES

There are several hypotheses that were taken into consideration which are:

- a) Alternative hypothesis : Aqueous *Clinacanthus nutans* extracts inhibits the HER2 cancer cell proliferation and migration.
- b) Null hypothesis : Aqueous *Clinacanthus nutans* extracts allows the HER2 cancer cell proliferation and migration

- c) Alternative hypothesis : Ethanolic *Clinacanthus nutans* extracts inhibits the HER2 cancer cell proliferation and migration.
- d) Null hypothesis : Ethanolic *Clinacanthus nutans* extracts allows the HER2 cancer cell proliferation and migration.

CHAPTER 2

LITERATURE REVIEW

2.1 BIOLOGICAL CHARACTERISTICS OF CLINACANTHUS NUTANS

2.1.1 Origin and distribution

Clinacanthus nutans is a remedial plant frequently used in Asian countries. In addition, it is originating in South China and South East Asia generally in Thailand, Malaysia and Indonesia (Yuann et al., 2012). It is widely grown in tropical Asia and has been used as important medicinal herb in China, Thailand and Malaysia (Yahaya et al., 2015). It is famously known as Sabah snake grass or belalai gajah in Malaysia. since it is originate in Sabah of East Malaysia. The plant is prevalently acknowledged as Saled Pangpon Tua Mea or Phaya-Yo or Phaya in Thailand. (Yuann et al., 2012).

2.1.2 Botany and taxonomy

Kingdom for *Clinacanthus nutans* plant known as a plantae. It is a magnoliophyta of phylum and from a class of magnoliopsida. The family Acanthaceae be made up of about 250 genera and 2500 species. They are from the taxon of dicotyledonous flowering plants. The plant are typically herbs and shrubs. The genus *Clinacanthus nutans* is consist of two classes which are *Clinacanthus nutans* (Burm. f.) Lindau and *Clinacanthus siamensis* (Fong et al., 2014). *Clinacanthus nutans* is related to *Clinacanthus siamensis* of the same family and reported that there are no discrete differences in their distribution and medicinal values (Fong et al., 2014). *Clinacanthus nutans* is a tall plant, where it grows up to 1 m height. The stems of *Clinacanthus nutans* are approximately slender with a circular tranverse section at ends. The stems are also striated and has smooth surface. It

has either oval-shaped or lancelike shape of leaves. It is about 2.5-13 cm in length. Width of leaves are roughly 0.5-1.5 cm with apex acute or acuminate. The margin of leaves are exsculptate, dentate and subentive. The leaf base are trigonal, obtuse rounded or shorten and habitually oblique. Moreover, the flowers are existing in solid cymes at the uppermost of branches, roofed with 5-alpha cymules and combined into a large lax, leafy panicle.



Figure 2.1: Flower of *Clinacanthus nutans*

Figure 2.2: Stem of *Clinacanthus nutans* plant

2.2 PHARMACOLOGICAL PROPERTIES AND MEDICINAL USES

2.2.1 Pytochemical of *Clinacanthus nutans*

Twenty-five known and unknown compounds and nine mixtures of compounds have been isolated from *Clinacanthus nutans* extracts. Six known C-glycosyl flavones (Zhang et al., 2008), two phytosterols, one triterpenoid, two glycolipids (Aslam et al., 2015) belutin (Ab Alim et al., (2017), five sulfurcontaining glycosides, and eight compounds related to chlorophyll and chlorophyllb were found. A mixture of nine cerebrosides and a monoacylmonogalatosylglycerol separated from the ethanolic leaf extract of *Clinacanthus nutans*.

2.2.2 Anti cancer studies

The leaf extracts of *Clinacanthus nutans* are shown to have anticancer characteristics in numerous cancer cells. According to the study done by Yong et al. (2013), *Clinacanthus nutans* of methanol and chloroform extracts has been studied for their anti-proliferative and antioxidant properties. Their study reported substantial anti-proliferative effects on K-562 and Raji cells at 72 h with 91.3% (IC₅₀:100 µg/mL) and 89.0% (IC₅₀: 47.31 µg/mL) respectively.

Comparison was done with effects of crude chloroform leaf extract in their study. Treatment with 100 µg/mL had more than 50% anti-proliferative action with 55.8% on human lung (NCI-32), and 56.7% on cervical cancer (HeLa) at 72 hours (Yong et al., 2013). In addition, comparison was done with the crude petroleum ether of *Clinacanthus nutans* leaf extract. Result of cytotoxicity showed almost similar effects against the HeLa and K-562 cells with IC₅₀ values of 18 and 20 µg/mL, respectively at 72 hours (Arullappan et al., 2014). However, methanol demonstrated lower cytotoxicity against the K-562 cells with a IC₅₀ value more than 100 µg/mL within the same duration. This was due to the high polarity of the solvent of the leaves extracted.

Therefore, the crude aqueous leaf extract was more potent because it had the anti-proliferative activity against the K-562 and Raji cells which was less than 50% (Yong et al., 2013). Furthermore, the bioactive compounds that acted against the specified cell lines may be non- to mid-polar in nature due to the polarity of solvents. Nevertheless, further studies on other cancer cell lines are needed to verify these outcomes. However, previous studies also did not examine the mechanism of actions of the extracts against the drug resistant cancer cells.

Anti-proliferative compounds utilised their anti-cancer effects through the hindrance of cell proliferation (Zulkipli et al., 2017). Apart from that, compounds may inhibit the progress, maturation or spread of oncogenic cells. Previous study indicated the existent of the potential anti-tumorigenic activity of *Clinacanthus nutans*. Effects of its ethanolic extract was equalled with control treatment and the discoveries showed significant decline of tumour development in a mouse HepA hepatoma model (Huang et al., 2016). Anti-tumorigenic effect in was found to be ominously better than that of fluorouracil which is a conventional chemotherapeutic drug.

Moreover, *Clinacanthus nutans* leaf extracts were proclaimed to prevent proliferation of a number of cell types as well as HeLa (Yong et al., 2013). The MTT assay has been practiced as a standard measure of cell proliferation experimental throughout all these studies. Few studies have tested for anti-proliferative effects of *Clinacanthus nutans* on primary cell types, although one study tested *Clinacanthus nutans* extracts on primary human gingival fibroblasts and detected no antiproliferative activity (Arullappan et al., 2014) against these cell lines.

CHAPTER 3

METHODOLOGY

3.1 SAMPLE PREPARATION

One kilogram of *Clinacanthus Nutans* leaf powder was purchased from Herbagus, Bertam in Kepala Batas. It was placed in a sealed bag and stored at room temperature prior to extraction step.

3.1.1 Soxhlet extraction of ethanolic *Clinacanthus nutans*

Solvent extraction was done using Soxhlet tool for 48 hours, where 250 mL of absolute ethanol was mixed with 25 g powder of *Clinacanthus Nutans* leaves. Heating temperature of ethanol extraction was fixed at 60°C for approximately 48 hours. Subsequently, extracts were filtered using rotary vacuum evaporation (Heidolph) at 65°C of 145 hPa to waterless and followed by freeze dry step.

3.1.2 Heating extraction of aqueous *Clinacanthus nutans*

Solvent extration was performed in a beaker for 72 hours. Twenty five gram of powder was dissolved in 250 mL of deionised water. It was heated at 60 degree celcius on top of a hot plate in fumehood chamber. Samples were mixed with magnetic stirring for the samples to be saturated with the solvent. Ultimately, this method will stimulate the diffusion of the solvent into the sample matrix and advances movement of active compounds into the solvent. Similar with ethanol extract, aqueous extracts were filtered with rotary vacuum (Heidolph) at 65°C of 160 hPa.

3.1.3 Percentage of yield

The percentage of yield for both dried aqueous and ethanol extracts were calculated as follows :

Yield (%) = (W1 × 100) / W2, where W1 is the weight of the extracts after lyophilization of solvent and W2 is the weight of the plant powder.

The extracts was weighed and kept in a refrigerator at 4°C until use.

3.2 CELL LINES

Human breast malignancy cell lines were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). This study involved the culture of parent UACC732 cell line, UACC 732 cell line and its lapatinib, trastuzumab and tamoxifen-resistant cell line ('Tam-R'). These cell lines were cultured in their particular growth media as recommended by ATCC.

3.2.1 UACC 732 cell line culture

Human breast malignancy UACC 732 cells were cultivated on 96 well cell culture plates from Corning (Corning, NY, USA). The incubator was sustained at 37°C with 5% CO₂ atmosphere and 95% humidity. UACC 732 cells were retrieved from liquid nitrogen storage and all cells were cultured in T25 that consists of RPMI-1640 medium (Gibco Thermo Fisher Scientific, USA) with 10% foetal bovine serum (FBS) and antibiotics of penicillin of 100 IU/mL and streptomycin of 100µg/mL bought from Gibco/Life Technologies. The culture medium of 5 mL in T25 flask was replaced every 3 days. The passage number used in this study was at 20 passage.

3.2.2 UACC 732 TAM-R cell line culture

This cell line is a lapatinib and transtuzumab resistant cell line. As recommended by The National Comprehensive Cancer Network guideline, resistant breast cancer cells are recommended to be subsequently treated with new therapies. Therefore, UACC 732 parent cells were pulse treated with tamoxifen. It is an endocrine therapy. Confirmation of tamoxifen-resistant of UACC 732 cells was done prior to this study. The UACC 732 Tam-R cells were cultured in phenol RPMI (Gibco Thermo Fisher Scientific, USA) with 10% foetal bovine serum (FBS) and antibiotics of penicillin of 100IU/ml and streptomycin of 100µg/mL from Gibco/Life Technologies.

3.2.3 Cell passaging step

Cell culture medium was removed from culture T25 flask and followed by rinsing step using 1mL of phosphate buffer saline. Next, the adhered cells were detached by using 1mL combination of trypsin (0.05%) and EDTA (0.02%). The cell culture T25 flasks were placed in an incubator for subsequent 5-7 minutes to allow for cells to disengage from the flask surface. Subsequently, trypsin was neutralised by the adding of 1 mL of complete medium. The cell suspension was centrifuged at $125 \times g$ for 7 minutes (Thermo Fischer Scientific) at 22-25°C. Later the supernatant was discarded and the cell pellet was resuspended homogeneously after adding of 1 mL of the complete culture medium. Cell suspension (50 uL) was seeded into a T25 flask with 2.265×10^6 seeding density.

3.2.4 Cell line cryopreservation

After cells were collected and pelleted by centrifugation at $125 \times g$ for 7 minutes. The cell pellet were resuspended in 1 mL growth medium. Dimethyl sulfoxide (DMSO) was used as cryoprotective agent to prevent formation of ice crystals and fragmentation

of the cell membrane (Langdon, 2003). DMSO was added to the cell suspension at the final concentration of 10% (Corsini, 2004). Finally, the cell stock was transferred into cryotubes and stored at -80°C in liquid nitrogen for two weeks.

3.3 CELL PROLIFERATION ASSAY

Cell proliferation assay was performed to measure half maximal inhibitory concentration (IC_{50}) of *Clinacanthus nutans* extracts via MTT assay kit. MTT assay kit is known as colometric technique to evaluate the cytotoxic effects of anti cancer medicines and additional toxic mediators and pharmaceutical complexes. This routine is constructed on the reduction of MTT tetrazolium composite by viable cells via dehydrogenase enzymes found in metabolically vigorous cells. It will produce coloured formazan product that is soluble in the cell culture medium. This product is directly proportional to the number of living cells. Quantification was done by measuring the absorbance at 570 nm wavelength and absorbance at 630nm and 750nm wavelength are using as references absorbances. The CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA) is a collection of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT).

For this experiment, UACC 732 cells were seeded in 96 well flat bottomed plate at a density of 4.4×10^6 cells with 200 μ L of complete culture media. Cells were incubated overnight to allow the adherence of cells to the surface of 96 well plate before treatment with *Clinacanthus nutans* extracts at a number of different concentrations which comprise of 0, 0.98, 1.95, 3.91, 7.81, 15.62, 31.25, 62.5, 125, 250, 500, 1000 μ M. Duration of *Clinacanthus nutans* treatment was 24 hours. After treatment, the medium in 96 well was discarded and 100 μ L of complete culture medium varied with 15 μ L

CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA) was pipetted into each well of the plate. It was left to incubate at 37°C at 2 hours in a 5% CO₂ atmosphere and 95% humidity incubator.

After incubation, 100 µL of the solubilisation solution was added to each well without removing the maintaining medium. The plate was placed in incubator by sealing with aluminium foil to prevent evaporation upon storage. Solubilization solution was added and left for an hour. After that, the medium content in each well was mixed using a multichannel pipette in order to obtain a homogeneously dyed solution. This step was done with care to prevent bubble formation. Bubbles could interfere with the accurate reading of absorbance values. Absorbance reading for samples were recorded at 570 nm using the ELISA microplate reader analysis.

In this study, the negative controls were supplemented with 0.2% of DMSO as well as for ethanolic *Clinacanthus nutans* extract. Each of the treatment was done in triplicates (n=3). Total amount of formazan MTT product was calculated at 570 nm absorbance and it is directly proportional to the number of viable cells in the samples. Percentage of cell viability was calculated as follows :

$$\text{Cell viability (\%)} = [(\Delta \text{ Treatment} - \Delta \text{ Blank}) / (\Delta \text{ Control} - \Delta \text{ Blank})] \times 100,$$

where Δ treatment is the absorbance of treated cells, Δ control is the absorbance of control cells and Δ blank is the absorbance of media without cells.

The *Clinacanthus nutans* concentration that reduced the viability of cell by 50% (IC₅₀) was presented by plotting the mean of triplicates data points over a concentration from 0 up to 1000 µM. The IC₅₀ of *Clinacanthus nutans* extracts was determined after 24 hours treatment.

3.4 CELL MIGRATION ASSAY

Cell migration assay was carried out to determine the cell migration and cell interactions. Cell migration based on wound healing assay has been used in studies collectively on migration due to its simplicity and capability of revealing the complexity of collective cell migration. The parent and resistant UACC 732 cell lines were seeded on 24 well plate. They had been grown at a density of 2.27×10^6 cells/well in 200 μ L of complete culture media of RPMI-1640 medium (Gibco Thermo Fisher Scientific, USA). Media was supplemented with 10% foetal bovine serum (FBS) and antibiotics of penicillin of 100 IU/mL and streptomycin of 100 μ g/mL from Gibco/Life Technologies. Plates were left at least for 24 hours to allow for cells to adhere to the surface of 24 well plate before treatment with *Clinacanthus nutans* extracts at the different time. *Clinacanthus nutans* treatment was done for 24 hours.

Next, the medium in 24 well plate was discarded and 100 μ L of complete culture medium was placed into each well. Then, the 10 μ g/mL of colcemid (Demecolcine) solution was mixed with the medium and been incubated for 2 hours. After 2 hours incubation, 200 μ L microliter tip were used to scratch at the center of each well. The pipette tips were kept under an angle of 45 degrees to ensure the scratch width were similar. Wells were washed with PBS to remove the remaining debris. Finally, the treatment was done with *Clinacanthus nutans* extracts. Movement of cells were captured with phase contrast microscope at 0 hour, 20 hours and 40 hours at 200x magnification. The migration rate can be expressed as the percentage of area reduction or wound closure. The closure percentage will increase as cells migrate over time. ImageJ was used to analyse the wound closure. Wound closure formula were calculated as follows:

Percentage of wound closure was calculated based on different of scratch area along the well. Measurements were taken in triplicates for control and treatment. Wound closure (%) = $[(A_{t=0h} - A_{T=\Delta h}) / A_{t=0h}] \times 100$, where $A_{t=0h}$ is the area of the wound measured immediately after scratching ($t=0h$) and $A_{T=\Delta h}$ is the area of the wound measured h hours after the scratch is performed.

CHAPTER 4

RESULT

4.1 YIELD OF EXTRACTION

A total of 0.6773 g of *Clinacanthus nutans* aqueous extract was acquired which gave the extraction yield of 2.7% from 25 g of freeze dried powder of *Clinacanthus nutans*. In the meantime, 25 g of dried powder yielded 0.6997 g for the ethanol extract. The yield percentage of ethanol extract presented was 2.8%. The yield percentage calculation was shown in Table 1.

Table 4.1: Percentage Yields of aqueous and ethanol extracts

Plant	Percentage of Yield [% / (W/W)]	
Clinacanthus Nutans (Dried Powder Leaves) 25 g	Ethanol extract	Aqueous extract
	2.8 %	2.7 %

4.2 CELL PROLIFERATION ASSAY

4.2.1 Effects of *Clinacanthus nutans* extracts on UACC 732 breast cancer cell morphology

The morphological changes following exposure to *Clinacanthus nutans* extracts were observed using phase contrast microscope. Both the parent and resistant UACC 732 breast cancer cells were incubated with 2 mg/mL and 100 mg/mL of aqueous and ethanol *Clinacanthus nutans* extracts. Results indicated *Clinacanthus nutans* caused alteration in morphology of resistant UACC 732 cells when observed under phase contrast microscope of 200x magnification.

4.2.2 Effects of *Clinacanthus nutans* extracts on cell proliferation

Cell proliferation was determined by using MTT assay kit. Cytotoxic effects of *Clinacanthus nutans* extracts was investigated using parent and resistant UACC 732 cells. In this study, the ethanol extract of *Clinacanthus nutans* exhibited cytotoxic effect compared to aqueous extract toward parent UACC 732. Results of cell viability at various concentrations is provided Figure 4.1. The IC_{50} value for 24 hours treatment was 837.98 μ g/mL. On the other hand, Figure 4.2 presents the cytotoxic effects of aqueous *Clinacanthus nutans* extract on parent was not obtained. However, it caused inhibition in resistant UACC 732 with IC_{50} of 923.98 μ g/mL for 24 hours treatment. Data are presented as mean from triplicates of experiment.

Cytotoxic effects of aqueous *Clinacanthus nutans* on parent UACC 732 and resistant UACC 732 cells viability at various concentrations are provided in Figure 4.3 and Figure 4.4 respectively. However, the cell lines had no significant cytotoxic effects compared to ethanol extracts for 24 hours treatment. Data were articulated as mean from triplicates experiments.

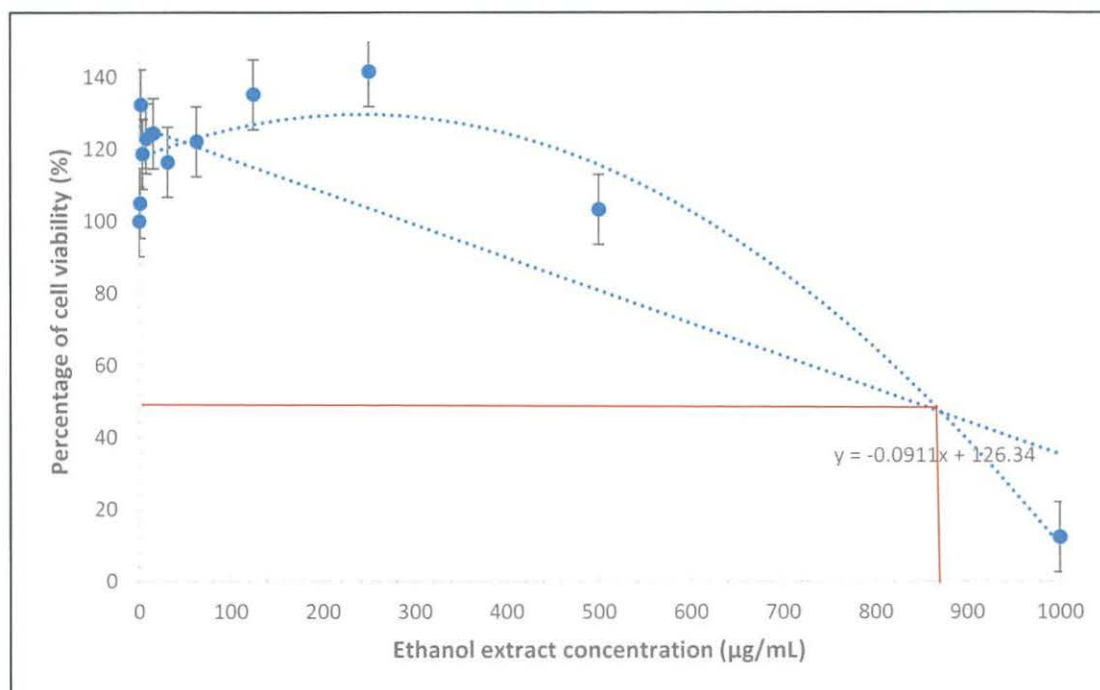


Figure 4.1: Cytotoxic effects of ethanol *Clinacanthus nutans* extract on parent UACC 732 cell viability at various concentrations. The IC₅₀ value for 24 hours treatment was 837.98 µg/mL. Data were expressed as mean from triplicate experiments.

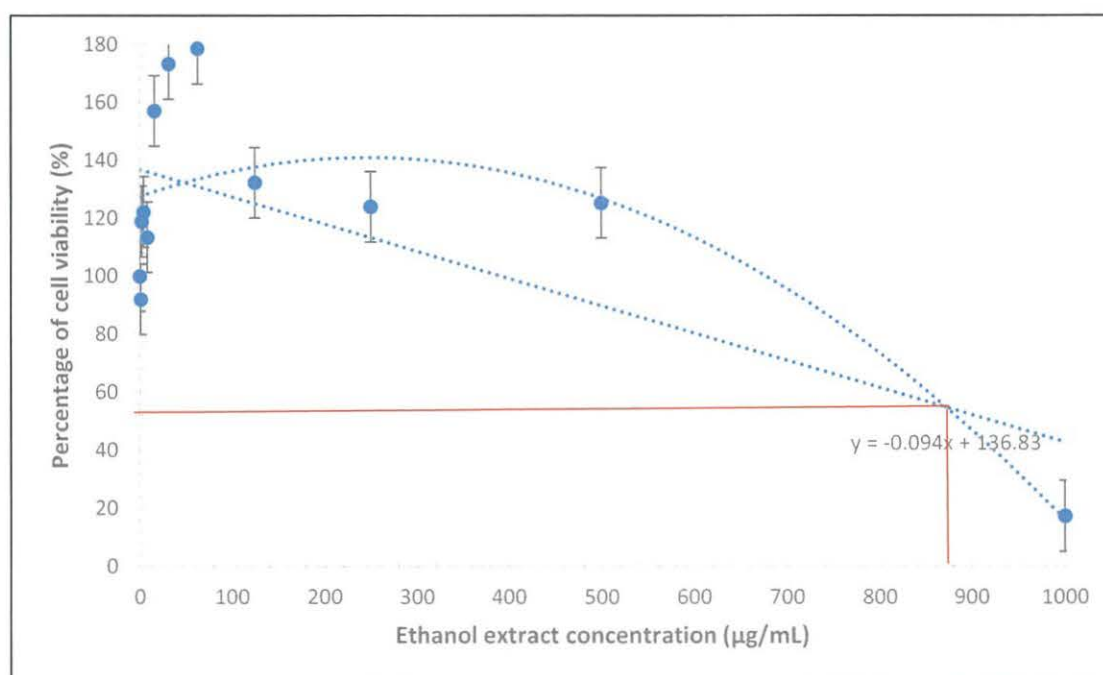


Figure 4.2: Cytotoxic effects of ethanol *Clinacanthus nutans* extract on resistant UACC 732 cell viability at various concentrations. The IC₅₀ value for 24 hours treatment was 923.98 µg/mL. Data were expressed as mean triplicate experiments.

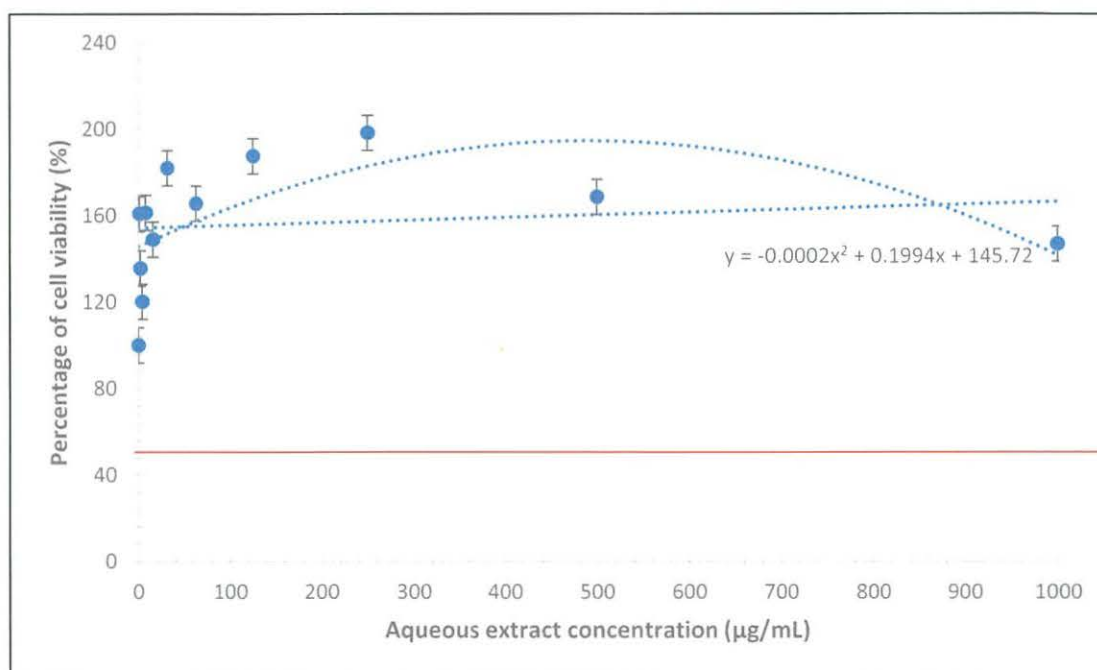


Figure 4.3: Cytotoxic effects of aqueous *Clinacanthus nutans* on parent UACC 732 cell viability at various concentrations. The cell line showed no significant cytotoxic effects with no IC₅₀ detected for 24 hours treatment. Data were expressed as mean from triplicates experiments.

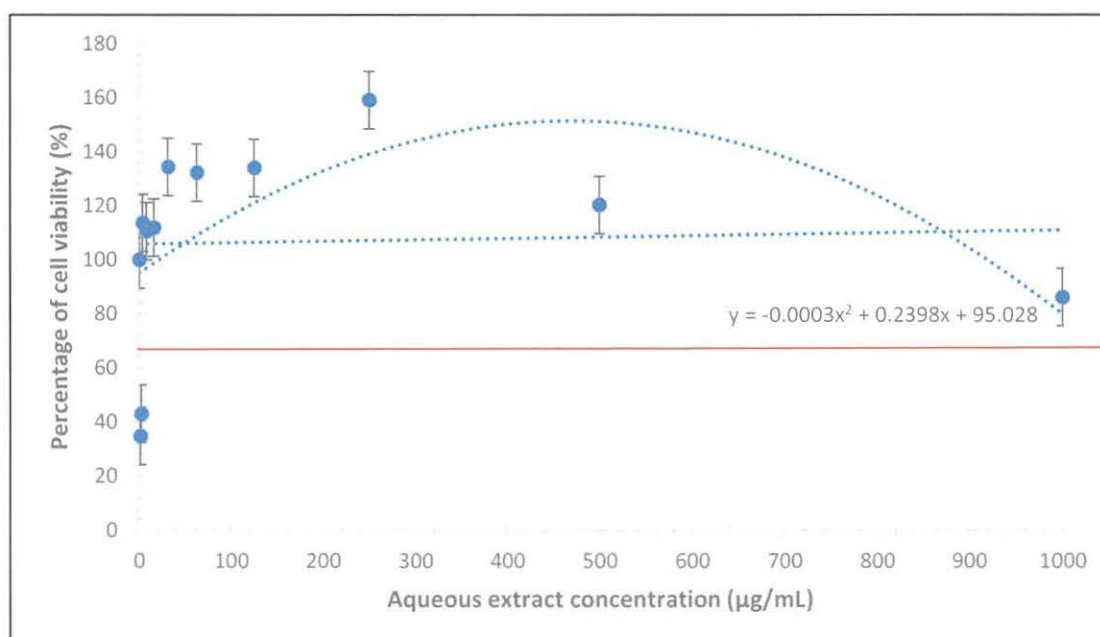


Figure 4.4: Cytotoxic effects of aqueous *Clinacanthus nutans* on resistant UACC 732 cell viability at various concentrations. The cell line showed no significant cytotoxic effects with no IC₅₀ detected for 24 hours treatment. Data were expressed as mean from triplicates experiments.

4.3 CELL MIGRATION ASSAY

4.3.1 Effects of ethanol *Clinacanthus nutans* extracts on cell migration

Cell migration assay was done to compare the effects of *Clinacanthus nutans* ethanolic extracts to inhibit migration of resistant UACC 732 cells. Effect of treatment on parent (Fig 4.5) and resistant UACC 732 cells (Fig 4.6) are provided. Generally, data obtained showed that the migration of both type of cells were affected particularly at 24 hours. Figure 4.5 presents images for the morphology and migration pattern of parent cell when treated with 100 mg/mL ethanol *Clinacanthus nutans* extract. Inhibition of migration occurred at 24 hours in treated cells compared to the parent with ethanolic extract as shown on the lower panel after 24 hours' treatment. However, later cells migrated until the closure of the wound area was noted.

Next, experiment was done to determine cell migration assay in resistant UACC 732 cells. This cell line was also treated with ethanol *Clinacanthus nutans* extract and cell migration is presented in Figure 4.6. Upper panel, shows the morphology of untreated resistant UACC 732 cells when treated with 100 mg/mL aqueous *Clinacanthus nutans* extract. The morphology of treated resistant UACC 732 cells are shown on the lower panel. Results indicated an inhibition of cell migration begin to occur at 24 hours.. Similar to parent UACC 732 cells, results at 40 hours indicated cells migrated rapidly and wound scratch were close completely. Images were captured at 200x magnification using phase contrast microscope.

Furthermore, wound closure of cell migration assay was measured to determine the percentage of wound closure for both parent and resistant UACC 732 cells. Table 4.1 shows comparison of wound closure with ethanol extract of *Clinacanthus nutans*. Results revealed an inhibition of cell migration due to the lower percentage of wound closure for

parent UACC 732 of treated cells which 13.786% at 40 hours comparing to the wound closure for resistant UACC 732 of treated cells which 42.107% at the same duration of time.

Table 4.2 : Comparison of wound closure with ethanol of *Clinacanthus nutans* extract

Hour	Control (Parent UACC 732)	Treatment (Parent UACC 732)
0	0	0
20	87.402	6.123
40	100	13.786
Hour	Control (Resistant UACC 732)	Treatment (Resistant UACC 732)
0	0	0
20	40.129	0.599
40	100	42.107