PHYTOCHEMICAL ANALYSIS OF *Eleutherine americana* MERR ETHANOL EXTRACT AND ITS ANTICANCER PROPERTIES AGAINST BREAST CANCER SPHEROIDS

By

FARA FARIZA ZAHAR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Chairman : Professor Rozita Rosli, PhD
Faculty : Medicine and Health Sciences

Prevalent among women, breast cancer is the most common form of cancer affecting women in Malaysia; about one in nineteen women in this country are at risk. Bioactive compounds extracted from plants (phytotherapy) have long been recognized as one of the sources of anticancer drugs, several of which have been validated to have potent anticancer properties such as Taxol (paclitaxel). Eleutherine americana (E. americana) possesses a number of phytochemical compounds with the potential to be developed as an anticancer drug. The objectives of this study were to evaluate the phytochemical properties of E. americana ethanol extract, to assess its cytotoxicity on breast cancer cells and to determine its anti-cancer properties on the spheroids and its cancer-associated fibroblasts. In the phytochemical qualitative analysis, E. americana's bulb extract demonstrated the presence of terpenoids, flavonoids, glycosides and steroids known as beneficial pharmacology bioactive compounds. In the quantitative analysis, the bulb extract displayed significant results on the total phenolics at 151.13 ± 0.027 mg GAE/g, total flavonoids at 22.02 ± 0.005 QE/g and total alkaloids at 2.26 mg ± 0.381/50 g. The extract also showed antioxidant properties with the DPPH assay results measured at 47.60 µg/mL. These results provide validation that the plant extract used in this study contains beneficial pharmacological properties. The invasion assay showed a significant reduction in the invasion area in both monoculture and co-culture treated with E. americana extract, indicating the anti-proliferative activity of the plant extract. In addition, the H&E staining of MDA-MB-231 spheroids treated with E. americana ethanol extract showed extensive damage to the nucleus, indicating cell death. This study applies the use of cell spheroids which are aggregated adherent

cells known to mimic the complex intercellular interaction within the tumour microenvironment. The cytotoxicity of the bulb extract was determined using the MTT assay for monolayer culture and the Alamar Blue® assay for the spheroids of breast cancer cells, respectively. MTT assay results determined the IC_{50} values for monolayer culture at 25.17 µg/mL for MCF7 cells and 25.23 µg/mL for MDA-MB-231 cells. The Alamar Blue® IC_{50} results on MCF7 spheroids were recorded at 37.90 µg/mL and MDA-MB-231 spheroids at 48.72 µg/mL at 72 hours post-treatment. Thus, these findings further substantiate the cytotoxicity of E. americana ethanol extract towards the spheroids of MDA-MB-231. In conclusion, E. americana bulb extract has the potential to be developed as a phytotherapy for breast cancer.

Keywords: phytotherapy, cancer-associated fibroblasts, breast cancer, Eleutherine americana.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANALISIS FITOKIMIA EKSTRAK ELEUTHERINE AMERICANA ETANOL MENTAH DAN SIFAT ANTI KANSERNYA MELAWAN SFEROID KANSER PAYUDARA

Oleh

FARA FARIZA ZAHAR

Januari 2023

Pengerusi : Profesor Rozita Rosli, PhD
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Lazim di kalangan wanita, kanser payudara adalah bentuk kanser yang biasanya menyerang wanita di Malaysia, kira-kira satu daripada sembilan belas wanita di negara ini berisiko menghidap kanser payudara. Sebatian bioaktif yang diekstrak daripada tumbuhan (fitoterapi) telah diiktiraf sebagai salah satu sumber ubat antikanser, sebilangan daripadanya telah disahkan mempunyai sifat antikanser yang mujarab seperti Taxol (paclitaxel). Eleutherine americana (E. americana) mempunyai sebatian fitokimia yang berpotensi untuk dibangunkan sebagai ubat antikanser. Objektif kajian ini adalah untuk menilai sifat fitokimia ekstrak etanol E. americana, untuk menilai ujian kesitotoksiannya terhadap sel-sel kanser payudara dan untuk menentukan sifat anti-kansernya pada sferoid dan fibroblas yang bersekutu dengan kanser. Kajian ini menggunakan penggunaan sferoid sel yang mimik interaksi antara sel yang kompleks dalam persekitaran mikro tumor. Kesitotoksikan ekstrak bebawang ditentukan menggunakan ujian MTT untuk kultur sel monolapisan dan ujian Alamar Blue® untuk sferoid sel kanser payudara, masing-masing. Keputusan ujian MTT menentukan nilai IC_{50} untuk kultur sel monolapisan pada 25.17 µg/mL untuk sel MCF7 dan 25.23 µg/mL untuk sel MDA-MB-231. Keputusan IC_{50} Alamar Blue® terhadap sferoid MCF7 direkodkan pada 37.90 µg/mL dan sferoid MDA-MB-231 pada 48.72 µg/mL pada 72 jam selepas rawatan. Dalam analisis kualitatif fitokimia, ekstrak bebawang E. americana menunjukkan kehadiran terpenoid, flavonoid, glikosida dan steroid yang diketahui mengandungi bahan bioaktif farmakologi yang bermanfaat kepada kesihatan manusia. Keputusan analisis kuantitatif ekstrak bebawang juga menunjukkan amaun bererti dengan jumlah kandungan fenolik pada

151.13 ± 0.027 mg GAE/g, jumlah kandungan flavonoid pada 22.02 ± 0.005 QE/g dan jumlah kandungan alkaloid pada 2.26 mg ± 0.381/50 g. Ekstrak juga menunjukkan sifat antioksidan dengan keputusan ujian DPPH pada sukatan 47.60 µg/mL. Keputusan ujian fitokimia ini mengesahkan bahawa ekstrak tumbuhan yang dihasilkan dalam kajian ini mengandungi sifat farmakologi yang bermanfaat. Keputusan ukuran kawasan invasif menunjukkan pengurangan ketara dalam kawasan invasif yang dipamerkan oleh sel monokultur dan sel kultur bersama yang dirawat dengan ekstrak E. americana menunjukkan aktiviti anti-proliferatif ekstrak tumbuhan. Di samping itu, pewarnaan H&E bagi sferoid MDA-MB-231 yang dirawat dengan ekstrak etanol E. americana menunjukkan kerosakan yang meluas pada nukleus, menunjukkan bukti kematian sel. Oleh itu, penemuan ini membuktikan keupayaan kesitotosikan ekstrak etanol E. americana terhadap sferoid MDA-MB-231. Kesimpulannya, ekstrak bebawang E. americana berpotensi untuk dibangunkan sebagai fitoterapi untuk kanser payudara.

Kata kunci: fitoterapi, fibroblas bersekutu kanser, kanser payudara, Eleutherine americana.

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LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
ACS	American chemical society
BCL-XL	B-Cell Leukemia/Lymphoma 2
BSE	Breast self-examination
CAF	Cancer-associated fibroblast
CO2	Carbon dioxide
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
EP	European pharmacopoeia
ePLA^{+}	Polylactic acid
ER	Oestrogen receptors
FBS	Foetal bovine serum
GAE	Gallic acid
GAE	Gallic acid
H&E	Hematoxilin and Eosin
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
ICC	Immunocytochemistry
IDC	Invasive ductal carcinoma

ILC	Invasive lobular carcinoma
iNOS	Inducible nitric oxide synthase
miRNAs	MicroRNA
MMDC	Minimum morphological deformation concentration
MMPs	Metalloproteinase family
MTT	Microculture tetralzolium assay/ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
NCI	National cancer institute
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PI	Propidium iodide
PR	Progesterone receptors
QE	Quacertin
RMS 9-4/8	Rat rhabdomyosarcoma
RPMI	Roswell park memorial institute medium
SD	Standard deviation
SLA	Stereo-lithography
TA	Total alkaloid
TF	Total flavonoid
TME	Tumour microenvironment
TP	Total phenolic
UBD	*Unit Biodiversiti*
WHO	World Health Organization
αSMA	Alpha-smooth muscle actin

CHAPTER 1

INTRODUCTION

Globally, breast cancer is recorded as among the highest cancer-related death. Evidence shows that metastasis is the dominant cause of death from the most common forms of cancer, including breast cancer. Approximately 90% of breast cancer deaths are associated with metastasis of cancer cells. Studies have shown that the tumour microenvironment (TME), especially cancer-associated fibroblasts (CAFs), influences these significant events (Hu et al., 2022). Fibroblasts associated with cancer cells (CAFs) are activated during inflammation and fibrosis in the tumours (Liu, Song, & Huang, 2019), it was also shown that cancer cells are able to reprogram fibroblasts to become CAFs through the actions of miRNAs (miR-31, miR-214, and miR-155) (Liu et al., 2019). Fibroblasts respond to mechanical changes such as various tissue damage signals and reacted by transforming into myofibrils mediated by the tumour ECM and their crosstalk with innate immune cells consequently resulted in the association of the fibroblast with the tumour (LeBleu and Kalluri 2018).

The favourable association between cancer cells and CAFs was proven in a study in which CAFs were isolated from human breast carcinoma and subcutaneously injected into immunocompromised (nude) mice. The growth rate of breast carcinomas treated with CAFs was significantly higher than that of breast carcinomas treated with normal fibroblasts (Chen & Song, 2019). Kojima et al. demonstrated that resident human mammary fibroblasts could convert to CAFs during tumour progression using a co-implantation tumour xenograft model (Kojima et al, 2010). Based on all the evidence and supporting data, conclusively makes breast cancer with its CAFs a suitable candidate for anticancer assessment in this study.

The search for alternative cancer treatments has been a central focus of scientific research. One such field in alternative cancer therapy is phytotherapy, which utilizes herbal plants. Generally, the original compositions and integrity of the herbal plants of interest are preserved (Falzon & Balabanova, 2017). Herbal plant extract can be used synergistically or as an adjuvant with existing chemotherapeutic drugs available in the market (Liu et al, 2019). Based on preclinical evidence, natural compounds such as curcumin, eupatorin and resveratrol have been proven to enhance the cytotoxicity of chemotherapeutic drugs towards cancer cells when utilized as an adjuvant in cancer therapy (Lin et al, 2020).

Eleutherine americana (E. americana) is a tropical American species that is widely cultivated in Asia. The plant is abundant in altitudes between 600 and 2000 metres above sea level and thrives in sulfuric soil (Insanu, Kusmardiyani, & Hartati, 2014). The plant is classified as belonging to the *Iridaceae* family, in which the common features include fan-like leaves blade and colourful flowers with 6 petals. In East Asia, the blossom is white. *E. americana*, locally known as *bawang hutan*, is frequently used in traditional medicine in Southeast Asia to treat cardiac arrest, diabetes, stroke, hypertension, and wound healing (Ieyama et al al., 2011). The plant bulb comes with a signature deep red colour and is known to be used by the Kalimantan people to treat heart disease. This is reflected in the plant's pharmacological activities, which have been revealed to contain immunostimulant, anti-inflammatory, anti-bleeding, and anti-cancer properties (Kuntorini & Nugroho, 2009). The plant's primary bioactive component, found in its red bulb, contributes to the plant's functional pharmacological capabilities.

Previous literature has shown that the known major functional bioactive compounds of *E. americana* or *bawang hutan* are divided into 3 main groups, naphthoquinones, naphthalene, and anthraquinones (Mahabusarakam et al. 2010). To optimize the elucidation of the bioactive compound, ethanol was chosen as the solvent for its high polarity and low toxicity (Gandhidas, 2017). Several studies have been done on the bioactive compounds of *E. americana's* pharmacological properties. These include antimicrobial, antiviral, anti-hypertension, anti-fungal, anti-HIV, wound healing, and anti-cancer (Qiu et al, 2005). To date, there is no clear evidence of the molecular mechanisms involved in these properties, and only a few detailed kinds of research have been carried out on its anti-cancer property, specifically on the extract of *E. americana.*

Phytochemical analyses were performed in this study to determine which part of the *E. americana* (root or bulb) contains the active component that has the potential properties as an anti-cancer. *In vitro* testing was performed on breast cancer cells (MDA-MB-231 and MCF7) and normal transformed breast cells (MCF-10A). After the IC_{50} values were obtained, it was further examined using MDA-MB-231 monoculture and co-culture of spheroids (with MRC5). Viability and invasion assays, immunocytochemistry, and immunohistochemistry were performed to corroborate the findings.

The spheroids culture technique was used to recapitulate an *in vivo* analysis involving cell culture models. Compared to the conventional 2D cell culture model, the 3D spheroid model is an improved representation of the structure and functions of *in vivo* tissues and organs (Ivanov & Grabowska, 2018). Moreover, cells in living tissues reside in 3D microenvironments with complicated nutrition and cell transport

dynamics and cell-cell and cell-matrix interactions (Shikov et al., 2014). These matrices allow the cells to migrate within the spheroid in a manner similar to how cells migrate in a live tissue (Smyrek & Stelzer, 2017). As a result, spheroids are better models for cell migration, differentiation, survival, and growth. Hence in this study, the 3D spheroid model was used to better assess the efficacy of *E. americana* extract's anti-cancer pharmacological property. On the other hand, standard 2D cell cultures, also known as monolayer cell cultures, are insufficient representations of a tumour in these environments; making them inaccurate predictions of the *in vivo* therapeutic efficacy and toxicity (Yakavets et al., 2020).

Previous cancer studies have focused mainly on how to delay metastasis, slow down progression, impede invasion and inhibit angiogenesis of cancer cells. It is believed that cancer cells are malignant and the stroma surrounding the cancer masses is not (Shiga et al., 2015). However, the tumour comprises cancer cells and their stromal associate (e.g., fibroblasts, vascular endothelial cells, and immune cells) and the extracellular matrix. The cancer cells' interactions with the normal cells form an environment that favours the progression and proliferation of cancer cells. In the "Hallmarks of Cancer" (Hanahan & Weinberg, 2011), it is stated that cancer cells recruit the surrounding normal cells to act as cancer associates. These cancer associates become actively involved in cancer metastasis and display some hallmark capabilities (Hanahan & Weinberg, 2011).

1.1 Problem Statement

Breast cancer persists as the most frequent malignancy in women worldwide (Basu et al. 2019). Breast cancer is an uncontrollable growth of epithelial cells originating in the ducts or breast lobules (Reilly, 2007). To combat breast cancer, current treatments such as chemotherapy are often accompanied by a list of unfavourable side effects due to systemic toxicity (Lin et al., 2017).

Hence, alternative therapies derived from natural-based resources with less toxicity, effective and economically sustainable are necessary to ensure cancer treatment is accessible to everyone. *Eleutherine americana (E. americana)* has been claimed to possess an array of phytochemicals that have the potential as an anticancer towards breast cancer. It was demonstrated that *E. americana* exhibits potent antioxidant and anticancer properties towards mouse lymphocytic leukaemia cell line (L1210) with IC_{50} of 9.95 ppm (Lestari et al., 2019).

An evaluation of the anticancer properties of *E. americana* bioactive compounds towards breast cancer cells in inhibiting the proliferation of breast cancer would provide a better understanding of its mechanism of

action. Studying the relationship between breast cancer cells and fibroblasts and their responses towards *E. americana* bioactive compounds and the positive control Cisplatin, would provide insights into the regulation of tumour progression and control of cancer.

To date, since limited data is available on the main bioactive compounds of this plant (naphthoquinones, naphthalene, and anthraquinones) towards breast cancer cells and its cancer-associated fibroblasts, detailed investigations would further add to the current knowledge and validate the phytochemical properties and the anti-cancer effects of *E. americana*. A customized approach will involve among others, the use of 3D spheroids and agarose array casts.

1.2 Hypotheses

In this study, it is hypothesized that *E. americana* ethanolic extract possesses bioactive compounds that may have potential anticancer properties. The extract can also suppress the proliferation of breast cancer cell lines MDA-MB-231 and MCF7 and does not impose adverse effects on normal cell lines, MRC5 and MCF-10A. It is also hypothesized that the *E. americana* ethanolic extract can restrict the proliferation of MDA-MB-231 invasive spheroids and their *in vitro* transformed fibroblasts (CAF).

1.3 Study Objectives

1.3.1 Main Objective

To determine the phytochemical properties of E. *americana* ethanol extract and assess its anticancer properties towards breast cancer cells, breast cancer spheroids and its cancer-associated fibroblasts (CAFs).

1.3.2 Specific Objectives

The objectives of this study were:

1. To determine both the quantitative and qualitative *in-vitro* phytochemical properties of the bulb and root parts of *E. americana* ethanol extract.

2. To evaluate the cytotoxicity of *E. americana* bulb ethanol extract on breast cancer cells (MCF7 and MDA-MB-231, normal

fibroblast (MRC5), normal transformed breast cells (MCF-10A), and breast cancer spheroids.

3. To determine the anti-cancer properties of *E. americana* ethanol extract towards breast cancer spheroids and it's cancer-associated fibroblasts.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Breast Cancer

Cancer is a Greek term that refers to malignant tumours and ulcerated malignant tumours (Hajdu, 2004). It is defined as a condition characterised by an excess or deficiency of blood, bile, mucus, and other body fluid secretions. Between 25 BC and AD 50, Hippocrates' successor, Aulus Celsus, demonstrated that advanced breast tumours had a proclivity for recurrence in the armpit, produce arm swelling known as lymphedema, and can cause mortality by metastasis to distant organs.

In 1632, based on a book entitled De Recondita, which illustrated benign and malignant tumours of different organs, an army surgeon Marco Aurelio Serverino depicted that cancers that were fixed to the chest wall were malignant breast tumours whereas the freely movable ones were benign glandular (fibroadenoma) (Lowe, 1597). Additionally, a physician from France (1684-1766) believed that inflammation contributed significantly to carcinogenesis by obstructing the excretory ducts of glands, with breast cancer serving as a representative example (Hajdu et al., 2015). A physician from France (1684-1766) believed that inflammation contributed significantly to carcinogenesis by obstructing the excretory ducts of glands, with breast cancer serving as a representative example (Hajdu et al., 2015). All the findings from the historical studies have shaped cancer research in the current timeframe. Earlier descriptions of cancer have shed light on understanding the disease and paved the direction for finding cancer treatment.

2.2 Current Overview of Breast Cancer

In modern times, cancer is generally interpreted as genetic mutations that transform normal cells into malignant cells (Recillas-Targa, 2022). This transformation causes the cells to grow out of control, become invasive, metastasize and thus result in mortality. The latest global cancer data (Figure 2.1) revealed that the number of estimated cancer incidences has risen to 19.3 million new cases, and there were 10.0 million cancer-related deaths in 2020 (World Health Organisation, 2020). From the estimated number, breast cancer in females was recorded as the highest occurrence at 11.7% and documented as the highest mortality rate in women worldwide.

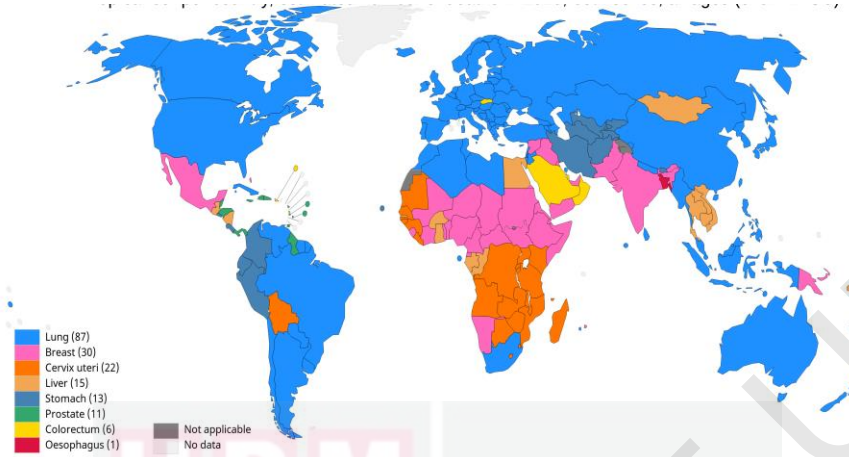


Figure 2.1 : Top Cancer per Country. Estimated age-standardized mortality rates (World) in 2020, both sexes, all ages. The incidence of breast cancer (in pink) was in second place after lung cancer worldwide. In the Asian continent, the high incidence of breast cancer (in pink) is in Southeast Asia. Adapted from the World Health Organization (2020)

Breast cancer was listed as one of the most prominent cancer incidences after lung cancer in terms of new cases worldwide and caused the highest death rate globally. Based on the figure, half of the breast cancer incidence was reported in Asia. This was owing to a lack of awareness among Asian people, and as a result, they sought medical assistance only after cancer had metastasized (Wang et al., 2022).

According to a study conducted, knowledge regarding breast cancer and awareness that the disease could also affect younger women were recorded to be lower among the younger women in Malaysia than the older women. This was because of the belief that only older women were affected by cancer, and due to a lack of awareness, younger women tend to present with a higher cancer stage, hence suffering more aggressive breast cancer compared to older women (Hadi et al., 2010). Figure 2.2 demonstrates the incidence of new cancer cases in Malaysia versus the Asia continent.

From the chart, breast cancer was recorded as the highest occurrence in both the Asian continent and Malaysia indicated by the blue bar. The mortality rate for breast cancer was also recorded as the first rank in the bar chart.

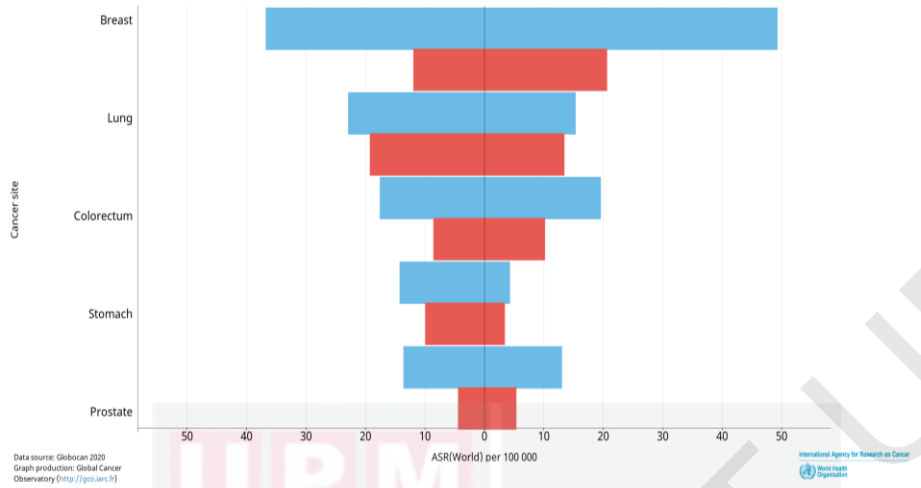


Figure 2.2 : Global Cancer (Asia vs. Malaysia) Estimated age-standardized incidence and mortality rates of both sexes and of all ages. The highest cancer incidence in Malaysia is breast cancer, indicated by the blue bar chart followed by the red bar chart beneath it, which signifies the mortality rate for breast cancer cases in 2020.Adapted from the World Health Organization (2020)

Breast cancer incidence in Malaysia was recorded as the highest cancer occurrence in 2020 (Figure 2.3) for both sexes and of all ages. Breast cancer exhibited the highest number of new cancer cases at 17.3%, followed by the highest number of mortalities. It was also documented as the most prevalent cancer among females in Malaysia by Globocan, in the year 2020.This could be due to cancer's late presentation at stages 3 and 4, which accounts for an astounding 50% to 60% of all breast cancer cases in Malaysia. Apart from a lack of awareness, ignorance towards the early manifestation of breast cancer is the main concern among Malaysian women. The shortage of treatment centres, limitations on access to competent medical treatments, unclear information about alternative treatments for breast cancer due to a lack of or inadequate information exchange, and geographical seclusion worsened the situation (Hisham & Yip, 2003; Lee et al., 2019).

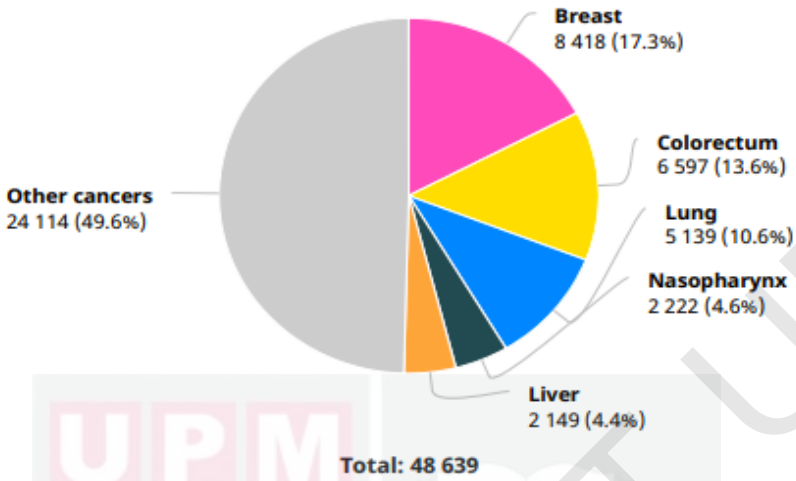


Figure 2.3 : Number of New Cancer Cases in Malaysia. Of 48,639 documented new cancer cases, 17.3% (8,418) were reported as breast cancer occurrences. It was also reported as the second highest mortality case after lung cancer in Malaysia. Adapted from the World Health Organization (2020)

Even though early detection could promote the survival rate of breast cancer patients, most breast cancer patients only seek medical attention once the cancer has metastasized and spread to distant organs. Breast cancer can be staged from 0 to 4. The staging system is based on the TNM system (tumour, nodule, metastases) (Reilly, 2016). Stage zero (0) refers to an *in-situ* carcinoma, in which the tumour only subsists at the area where it was first formed. At stage one (1), the primary breast cancer has no metastases occurring and no nodal involvement. At stage two (2), the primary tumour shows a significant increase in size, approximately 1 – 5 cm. However, there is no nodal involvement; if the tumour size measures more than 5 cm but with no nodal infiltration, it will still be categorized as stage two (2). Primary breast cancer that comes with infiltrated nodes and which already adheres to other structure(s) or organ(s) will be categorized as stage three (3). Once the cancer cells have already metastasized to a distant organ(s), they will be in late-stage four (4).

In Malaysia, approximately 50% to 60% of breast cancer cases were presented at advanced stages 3 and 4 where any therapy given could only provide little or no benefit to the patients' (Akhtari-Zavare et al., 2015). This might be due to poor awareness among Malaysian women regarding breast cancer in terms of early detection through breast self-examination (BSE). Although BSE may not be adequate as a tool for early detection, it can still be one of the important tools in the early detection

of breast cancer that does not cost anything and does not require any advanced machinery and medical knowledge. It is practical for developing countries (Girihadra et al., 2013).

The mutation of BRCA genes increases the risk of breast cancer and ovarian cancer in the human (Feng et al., 2018). Germline mutations of BRCA1 and BRCA2 are the common cause of breast cancer by genetic inheritance, accounts between 5 – 10% of all breast cancer cases (Barzaman et al., 2020). Conversely, the majority of breast cancer cases are sporadic, resulting from mutations in somatic genes without any occurance of germline mutation occurrence (Jayasekera et al., 2023; Kenemans et al., 2008). Treatment responses were different among patients due to the diverse mutational makeup of breast cancer. A condition is known as chemo-resistance and radio-resistance results in therapeutic resistance and the failure of targeted therapy for individual patients. This condition is contributed by cancer stem cells or tumour-resistance cells within the tumour (Cianciosi et al., 2018). Due to the mutational diversity of breast cancer, treatment regimens and treatment outcomes should be based on its major subtypes

Invasive breast cancer is classified into two major subtypes: invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) (Barroso-Sousa & Metzger-Filho, 2016). Accounting for approximately 75% of all breast cancers, the IDC is described as a group of malignant epithelial tumours which invade adjacent tissues and are prone to metastasize to lymph nodes and distal organs (Miki et al., 2009). On the other hand, invasive lobular carcinoma (ILC), represents a minor number of breast cancer incidence at as low as 5% to 10% (Barroso-Sousa & Metzger-Filho, 2016). Although ILC represents generally only 5% -10% of cases, it has been proven more destructive than IDC. The ILC produce worse outcomes than the same stage diagnosed IDC. This is due to the morphology of the ILC in which the lack of E-cadherin expression that controls cell to cell adhesion properties become small, non-cohesive cells that can penetrate the stromal in a single-file pattern (Reed et al., 2015).

2.3 Breast Cancer Progression Mechanism

Cancer cells cannot exist in isolation within a tumour. Similarly, in breast cancer, an extracellular matrix and a variety of neighbouring cell types, in addition to the cancer cells, constitute a tumour microenvironment (TME) that promotes tumour survival, growth, and resistance to treatment. Hanahan and Weinberg (2011) stated that tumours are not simply masses of proliferating cancer cells, but rather complex tissues made of multiple unique cell types that interact heterotypically with one another (Hanahan & Weinberg, 2011). This heterogeneous population that makes up the TME mainly includes

endothelial cells, fibroblasts, as well as various inflammatory and immune cells. The TME components strongly influence the behaviour of tumour cells such as invasiveness as well as survival capability via cell-cell or cell-matrix interaction (Heylen et al., 1998; Noël et al., 1993).

Fibroblast cells that enhance tumour cell motility are also known as cancer-associated fibroblasts (CAFs). Based on previous research and histological studies, fibroblast cells that were identified surrounding the cancer cells could enhance the survival of the cancer cells in their host. It has been reported that the fibroblasts associated with cancer promote the tumorigenicity and metastatic behaviour of malignant cells (Chung, 1991) by encouraging an oxygen-rich environment, enhancing the immune-suppressive, and proinflammatory microenvironment (Jena et al., 2020). The theory was further established by a study on an *in vivo* assay of RMS 9-4/8, rat rhab-domyosarcomal cells, a type of weak invasive lung tumour cells, that were co-inoculated with fibroblasts in nude mice, in which the co-inoculation successfully increased cell colonization of lung tumour in the nude mice (Heylen et al., 1998). In particular, several studies that focused on the role of fibroblasts in metastasis showed their vital role in promoting tumour cell motility such as in B16 melanoma cells and MCF7 breast cancer cells (Saiki et al., 1994), (Rossi et al., 1994). As mentioned earlier, Figure 2.4 illustrates the composition of tumour masses including the cancer associated-fibroblast as described by Hanahan & Weinberg., 2011.

2.4 3D Spheroids

Cell culture is a process of cultivating cells in a controlled environment for the observation and investigation of the response of the cells towards their surroundings (Edmondson et al., 2014). Nowadays, various forms of cell culturing techniques can be utilised, each with its own set of characteristics and applicability for specific applications. As a result, certain techniques of cultivation are chosen over others. Due to its innovative and practical qualities in comparison to prior cell culture methods, the 3D spheroid is becoming increasingly popular. 3D spheroids are a process of growing living cells in micro-assembled devices and are supported by three-dimensional geometries that mimic the microarchitecture of tissues and organs (Edmondson et al., 2014). It is practical to use 3D spheroids in research because it allows biological cells to grow and interact in an artificially constructed environment which allows researchers to explore and measure diverse parameters that can't be achieved by using 2D cell culture. Unlike 2D monolayer culture, a 3D spheroid environment enables cells to proliferate in all directions *in vitro* (Chen & Song, 2019).

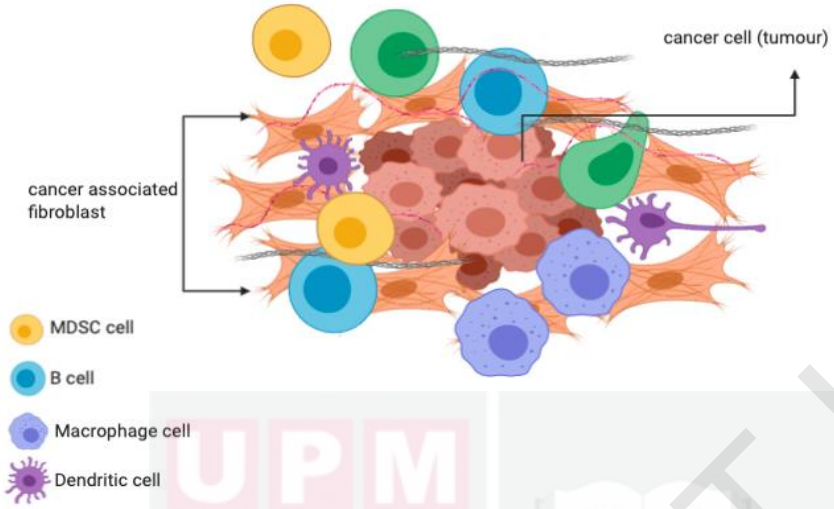


Figure 2.4 : Tumour Microenvironment (TME) of Cancer Cells. The illustration above portrays the interactions of cancer masses with surrounding normal cells. Fibroblast cells that were found surrounding the cancer cells were fibroblast associated with the cancer cells and, consequently, enhanced the survival of the cancer cells in its host

Thus, scientists worldwide have been researching the expansion of the 3D culture technique for application on different cell types including stem cells, autologous cells, allogenic cells, xenogenic cells, progenitor cells, and multipotent cells. In contrast to 2D monolayer cell culture, 3D spheroids may almost fully mimic *in vivo* cell behaviour and organization (morphology and physiology) (Huang et al., 2020). Using scaffolds or non-scaffolding technologies, multi-layer 3D spheroids can be assembled. Whether or not scaffolds were used, the resultant cells that make up spheroids could still interact directly and indirectly with the matrix or support due to cell junctions and soluble factors (Fang & Eglen, 2017). Many applications nowadays, such as tissue engineering, are based on 3D spheroids, which also helps to improve drug testing such as in the application of microfluidic organ-on-chips (Ivanov & Grabowska, 2018). Nonetheless, scientists are still striving to figure out the vast uses of 3D spheroids.

2.5 Cancer-Associated Fibroblasts (CAFs)

Cancer-associated fibroblasts can be identified nearly in all carcinoma including breast cancer, and encompass 80% of the tumour stroma (Smith et al., 2013). In the tumour microenvironment of breast cancer, CAFs acts as the predominant stromal cell type, and its crosstalk with tumour cells promotes tumour progression and metastasis through

immune response regulation, extracellular matrix remodelling, and various secretory proteins release (Hoshino, 2011)(Alguacil-Núñez et al., 2018). It has been documented that CAFs also play a major role in tumour resistance towards chemotherapy treatment (Seben & Schafer, 2012). Kojima et al. demonstrated that resident human mammary fibroblasts are able to convert to CAFs during tumour progression using a co-implantation tumour xenograft model (Kojima et al, 2010). Studies have shown that cancer cells reprogram fibroblasts to become CAF through the actions of miRNAs (miR-31, miR-214, and miR-155) (Pang et al., 2015). miR-31 and miR-214 were downregulated, whereas miR-155 was upregulated when compared with normal or tumour-adjacent fibroblasts. Mimicking this deregulation by transfecting miRNAs and miRNA inhibitors induced a functional conversion of normal fibroblasts into CAFs (Mitra et al., 2012).

Other studies conducted have shown that cancer cells can benefit from the association with fibroblasts. For example, when CAFs were extracted from human breast cancer and subcutaneously implanted into immunodeficient (nude) mice, the breast carcinoma grew rapidly in comparison to normal fibroblast-containing breast carcinomas, owing to increased release of stromal cell-derived factor 1 (SDF-1) (Orimo et al., 2005). CAFs also play a role in ECM remodelling by expressing members of the matrix metalloproteinase family (MMPs). MMP-2 and MMP-9 selectively degrade type IV collagen and laminin, which constitutes the basement membrane, and contribute to tumour proliferation. MMP-9 was activated by MMP-3 and MMP-13 (Knäuper, Smith, López-Otin, & Murphy, 1997). Through overexpression of the anti-apoptotic protein BCL-XL (Kharaziha et al., 2012), CAFs also protected prostate cancer cells from the cytotoxic effect of sorafenib (multi-tyrosine kinase inhibitor).Therefore, based on cited scientific evidence of CAFs promoting cancer survival, CAFs or fibroblast associates may have the potential to become a target for tumour therapy.

2.6 Early Middle Ages Breast Cancer Treatments

Before the 19th century, the 'Egyptians utilized arsenic paste to treat tumours and cancer together with cautery, knives, and salts (Hajdu, 2011). In addition, herbal remedies such as tea, fruit juices, figs, and boiled cabbage were practised by the Sumerians, Chinese, Indians, Persians, and Hebrew in an attempt to treat cancer, and they even resorted to chemical pastes of iron, copper, sulfur, and mercury in advanced cases of cancer (Petrovska, 2012). Interestingly, in *Materia Medica,* a compilation of remedies for cancer by Pliny the Roman (AD 23-79), it was recommended that herbal compounds be used internally in advanced cancers before and after surgery (Hajdu, 2005). Similarly, breast cancer treatments suggested by *Ambroise Pare* (1510-1590) included the use of a sheet of lead covered with quicksilver (mercury)

placed on the tumour, palliative treatment, purgatives, and diets rich in fruits and broths (Hajdu, 2004b).

Later on, Oribasius of Baghdad (325-403) reported that cancer could only be treated by amputating the cancerous and surrounding healthy tissues, but he was unsuccessful in treating ulcerated cancer. Similarly, Aetius (527-565), who served as a physician under Emperor Justinian in Constantinople, offered mastectomy as a treatment for breast cancer, but only for non-ulcerated lesions of the cervix, labia, and anus with cautery, but not for ulcerated breast cancer (Hajdu, 2004). Johannes Scultetus (1595-1645), a German surgeon, also proposed removing infected breasts via excision and amputation, which he depicted and documented in 1655 in his book *Armamentarium Chirurgicum* (Figure 2.5)(Hajdu, 2011). He recommended that following the removal of the diseased breast, cauterization be used to control bleeding and prevent recurrence (Hajdu et al., 2015).

Other breast cancer treatments include polypectomy designed by Avicenna of Persia (980-1037) in which a wire loop was made tighter each day until the tumour fell off and also the practice of bloodletting prior to surgery for tumour removal by the first Muslim physician in Europe, Albucases (1013-1106) (Gruner OC., 1930; Hajdu, 2004a). It was not only until the 16th century that the systematic therapeutic use of chemicals was instigated, as described in *De Grandibus*, a book published in 1567 by Paracelsus, a pioneer in chemistry and chemotherapy. The chemicals used as internal medications for cancer include mercury, lead, sulphur, iron zinc, copper, arsenic, iodine and potassium (Hajdu et al., 2015).

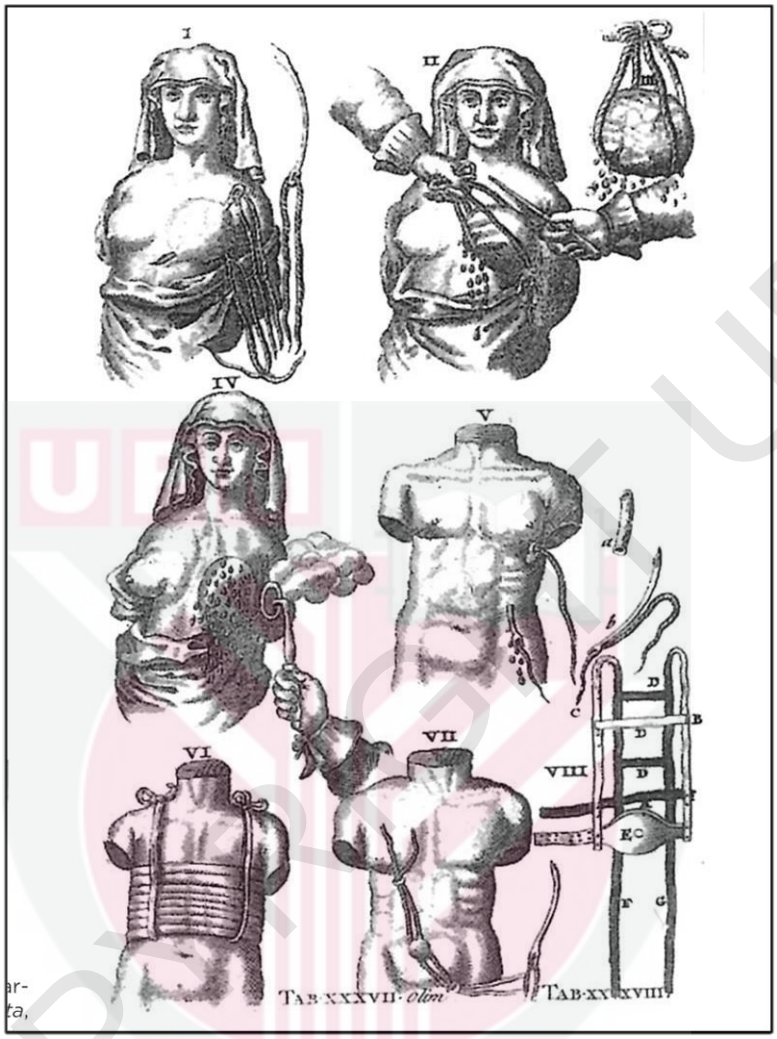


Figure 2.5 : Mastectomy in the Year 1655. Diagram illustrates the procedure of mastectomy and post-operative care in the year 1655 Adapted from Hajdu et al., 2015

It was not only until the 16th century that the systematic therapeutic use of chemicals was instigated, as described in *De Grandibus*, a book published in 1567 by Paracelsus, a pioneer in chemistry and chemotherapy. The chemicals used as internal medications for cancer include mercury, lead, sulphur, iron zinc, copper, arsenic, iodine and potassium (Hajdu et al., 2015).

2.7 Modern Breast Cancer Treatment

Current treatment for breast cancer varies based on the pathological stage at the time of detection and diagnosis of the disease and includes surgery, radiotherapy, and chemotherapy (Maughan et al., 2010). For instance, patients with early stages of breast cancer are commonly treated with surgery (mastectomy) and radiotherapy. Meanwhile, for patients with advanced stages (stage 3 and stage 4) of the disease, chemotherapy is the main treatment option.

2.7.1 Surgery

Almost all women diagnosed with breast cancer are suitable candidates for masectomy, or breast cancer removal surgery (Bloom & Manasseh, 2009). Additionally, it was the most frequently used treatment for early-stage breast cancer. As previously stated, surgery for breast cancer has been practiced since the dawn of human history (Hajdu, 2011). Since the nineteenth century, mastectomy has been refined and enhanced to assure the treatment's efficacy. The development of the mastectomy procedure is shown in Table 2.1.

Table 2.1 : Mastectomy Approaches

Mastectomy approaches	Surgical Procedure	References
Modified radical mastectomy (MRM) I	Removal of entire breast, nipple-areolar complex and axillary lymph nodes	(Auchincloss, 1963)
Skin-sparing mastectomy (SSM)	Removal of breast parenchyma, the NAC, removal of the skin surrounding the lesion and preserved breast envelope.	(Toth BA, 1991)
MRM II	Preservation of axillary nodes if sentinel lymph node biopsy negative.	(Manasseh D, 2006)

However, there has been an increased number of incidents of breast cancer recurrence in recent years. The effectiveness of surgery as the treatment for breast cancer has been debated among medical practitioners since the 19th century (Nielsen, Overgaard, Grau, Jensen, & Overgaard, 2006). Failure of the surgery treatment outcomes is significantly linked to an increased risk of metastases to the distal organs (Miki, Suzuki, & Sasano, 2009). Recurrence from surgical treatment at the local site of the surgery area after radical mastectomy

was at 5% and 8%, and for regional recurrence, 4% and 8% in node-negative and node-positive cancers, respectively. Skin and chest wall recurrences were the most common, representing 49% to 83% of the recurrence (Wapnir et al., 2018). By comparison, nodal recurrences were less frequent, constituting 15% to 52% of incidents. Conversely, mastectomy combined with systemic adjuvant treatment has improved overall outcomes and reduced the frequency of recurrence (Gibbs & Kent, 1998). Thus, finding effective adjuvant treatments such as phytotherapy could potentially reduce or eliminate breast cancer recurrence after mastectomy surgery.

2.7.2 Radiotherapy

Radiotherapy treatment has become one of the most important approaches to combat cancer (Braunstein & Bellon, 2020). It utilizes ionizing radiation that forms electrically charged particles that bombard the targeted cells with high energy. The radiation produces high-intensity energy that causes genetic changes in the targeted cells resulting in cancer cell death and shrinkage in tumour size (Baskar et al., 2012). Radiation therapy (radiotherapy) can be given to cancer patients to treat the tumour or to alleviate the symptoms caused by cancer. Radiation can also be used as neoadjuvant treatment (before the surgical procedure) to reduce the tumour size or adjuvant treatment after the surgical procedure to fully eliminate any remaining cancer cells (Majewski et al., 2005).

Two delivery modes of radiation are available for targeting the tumour's exact location on-site (Baskar et al., 2012). Aiming the high ionizing radiation rays (radiation particles or photons) at the tumour's exact location outside of the patient's body is known as external beam radiation. Medical practitioners commonly utilize external beam radiation to treat cancer. Another delivery mode of radiotherapy practice is internal radiation, also called brachytherapy; the radiation's energy is sealed in catheters or seeded directly into the tumour site (Nielsen et al., 2006).

As effective as radiotherapy in combating cancer, it also has side effects. Among the adverse effects of the treatment is painful skin at the application site, fatigue, hair loss, changes in breathing rhythm, and swallowing difficulties. Gastrointestinal morbidity, cardiac morbidity, and fertility problems are also frequently reported (Majewski et al., 2005).

2.7.3 Chemotherapy

Chemotherapy is a method of treating cancer that uses strong chemicals at the highest doses tolerated to control, decrease, or kill cancer cells (Takei et al., 2017). In contrast to radiotherapy and surgery, chemotherapy affects the entire body. Chemotherapy is effective because it targets fast-growing cells; however, chemotherapy also harms normal cells, including hair follicles, stomach lining, bone marrow, and skin (Breen et al., 2017). As a result, hair loss, changes in appetite, anaemia, and easy bleeding and bruising have been reported. Chemotherapy treatment regimens differ according to the type of cancer. Depending on the type and stage of cancer, a single chemo-drug treatment or a combination of chemo-drug treatments may be applied (Leal & García-Perdomo, 2019). To further enhance the chemo-treatment, chemotherapy can also combine with radiotherapy. Several commercial synthetic drugs available for chemotherapy are cisplatin, tamoxifen, paclitaxel, and doxorubicin which have distinct mechanisms of action (Pádua et al., 2015).

However, the failure of chemotherapy for cancer treatment has been reported due to genetic and epigenetic changes resulting in drug resistance, despite attempts to develop more specific and effective drugs. For example, the regulation of epithelial–mesenchymal transition triggered by cancer metastasis contributes to tolerance towards chemo-drugs (Ponnusamy et al., 2019). Thus, the race to discover the novel chemical and natural compounds as potential breast cancer treatment commences.

2.8 Phytotherapy

Phytotherapy is a niche in medicine that uses plants to treat ailments or as health-promoting agents (Falzon & Balabanova, 2017). Mankind's search for the cure of illnesses through plant medicine is dated as old as mankind's history itself. The first written proof of the use of the plant as medicine dated as far back as 5000 years ago, was identified on a Sumerian clay slab, comprising 12 recipes of drug preparation referring to over 250 types of plants (Asghar et al., 2015). In addition, the Emperor of China, Emperor Shen Nung wrote a book on roots and grasses circa 2500 BC (Shikov et al., 2014). There were 365 types of dried parts of medicinal plants still used today, such as ginseng, cinnamon bark, jimson weed, and ephedra (Christophe, 2007).

In Indian holy books, the Vedas mentioned treatments using medicinal spice plants, including nutmeg, pepper, clove, and many more (Falzon & Balabanova, 2017). Circa 1550 BC, The Ebers Papyrus scripted collections of 800 prescriptions using 700 types of medicinal plant

species such as pomegranate, castor oil, garlic, onion, coriander, juniper, common centaury, and countless more (Hajdu, 2004). The renowned physician of Greek, Hippocrates (459-370 BC), also worked on 300 medicinal plants and perfected the art of using plants as medicament by categorizing the plants based on their physiological actions. For example, wormwood and common centaury were used against fever, oak, and pomegranate as astringent, sea onion, celery, parsley, asparagus, and garlic as diuretic agents, and deadly nightshade and mandrake were used as narcotics (Petrovska, 2012).

Numerous scientific pieces of evidence have shown the potency of herbal plants in treating various illnesses. In recent years, the combination of anticancer drug treatment with the natural product has beensuccessfully reported to offer less invasive treatment and reduce the doses of administered drugs, which will decrease the drug toxicity and medical side effects (Lin et al., 2019). The natural products which include those extracted from plants, fruits, and vegetables, were reported to possess chemo-preventive and/or chemotherapeutic properties (Kalimuthu et al., 2013). Moreover, several studies have revealed that high consumption of plant-based sources with anticancer drug treatment leads to a decrease in cancer incidence (Giampieri et al., 2017). This finding may suggest that plant-based sources might contain bioactive compounds capable of hindering carcinogenesis and/or increasing the potency of chemotherapy and radiotherapy as a treatment against tumour (Giampieri et al., 2017). Therefore, researchers have focused their studies on using herbal plants to replace or to be used concurrently with commercial cancer drugs for cancer therapy.

Herbal plants have also been traditionally used as medicine in Malaysia too. Several medicinal plants in Malaysia have been studied and proven to display antiproliferative properties towards the breast cancer (Mainasara et al., 2018). Belalai Gajah with the scientific name *Clinacanthus nutans* is one of the local herbal plants that had been proven to possess anticancer properties (Alam et al., 2016). In fact, a long list of herbal plants in Malaysia has been extensively studied for their medicinal values, including *Tongkat Ali* proven to have an antimalarial, aphrodisiac, anti-diabetic, antimicrobial and antipyretic activity (Bhat & Karim, 2010), *Pegaga* with an injury recovering effect, anticancer properties, antimicrobial properties, memory enhancing, asthma preventive and is capable to treat ulcers and leprosy (Abu Bakar et al., 2022) and, *Hempedu Bumi* possesses antibacterial, antifungal, antiviral anti-neoplasm, hepatoprotective, hypoglycemic, hypocholesterolemic and energetic effects (Alipanah-Moghadam et al., 2021).

2.8.1 *Iridaceae* Family

Translated as the rainbow in Irish verbal, Iridaceae plants are known to have an extensive array of colours flowers. Categorized under the *Asparagales* order, it is known to have 66 accepted genera from a total of 2244 species worldwide (Christenhusz & Byng, 2016). The plants are mainly identified in tropical and temperate climate, and they could be identified as perennial plant that grows erected from the ground and with grass-like leaves. The leaves are the unique features of the Iridaceae family, stated as "isobilateral equitant and overlapping transversely with each other to form fan-like arrangement" (Singab et al., 2016). Other features identifiable with the plant are the underground storage, generally in the form of rhizomes, bulbs, or tuber (Singab et al., 2016; Glimn-Lacy and Kaufman, 2006).

Iridaceae plants are known for their phytochemical properties that cover a wide range of usage and medicinal benefits. Its broad category of secondary metabolites comprises flavones, flavanones, triterpenes, quinones, stilbenes, peltogynoids, and isoflavones (Ayouba et al., 2015). Different taxa of the Iridaceae family produce distinctive secondary metabolites with beneficial pharmacology properties. The significant pharmacological benefits include antimicrobial, anti-inflammatory, antioxidant, anti-diabetic, phytoestrogens, hepatoprotective, and hypolipidemic activities (Chen et al., 2019). There is also evidence of anticancer activities, antifungals, HIV inhibitors, and wound healing. Topoisomerase II inhibitors and prothrombin have also been documented (Ayouba et al., 2015; Chen et al., 2019). A few members of the Iridaceae family have been the focus of the research community based on their significant pharmacological benefits including *Moraea*, Cipura, Belamcanda, Gladiolus, and *Eleutherine*

2.8.2 *Eleutherine americana (E. americana)*

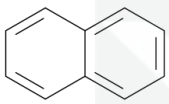
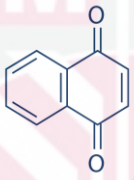
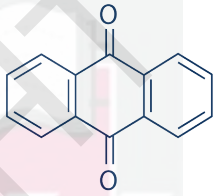
Eleutherine americana or *Bawang Dayak* in Malay belongs to the Iridaceae family and can be identified scattered broadly across Southeast Asia, South Africa, South America, and Hainan Island South China (Insanu et al., 2014). In the Dayak tribe, *E. americana* is used as a breast-milk booster. It has also been used as a traditional remedy to treat hypertension, diabetes, stroke, sexual disorders, cardiac diseases, particularly coronary disorders and breast cancer (Ieyama et al., 2011). There are also records stating the pharmacological properties of *E. americana*, such as purgative, antifertility, antihypertension and wound healing (Villegas et al., 1997). Mature plants bloom with white flowers with six petals and yellow stamen as depicted in Fig 2.6

Figure 2.6 : Mature *E. americana Plant (Flower and Leaf Segment.* The unique characteristic of the *Iridaceae family* is the fan-like structure of the leaf. *E. americana* comprises white flowers with six (6) petals and yellow stamen

2.9 Major Bioactive Compounds of *E. americana*

Based on a study by Ieyama et al. (2011), three (3) major bioactive compounds were discovered in *E. americana,* which are naphthalene, naphthoquinones, and anthraquinones (Table 2.2) (Ieyama et al., 2011). Each bioactive compound group (Naphthalene, Naphthaquinone, and Anthraquinone) consists of a sub-group bioactive mixture and each sub-group has specific pharmacology properties.

Table 2.2 : Chemical Structures of Three Major Bioactive Compounds of *E. americana* Ethanol Extract

		
Naphthalene	Naphthaquinone	Anthraquinone

(Adapted from Ieyama et al., 2011)

2.9.1 Naphthalene

One of the key groups of bioactive compounds for *E. americana* is Naphthalene, and it could be divided into a list of subgroups. *Eleuthero, Hongconin, Eluthoside A, Eleutherinoside A* were identified as derivatives(Xu et al., 2006). Naphthalene derivatives were reported to possess anti-inflammatory effects based on *in-vitro* evaluation, by inhibiting inducible nitric oxide synthase (iNOS) production and nitric oxide overproduction (Song et al., 2009). Table 2.3 summarizes the naphthalene derivatives and their pharmacological activities based on reported studies.

Table 2.3 : Naphthalene Derivatives and Their Pharmacological Properties

Compound	Pharmacological activities	Reference
Eleutherol	Increase of coronary flow in guinea pig	(Song et al., 2009)
Hongconin	MMDC = 130 µg/mL on *Pyricularia oryzae* Inhibition of proliferation of K562, IC_{50} = 174 µg/mL Inhibition of RAW 264.7 lipopolysaccharide-activated mouse macrophage cells with IC_{50} = 19.8 µM	(An et al., 2008)
Isoeleutherol	Inhibition of HIV replication IC_{50} = 100 µg/mL	(Insanu et al., 2014a)
Eleutherinoside A	β-glucosidase inhibitor with IC_{50} =0.5 mM Inhibition of iNOS expression at 30 µM with 98% inhibition.	(Song et al., 2009)

*MMDC - minimum morphological deformation concentration

2.9.2 *Naphthaquinones*

The sub-group *Napthaquinones*; namely eleutherine and isoeleutherine, have been proven to contain anti-inflammatory and antihypernociceptive activities based on *in-vivo* analysis, which displayed positive results in the inhibition of carrageenan-induced paw oedema (Mahabusarakam et al., 2010), (Song et al., 2009), (Paramapojn et al., 2008). Extensive studies have been performed on naphthoquinones. The compound displays chromatic pigmentation when extracted, due to the production of a secondary metabolite in the plants. Interestingly, this only occurs in 17 families of plants; *Plumbaginaceae, Juglandaceae, Ebenaceae, Boraginaceae, Dioncophyllaceae, Ancistrocladaceae,* ***Iridaceae****, Verbenaceae, Scrophulariaceae, Avicenniaceae, Balsaminaceae, Bignoniaceae, Gentianaceae, Droseraceae, Nepenthaceae, Lythraceae, Euphorbiaceae* (Republic, 2005). Table 2.4 summarizes the derivatives of naphthoquinones and their pharmacological activities.

Table 2.4 : Naphthaquinones Derivatives and Their Pharmacological Properties

Compound	Pharmacological activities	Reference
Eleutherin	Inhibition of proliferation of K562, IC_{50} = 49 µg/mL MMDC = 30 µg/L Inhibition of RAW 264.7 lipopolysaccharide-activated mouse macrophage cell with IC_{50} = 11.4 µg/mL	(Mahabusarakam et al., 2010)
Isoeleutherine	Inhibition of HIV replication in Hg lymphocytes, IC_{50} = 8.5 µg/mL Inhibition of RAW 264.7 lipopolysaccharide-activated mouse macrophage cell with IC_{50} = 7.7 µM MMDC = 30 µg/mL on *Pyricularia oryzae*	(Paramapojn et al., 2008)
Elecanacin	Inhibition of HIV replication IC_{50}= 100 µg/mL	(Mahabusarakam et al., 2010), (Song et al., 2009)
Eleithinone A	Inhibition of RAW 264.7 lipopolysaccharide-activated mouse macrophage cell with IC_{50} = 21.7 µg/mL	(Paramapojn et al., 2008)

*MMDC - minimum morphological deformation concentration

2.9.3 *Anthraquinones*

Due to the fact that the majority of the derivatives in this group were recently synthesised, the only data available is from an antibacterial test that claimed weak activity against the target microorganisms. The compounds and pharmacological characteristics of these compounds are summarised in Table 2.5.

Table 2.5 : Anthraquinones Derivatives and Their Pharmacological Properties

Compound	Pharmacological activities	Reference
4,8-dihydroxy-3-methoxy-1-methyl antraquinone-2-carboxylic acid methyl ester	MMDC = 55 µg/mL on *Pyricularia oryzae*	(Mahabusarakam etal.,2010),(Jinzhong, Feng, Wenjuan, & Gexia, 2006)
8-hydroxy-3,4-dimethoxy-1-methylantra quinone-2-carboxylic acid methyl ester	MMDC = 170 µg/mL on *Pyricularia oryzae*	(Paramapojn et al., 2008), Song et al., 2009)
3,4,8-trimethoxy-1-methylantra quinone-2-carboxylic acid methyl ester	MMDC = 170 µg/mL on *Pyricularia oryzae*	(Mahabusarakam et al., 2010), (Song et al., 2009)
2-acetyl-3,6,8-trihydroxy-1-methyl anthraquinone	Weak activity against the growth of *Pyricularia oryzae*, MMDC = 124.8 µg/mL	(Jinzhong et al., 2006)

*MMDC - minimum morphological deformation concentration

Limited information and research have been carried out on the anticancer property of *E. americana* setting the groundwork for this study. Documented to possess various phytochemical compounds that were suggested to have the ability to impede the progression of cancer, *E. americana* is a promising candidate for an anticancer study on breast cancer cell lines.

CHAPTER 3

MATERIALS AND METHODS

3.1 General Experiment Procedures

The laboratory work for this research is divided into three stages, which are:

1) phytochemical analysis and evaluation, 2) preliminary screening in 2D cell cultures, and 3) the effects of EA extract on cell culture spheroids (3D spheroid model) and its cancer-associated fibroblasts. In the phytochemical evaluation stage, two parts of the plant (the root and bulb) were used to prepare the ethanolic extract.

Preliminary studies on the phytochemicals from both ethanolic plant extract were conducted for both qualitative and quantitative evaluations. The preliminary screening stage involved determining the cytotoxicity and minimal inhibitory concentration (IC_{50}) of the extract against two (2) distinct breast cancer cell lines (MDA-MB-231 and MCF7) and two (2) normal cell lines (MCF-10A and MRC5) using two (2) main sections of the plant's ethanolic extract (root and bulb).

The results from this stage set the groundwork for the subsequent study phase. Assays such as viability assay, invasion assay and H&E staining was performed on 3D cell culture model to acquire sufficient data to support the analysis and other critical tests. Multiple t-tests and ANOVA were then used to demonstrate statistical significance.

3.2 Materials

3.2.1 Cell Culture Procedures

The four types of cell lines utilised in this research were obtained from the UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience UPM collections. The cell lines were MDA-MB-231, MCF7, MCF-10A and MRC5. The cells were maintained in various culture media and incubated at 37°C in a 5% CO_2 atmosphere.

3.2.2 Cell Culture Media Preparation Procedures

Each cell culture was maintained in different specific culture media. The concoction of media and the supplement(s) used for each cell culture can be referred to in Appendix A.

3.2.3 Chemicals

All chemicals utilised in this study were of laboratory grade. Distilled water was collected from a Millipore Merck ultra-pure water and distilled water was collected from MILI-Q Rios8 (Merck). The media and supplements were of a quality suitable for research and cell culture grade.

3.2.4 Plant Sample (*E. americana*)

Plant samples *E. americana* was provided by Dr Nurmawati Syakroni from Cyberjaya University College of Medical Science. Plant samples that were provided included dried plant parts and seedlings of *E. americana.* The specimen number of the plant is MFI 0055/19. The plant identification was conducted by a Research Officer at the Biodiversity Unit (UBD), Institute of Bioscience, UPM. The seedlings were replanted and cultivated for morphological observations. From the plant sample provided, 500 grams of the dried plant parts were macerated in 1 L of 99% ethanol for 72 hours. The maceration technique with ethanol was chosen since ethanol is identified as a high-polarity solvent that allows it to dissolve both polar and non-polar substances of the bioactive compounds. Thus, ethanol maceration can produce the maximum concentration and number of bioactive compounds that could be extracted from the plant sample. The ethanolic extraction of *E. americana* was then concentrated using a rotary evaporator (Buchi). It was then stored at 4°C and resuspended with dimethyl sulfoxide (DMSO) with a concentration of less than 5% for further use.

3.3 Methods

3.3.1 Phytochemical Quantitative Analysis

3.3.1.1 *In-vitro* Antioxidant Assays (DPPH Assay)

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was used in assessing the antioxidant content of bulb and root ethanol extracts of *E. americana*. A 0.2 mmol/L of DPPH (2,2-diphenyl-1-

picrylhydrazyl) was diluted in ethanol. The serial dilution of extract of *E. americana* (bulb and root separately) starting from 50 ul, 100 ul, 150 ul, 200 ul, and 250 ul were then dispensed into 500 ul of DPPH 0.2 mmol/L solution. The mixture was then mixed briskly and incubated in the dark and at room temperature for 30 minutes. Blank DPPH 0.2 mmol/L solution was used as a positive control and Quercetin as a negative control. Absorbance readings were taken at 540 nm using a microplate reader (Tecan Infinite M200Pro). The radical scavenging activity of both bulb and root sections of *E. americana* was calculated using the following formula:

Percentage radical scavenging activity (RSA)

$$= \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100\%$$

The concentration of the sample that scavenged 50% of DPPH was determined based on a linear regression graph and recorded as IC_{50}. The linear regression graph can be referred to in Appendix B.

3.3.1.2 Total Phenolic Content Estimation

In this procedure of total phenolic estimation, a Folin-Ciocalteau reagent method was applied. About 20 µg of the bulb and root extract were weighed separately and diluted in 1 mL of distilled water. The Folin-phenol reagent was then prepared fresh prior to the procedure by diluting it with distilled water at the ratio of 1:1. Then mix 500 µL of diluted Folin-phenol reagent with the plant extract (root and bulb separately) and 2.5 mL of sodium carbonate Na_2CO_3 (at 20% concentration). Subsequently, the mixture was incubated in dark conditions, estimated around 30 minutes to 40 minutes for the development of colour. The absorbance value was measured at 725 nm, taken after the incubation time ended. The total phenolic content was expressed as equivalent to gallic acid (mg/GAE/g) of the extract by using the standard curve of gallic acid. The standard curve of gallic acid can be referred to in Appendix B.

3.3.1.3 Total Flavonoid Content Estimation

For estimating the total flavonoid, in a separate container, 1 mL extract of *E. americana* bulb and root segments were diluted separately in 200 µL of distilled water, then 150 µL of sodium nitrate, $NaNO_3$ (at 5% concentration) was added and incubate for 5 minutes before adding 150 mL of aluminium chloride, $AlCl_3$ (at 10% concentration). The mixture was incubated for another 6 minutes. Finally, 2 mL of sodium hydroxide, NaOH (at 4% concentration) and distilled water were added to form a total volume of 5 mL. The mixture was then allowed to settle for 15 minutes at room temperature before taking the absorbance reading at 510 nm. The data was represented as Quercetin (mg QE/g) extract using the standard curve on a dry weight basis. The standard curve of Quercetin can be referred to in Appendix B.

3.3.1.4 Total Alkaloid Content Estimation

To perform an estimation of the total alkaloid content, in a separate container, 5 g of plant sample in powder form (bulb and root) was added to 200 mL of acetic acid, and the mixture was left steeped for 4 hours. Following that, both mixtures were filtered and immersed in the water bath until the mixtures were reduced to one-quarter of their original volume. A few drops of concentrated ammonium hydroxide were added resulting in precipitation. The ammonium hydroxide was continuously added onto the mixture until precipitation was completed. The mixture was filtered, and the precipitate was collected and allowed to dry. Once dried, the weight and total alkaloid content were calculated using the following formula:

$$\text{Percentage (\%) of alkaloid content} = \frac{\text{Weight of residue/precipitate}}{\text{Weight of sample taken}} \times 100\%$$

3.3.2 Phytochemical Qualitative Analysis

3.3.2.1 Analysis of Flavonoids

For flavonoids analysis, then 2 mL of 2.0% Sodium Hydroxide (>98% pellet, Sigma Aldrich reagent grade) were added to the diluted plant extract. Sodium hydroxide solution produced an intense yellow colouration when it was mixed together with the plant extract. After the

mixture has completely incorporated, 2 drops of diluted acid were added to the mixture. Flavonoids are present in the plant extract, when the bright yellow mixture turns colourless.

3.3.2.2 Analysis of Terpenoids

In the estimation of terpenoids, 1 mL of plant extract was prepared and then 0.5 mL chloroform was added (>99% solution, Sigma Aldrich analytical grade). Concentrated sulphuric acid (95.0-98.0%, Merck ACS reagent) was titrated to the mixture one drop at a time. The mixture was monitored for the formation of a reddish-brown precipitate indicating the presence of terpenoids.

3.3.2.3 Analysis of Glycosides

The phytochemical analysis for glycosides required the preparation of 5 mL of plant extract of root and bulb separately, then 2 mL of acetic acid (>99%, Sigma Aldrich ACS reagent) and 2 mL of chloroform (>99% solution, Sigma Aldrich analytical grade) was added into the plant extract. The mixture was swirled gently. The combination of this solution produced heat, therefore letting the mixture rest at room temperature to cool down. Once the mixture has cooled down, a concentrated sulphuric acid (95.0-98.0%, Merck ACS reagent) was added dropwise. When the mixture developed a green-coloured solution, it suggests the presence of glycosides in the plant extract.

3.3.2.4 Analysis of Steroids

In order to analyse the presence of steroids, 5 mL of plant extract from each bulb and root plant segment was prepared by dissolving it with distilled water. Once the mixture was completely diluted, 2 mL of chloroform (>99% solution, Sigma Aldrich analytical grade) and 2 mL of concentrated sulphuric acid (95.0-98.0%, Merck ACS reagent) were added to the mixture and left to sit for 10 minutes. The observation was then made for the formation of a red-coloured layer indicating the presence of steroids.

3.4 Cell Culture Preparation

3.4.1 2D Cell Culture Model

Normal transformed breast cell lines MCF-10A were grown in DMEM F-12 media supplemented with 5% (v/v) Horse Serum, Insulin 10 mg/mL,

EGF 20 ng/mL, Hydrocortisone 0.5 mg/mL, cholera toxin 1 mg/mL and 100 µg/mL Streptomycin/Penicillin. MRC-5, a normal lung fibroblast cell, and MCF-7 breast cancer cells were cultured in RPMI supplemented with 10% FBS, 1% Penicillin/Streptomycin, and 0.5% Amphotericin B. For invasive breast cancer cells, the MDA-MB-231 culture medium used was DMEM (High Glucose) supplemented with 10% FBS, 1% Penicillin/Streptomycin, and 0.5% Amphotericin B.

The media was replaced every 3-4 days and the cells were passaged upon reaching a confluency of 70% to 80%. The passaging procedure started with old media being aspirated and the cells rinsed with phosphate buffer saline (PBS). After the PBS was discarded, trypsin (0.05% (w/v)/EDTA) solution was added and the trypsinized cells were incubated for 5 min in incubator at 37 °C. Trypsin was then inactivated by adding an equal amount of the appropriate fresh cell culture media. Cells were collected by centrifugation (Kubota) at 1000 rpm for 5 minutes and seeding was done in a T25 flasks. The flasks were then incubated at 37 °C and 5% CO_2 (CelCulture CO_2 ESCO)

3.4.2 Cell Counting

After the trypsinization protocol for cell culture was completed, the cell suspension obtained was placed in a 15 mL Falcon tube for centrifugation at 1000 rpm for 5 minutes. The pellet was then resuspended in fresh cell culture media at a ratio of 1:5. Ten microliters of the cell suspension were then loaded on both counting chambers of a hematocytometer (Neubauer, 0.0025mm^2). The viable cells (bright coloured) were then counted under the microscope (Olympus CKX53) at 40x magnification. The total viable cell number can be calculated using the formula below:

$$\text{Total of viable cells} = \frac{\text{\# viable cells calculated} \times 10^4}{\text{Dilution volume}}$$

3.4.3 Preliminary Cytotoxicity Screening on 2D Cell Culture Model

Preliminary screening for cytotoxicity of the plant extract of the bulb and root segment was performed on a monolayer (2D cell culture model) separately. Cells were seeded at 3 x 10^4 in a 96-well plate, incubated for 24 hours prior to treatment with plant extract at six (6) initial concentrations starting from 1.56 µg/mL, 3.13 µg/mL, 16.25 µg/mL,

12.5 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL. Three (3) time intervals were applied for the procedure at t 24, 48, and 72 hr. MTT assay was used to determine the percentage of cell viability after treatment with the plant extract. Untreated cells and DMSO-treated cells were used as the negative controls, and cells treated with Cisplatin were used as the positive control. Cisplatin is a well-known chemotherapeutic drug that has been used to treat a number of human cancers including breast cancer. The ability of Cisplatin to form a crosslink with the purine bases of DNA causes interference in the DNA repair mechanism and ultimately induces apoptosis in cancer cells. Cisplatin was chosen as a positive control in this study due to its versatility and easy access as it is commonly used in the laboratory research setting**.** The IC_{50} values for cell viability after the plant extracts treatment was determined using Graph Pad Prism 9®.

3.5 3D Spheroids Formation

After the determination of the IC_{50}, further assays were performed using a 3D spheroids model which includes the alamarBlue™, double staining, invasion assay and H&E staining. The procedure used to form spheroids was the hanging drop technique and followed by the use of agarose-coated 96 well plates (promoting low attachment of cells). The agarose coating should allow spheroids to form as it generates low cell attachment and promotes cells to clump and form spheroids, producing a single spheroid in each individual well of 96 well plate. The procedure requires seven (7) days to form spheroids of at least 300 µM in size in order to mimic an *in-vivo* environment via micrometastases; thus, they can recapitulate certain characteristics of an *in-vivo* tumour tissue, such as oxygen gradients and tumour proliferation (Huang, Yu, & Tang, 2020; Vinci et al., 2013)

3.5.1 Single Cell Suspension Preparation

Once the cultured cells reach 90% confluency, the cells were washed with PBS, trypsinized, and incubated at 37 °C and 5% CO_2 until all cells were detached. Trypsin was neutralized with fresh media, and cells were centrifuged at 1000 rpm for five (5) minutes. The supernatant resulting from the centrifugation was discarded, fresh media was added, and the pellet was resuspended gently until no cell clumping was observed. Using the haematocytometer, the number of viable cells was calculated. From the calculated number of viable cells, single-cell suspension was prepared at a concentration of 1.0×10^4. The same method was used to prepare both MRC5 and MDA-MB-231 single-cell suspensions. For co-culture (MRC5 + MDA-MB-231), the ratio of 3:1 (3 parts cancer cells: 1-part fibroblasts) was used to produce spheroids.

3.5.2 One Per Cent (1%) Agarose Preparation and 96-Well Plate Coating Procedure

For the preparation of 1% agarose, 0.01 g agarose powder (>98% powder, Sigma-Aldrich) was weighed and diluted with distilled water. The mixture was then gently stirred to prevent clumping and subsequently autoclaved to sterilise the mixture. A total of 50 µL of warm agarose was pipetted into each individual well of 96-well plates after the temperature of the sterilised agarose has cool down to 60°C or 50°C . The agarose was allowed to solidify at room temperature. Each agarose-coated well-plate can be stored at 4 °C for up to two (2) weeks. To prevent precipitation in well plates, the agarose-coated 96 well plates were left to sit until the agarose was completely solidified and cooled down to room temperature before refrigeration.

3.5.3 Hanging Drop Technique

This technique applied the use of a Tissue-Culture-Treated Dish (Corning). Prior to cell seeding, 10 mL of PBS was added to the bottom of the Tissue-Culture-Treated Dish to act as a hydration chamber. The upper lid of the culture dish was placed in an inverted position and 20 µl drops of the previously prepared single-cell culture suspension were pipetted, ensuring that every single drop was sufficiently apart from each other; a maximum of 20 to 30 drops were recommended in one Tissue-Culture-Treated dish. After completing the procedure, the lid was carefully inverted onto the bottom chamber and incubated for 72 hr at 37 °C and 5% CO_2. After 72 hr, the cells will start to aggregate and form a plate-like structure. The aggregated cells were then transferred to a 96-well microplate ULA (ultra-low attachment) or 1% agarose coated and incubated for an additional 4 days to allow the spheroids to reach the desired size of 300 µm.

3.5.3.1 Spheroids Formation (Monoculture)

Using the hanging drop technique, MDA-MB-231 cells were seeded at an initial concentration of 1 x 10^5 in total cells per drop. The initial incubation period was at 72 hours. The mono-culture spheroids were then harvested at 72 hours as the cells start to aggregate or form cell sheets and transferred into a 96 well-plate coated with 1% agarose and cultured for another 4 days. On day 7, the spheroids were harvested for further treatment with the plant extract or fixed with 4% PFA for histology staining and immunohistostaining.

3.5.3.2 Spheroid Formation (Co-Culture)

For co-culture spheroids formation, the cells were seeded at a ratio of 3:1 of cancer cells (MDA-MB-231) to normal fibroblast cells (MRC5) to a final concentration of 1 x 10^5 in total cells per drop. The initial incubation period was at 72 hours. The spheroids were then harvested and transferred into a 96 well-plate coated with 1% agarose and cultured for another 4 days. On day 7, the spheroids were harvested for further treatment with plant extract or fixed with 4% PFA for histology staining and immunohistostaining.

3.6 Cytotoxicity of Plant Extract towards 3D Spheroids

Four (4) different concentrations were used to assess the toxicity of the plant extract toward the 3D spheroids. As mentioned earlier, the cell spheroids used were MDA-MB-231, MRC5 (monoculture), and. co-culture spheroids of MRC5 and MDA-MB-231. The IC_{50} values obtained from the preliminary cytotoxicity screening for the 2D cell culture were used as the lowest concentration at 25 µg/mL, followed by 50 µg/mL, 100 µg/mL, and 200 µg/mL. Based on the cytotoxicity screening results on the 2D cell culture model, the time taken to obtain the IC_{50} was at 72 hours after treatment, hence the same time frame was also applied for the 3D spheroids study. The cytotoxicity of the plant extract on the spheroids was analysed using the alamarBlue™ assay.

3.6.1 Alamar Blue® Assay Analysis

After 72 hours of incubation with the plant extract (in a 96-well plate),

About 100 µl of old media was decanted from each well. Then, add 20 µl of Alamar Blue® solution at 0.15 mg/mL concentration was added to each well and incubated for 4 hours at 37 °C. Absorbance was taken using Synergy H1Multi-Mode Reader (Biotek) at 570 nm, and 600 nm was used as a reference. Reference wavelength data collection was necessary for the calculation of the reduction of the Alamar Blue® assay. The percentage of cell viability of the spheroids was determined using the formula given below:

Percentage of reduction of Alamar Blue

$[A_{LW} - (A_{HW} \times R_o)] \times 100\%$

where

A_{LW} = absorbance at lower wavelength minus the blank media
A_{HW} = absorbance at higher wavelength minus the blank media
R_o = correction factor

Correction factor calculation: $Ro = AO_{LW} / AO_{HW}$

where

AOLW = absorbance of AB in media - absorbance of media only
AOHW= absorbance of AB in media - absorbance of media only

Data is presented in bar charts and analysed using Graph Pad Prism 9® software.

3.7 Transformation *In-vitro* Normal Fibroblast as Cancer Associate

Based on previous research and histological studies, fibroblast cells that are found surrounding the cancer cells are fibroblasts that are associated with the cancer cells, consequently enhancing the survival of cancer cells in the host (Chandra Jena et al., 2021). The lung is an organ which is known to have an abundant number of fibroblasts (Ding et al., 2019). One of the resident fibroblasts is MRC5, which is also commonly used in the laboratory setting to study the interaction of normal fibroblasts with cancer cells. Breast cancer is also known to commonly spread to the lung (Lin et al., 2023). With the abundance of fibroblasts in the lung- the breast cancer cells are able to progress invasively through the secretion of growth factors, depositing ECM and recruiting cancer-associated macrophages (Ding et al., 2015). This interaction goes bidirectional, cancer cell interaction with normal fibroblasts facilitates the activation of the transformation of fibroblasts into CAFs (Awaji et al., 2019). Previous literature study identifies lung-specific immunosuppressive fibroblasts commandeer by breast cancer cells to facilitate metastasis (Gong et al., 2022). Cancer cells via paracrine signalling transform normal surrounding fibroblasts into affiliates known as Cancer-Associated Fibroblasts (CAFs). This research attempts to mimic the association of normal fibroblast (MRC5) with breast cancer cells *in-vitro* by three (3) methods, indirect co-culture, direct co-culture, and culture with conditioned media from an invasive breast cancer cell line (MDA-MB-231).

3.7.1 Conditioned Media Preparation

To prepare conditioned media, MDA-MB-231 cells were cultured in a T75 flask and incubated at 37°C with 5% CO_2 until it reaches 80% confluency. Once confluent, the old media was discarded, and the cells were washed with PBS and changed the culture media to 50% RPMI + 50% DMEM (with serum) for 24 hours. After 24 hours, the cells were rinsed again with PBS, and fresh media of 100% RPMI (blank/ without serum) was added and incubated for another 24 hours. The RPMI media (blank/ without serum) was collected after 24 hours and the media was syringe filtered for sterilization and stored at -80°C until further use. The conditioned media can be kept for up to 2 weeks.

3.7.1.1 Cultured with Conditioned Media (CM)

For this procedure, $1x10^4$ of MRC5 were seeded and cultured in a 6-well culture plate, then incubated for 24 hours to stabilize the growth. After 24 hours, the cells were then rinsed with PBS and fresh complete growth media RPMI with serum was added together with the conditioned media as a supplement at the ratio of 1:1 (v/v). The cells were incubated at 37 °C with 5% CO_2. The incubation was timed and observed at time frames of 24 hr, 48 hr, 72 hr, and seven (7) days. The morphological changes were observed on a daily basis. After 7 days, Immunocytochemistry (ICC) was carried out to examine the transformation of MRC5 into cancer-associated fibroblasts.

3.7.1.2 Indirect Co-Culture

Trans-well with a pore size of 3 μm was used in this method. The MRC5 cells were seeded at the bottom part of the well at a concentration of 1×10^4 total cells per well. Meanwhile, the MDA-MB-231 spheroids were deposited in the trans-well insert at (3:1 ratio) which was at 3×10^4 total cells per well. The trans-well was then incubated at 37 °C with 5% CO_2 for seven (7) days. The regular media was changed every three (3) days to ensure optimal cell proliferation. On day 7^{th}, Immunocytochemistry (ICC) was carried out to investigate the transformation of MRC5 into cancer-associated fibroblasts.

3.7.1.3 Direct Co-Culture

For the direct co-culture method, MDA-MB-231 and MRC5 cells were seeded together in the same well (at a 3:1 ratio) on a 6 well-plate. The total cells per well for the MDA-MB-231 was 3×10^4 and at 1×10^4 for

the MRC5. The plate was then incubated at 37 °C with 5% CO_2 for seven (7) days. The media was changed every three (3) days. Similar to the other co-culture methods, on day 7th, Immunocytochemistry (ICC) was carried out to investigate the transformation of MRC5 into cancer-associated fibroblasts

3.7.1.4 3Immunocytochemistry (ICC)

This procedure was performed to validate the transformation of normal fibroblasts to cancer-associated fibroblasts (CAFs). Transformed fibroblasts could be detected through specific markers; one of which is α-Smooth Muscle Actin (α-SMA)(Awaji et al., 2019). On day 7, the cells were fixed with cold 4% PFA prior to incubation for 20 minutes at room temperature. After 20 minutes of incubation, the cells were then rinsed twice with PBS, followed by rinsing the cells with wash buffer twice (wash buffer; 0.1% BSA in 1x PBS). To block non-specific staining, a blocking buffer (10% FBS + 0.3% TRITON® X-100) was subsequently added and followed by incubation for another 45 minutes at room temperature. The blocking buffer was aspirated after 45 minutes, and immediately add the diluted Human alpha-Smooth Muscle Actin Alexa Fluor® 488-conjugated Antibody (R&D System, catalogue # IC1420G) was added. To complete the procedure, the cells was incubated for an hour at room temperature. For optimization, an overnight incubation time at temperatures 2 °C to 4 °C was also applied.

Prior to visualization under a fluorescence microscope (Zeiss AxioVert A1 FL-LED), the diluted DAPI was incorporated for counterstaining. Data was collected in the form of fluorescence images. The fluorescence wavelength was set at Excitation wavelength = 488 nm & Emission wavelength= 545 nm in accordance with the Human alpha-Smooth Muscle Actin Alexa Fluor® 488-conjugated Antibody (R&D System, catalogue # IC1420G) protocol sheets.

3.8 Invasion Assay on 0.1% Gelatine

Cancer cell invasion contributes to its progression from the primary site to the distal organs. In this invasion assay, an analysis of the effect of *E. americana* towards the cancer cell spheroids invasion on gelatine, an extracellular matrix (ECM) protein was performed to further analyse the *E. americana* ethanol extract anti-cancer properties. The assay utilizes gelatine (Nacalai Tesque, EP grade cat#16605-55) as an extracellular matrix (ECM) protein coating to initiate an invasion of MDA-B-231 monoculture and co-culture.

3.8.1 Gelatine (0.1%) Coating in 96 Well-Plate

For the ECM coating procedure, 0.1% (w/v) of gelatine powder was prepared by diluting it with double deionized water (ddH_2O) and the mixture was then autoclaved to ensure all the powder was dissolved entirely and sterilized the mixture. The mixture was then cooled down to 40 °C before dispensing 50 µL of gelatine mixture into each well in 96 well-plate. The side of the well plate was then gently tapped for an even distribution of the gelatine. Then the plate was incubated at room temperature until the gelatine solidifies. The wells were then washed twice with cooled PBS and blocked with 1% BSA in PBS, and further incubated for another 1 hour at room temperature. Finally, proceed with the invasion assay or the well plates can be stored in a 4 °C chiller for up to 2 weeks.

3.8.2 Invasion Assay

For this procedure, a total of 200 µL of complete growth media was added into each well of gelatine pre-coated 96 well plates. Then, 100 µL volume of 4-days spheroid was transferred in into each of the ECM pre-coated well. The plates were then incubated for 30 to 40 minutes to allow the spheroids to adhere to the gelatine coating. The observation of invasion started at 0, 24, 48 and 72 hours. Untreated spheroids were used as negative control and the spheroids treated with Cisplatin were used as a positive control. The treatment was applied on day 0. The data was collected in image format and was viewed using a bright-field microscope (Zeiss AxioVert A1 FL-LED). The surface area of invasion was later calculated using the given formula.

$$\text{Surface area of invasion} = \frac{X}{\text{Invasion area at t=0}} \times 100$$

X = surface area of invasion at t (24, 48 and 72 hr)

3.9 Histology Staining Hematoxylin and Eosin (H&E) Staining

This protocol was carried out to examine the structure of spheroids of MDA-MB-231 cancer cells that were treated with the extract of *E. americana* compared to untreated MDA-MB-231. The H&E staining was applied to detect any morphological changes in monoculture spheroids before and after treatment with E. *americana* ethanol extract. However,

the spheroids formation method used in this study produced fragile spheroids that needed a special preparation procedure before histology staining (H&E). An agarose array method was developed to optimize sample collection and overcome the fragility of the spheroids.

3.9.1 Cast for Agarose Array

The cast was designed to construct an agarose array that overcame the problem posed by the fragility of spheroids produced in this study. The blueprint of the cast was constructed using OpenSCAD software and the cast was printed with a 3-D SLA printer model Creality Id002h. ePLA+ was used for the material. The size of each spike was customized to 2 mm in diameter, and the peak of the spike (cone shape) diameter was set to 1 mm in dimension. The spike's dimension created a well in an agarose gel that allows the loading of a spheroid with a size up to 2 mm in diameter in each well. Cast customization is shown in 4 (four) different angles (Figures 3.1, 3.2, 3.3 a and 3.3 b)

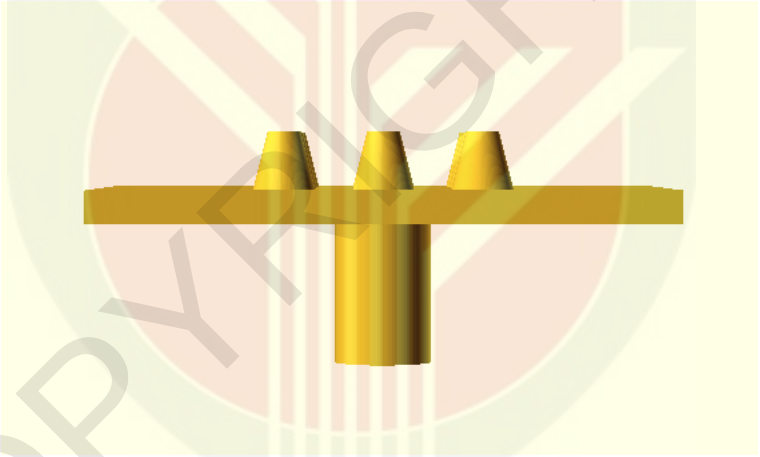


Figure 3.1 : Agarose Array Cast Side View. The side view of the cast. The cast is suitable for histology stainless steel tray at 30 x 24 x 7 mm

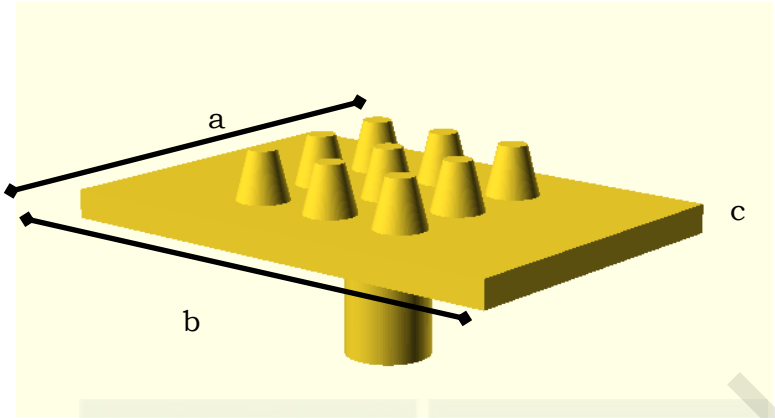


Figure 3.2 : Agarose Array Cast Z-Plane. The z-plane view of the cast. The overall dimension of the cast (a) 24 mm, (b) 35 mm, (c) 2 mm

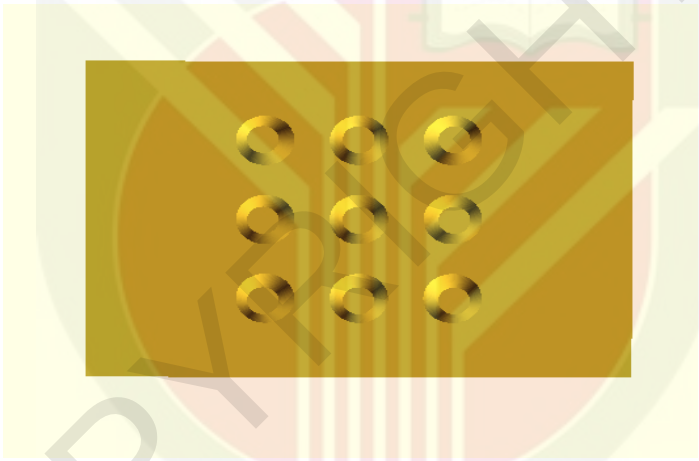


Figure 3.3a : Agarose Array Cast Top View. The top view displays the 9 spikes used to form well(s) on the agarose array

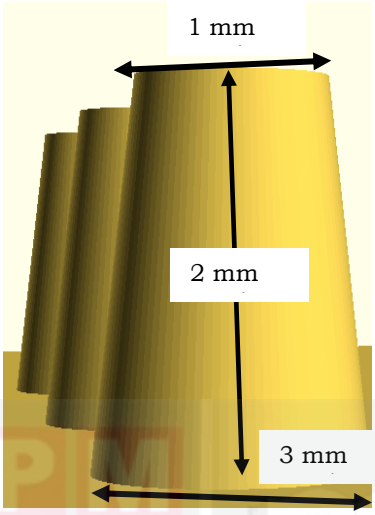


Figure 3.3b : Agarose Cast Spike(s) View showing the close-up structure of the spikes. The spikes' dimensions are 2 mm in diameter at the bottom of the spike(s) and 1 mm on the top of the spike(s) and the height of the spike(s) was set at 3 mm each

3.9.2 Agarose Array and Spheroids Sample Preparations for H&E Procedure

Using a microwave oven, a 2% agarose mixture was prepared. Once the agarose is completely dissolved, 3 mL of warm agarose was then poured onto a histology slide of size 35 x 24 x 7 mm. The agarose cast was then immediately placed on the warm agarose and gently pressed to prevent bubble formation and allowed it to solidify at room temperature. The cast was removed gently once the agarose had solidified completely. The fixed spheroids were carefully pipetted into each well. To secure a proper collection of spheroids, the top section of 100 µL tips was cut prior to usage.

The bottom of the histology tray was tapped gently to ensure the spheroids sank to the bottom of the well. Before the 'seal' were added to the agarose array, 10-minute intervals were applied for the spheroids to completely sink to the bottom of the well. In order to seal the spheroids inside the agarose array, a 1% agarose mixture was prepared using the microwave oven. The warm agarose mixture was added gradually from the side of the histology slide to avoid the spheroids dispersing from the bottom of the well. Once solidified, the agarose array was removed from the histology slide and transferred to the histology cassette. The agarose array was then placed in the tissue processor overnight (Leica ASP300s).

3.9.3 Agarose Array Sectioning

After the agarose array completed the treatment cycle in a tissue processor for overnight, the agarose arrays were then sectioned in 4 µm thickness using a fully motorized microtome (Leica RM2255). The sectioned film was then placed onto a poly-lysine-coated slide and allowed to dry before conducting the H&E staining procedure.

3.9.4 H&E Staining Protocol

The H&E staining was applied with a few routine modifications. The modifications involved the dipping step at 3 minutes during the Hematoxyline and eosin dye step. In a normal routine of H&E, the dipping step takes 5 minutes. The slides which have completed the H&E staining were then visualized using a light microscope (Olympus BX53) at 20 x, and 40 x magnification. Data was collected are in image format.

3.10 Methods of Computation and Data Representation

The calculations of raw data for the MTT assay and calibration graphs for this study were performed using Microsoft® Excel® 2019. Statistical calculations were performed using Prism 8®, which automatically constructed a graph and provided statistical analysis calculation options. The ANOVA and independent t-test was applied in determining the significance of treatment of *Elutherine americana* towards monoculture and co-culture of MDA-MB-231 (+MRC5). Analysis was performed in triplicates and mean ± SD of three parallel measurements. Data for invasion assay was collected in image format and analysed using ImageJ software. All data collected were then used to evaluate the effects of *E. americana* extract on cancer cell lines (MDA-MB-231 and MCF7) and its spheroids.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Estimation of Phytochemical Compounds

Phytochemicals are the by-product of photosynthesis; they can be categorised into 3 main components: primary metabolites, secondary metabolites, and biopolymer (Thiagarajah et al., 2019). Since *E. americana* is categorised in the *Iris spp.*, both bulb and root segments of the plant species have been documented to contain valuable sources of phytochemicals compounds (Singab et al., 2016). The properties of the phytochemicals compound that were measured include terpenoid, flavonoid, glycoside, and steroid, which are catalogued as polyphenol compounds. The phytochemicals display important roles as antioxidant, antibacterial, anti-inflammatory, anti-allergic, antispasmodic, chemo-preventive, hepato-protective, hypolipidemic, neuroprotective, hypotensive, immuno-modulator and carminative agents; prevent ageing, diabetes, osteoporosis, cancer, and heart diseases; induce apoptosis; act as diuretic, CNS stimulant, analgesic; and protect from UVB-induced carcinogenesis, and many more (Thakur et al., 2020).

A qualitative and quantitative phytochemical assay aims to reveal the presence or absence of a phytochemical with pharmacological benefits in the plant of interest (Egbuna et al., 2019). The qualitative phytochemical results that established the presence of a beneficial pharmacological benefit compound propose the anti-cancer potential of the plant. Further quantification of the presence of pharmacological compounds was performed through quantitative analysis to provide more comprehensive and more useful results to establish the anti-cancer potential of the plant of interest, the *E. americana.* The application of the qualitative phytochemical in this study facilitates determining the reactive segment of *E. americana*. To further support the finding from the qualitative analysis, quantification of the pharmacological compound of *E.americana* were performed and the results were compared to existing literature. Both qualitative and quantitative preliminary phytochemical analyses contribute significantly to this study.

Eight (8) parameters were measured for the phytochemical assessment assay. It comprises four (4) quantitative and four (4) qualitative procedures. The antioxidant property of the plant extracts was evaluated in terms of their total phenols (TP), total flavonoids (TFA), total flavanols (TFO), phenolic acids, catechins, lignans, and tannins (Cai et al., 2004; Djeridane et al., 2006). Tabulated below are the data obtained from the qualitative evaluation of both the root and bulb of *E. americana.*

Table 4.1 : Preliminary Qualitative Phytochemical Evaluations of the Bulb and Root Segments of *E. americana*

Phytochemical assay	Ethanolic extract	
	Bulb	Root
Terpenoids	++	-
Flavonoids	++	+
Glycosides	+	-
Steroids	+	-

(++) highly present, (+) moderately present, (-) absent

The qualitative evaluations revealed that the bulb segment of *E. americana* exhibited the presence of secondary metabolites; terpenoids, flavonoids, glycosides, and steroids, which are denoted by the (+) symbol. These qualitative tests were performed using the standard analytical technique. Observation of colour changes and the formation of precipitation denote a positive response to the presence of the targeted chemical compounds. The (++) symbol indicates a higher amount of precipitation or colour changes exhibited by the plant segment tested. As shown in Table 4.1, the root segment of the plant displayed the presence of flavonoids out of four of the phytochemical compounds tested. Whereas the bulb segment of the plant displayed the presence of all phytochemical compounds of interest. The results can be further supported by a previous study by Kuntorini & Nugroho in 2009, which presented data that established the bulb segment of *E. americana* extract to contain an array of secondary metabolites. Based on the qualitative results, it can be determined that the plant's bulb segment is the reactive part of the plant compared to the root segment. Hence, the bulb segments of *E. americana* are expected to have potential pharmaceutical benefits.

To further support the finding of the bulb segment reactivity, quantitative phytochemicals assays were also performed in this study to compare the bulb and the root segments. The data was presented in Table 4.2 below:

Table 4.2 : Quantitative Phytochemical Assays of *E. americana*

Phytochemical assay	Ethanolic extract	
	Bulb	Root
Total Phenolics	151.13 ±0.027[1]	73.31 ± 0.002 [1]
Total Flavonoids	22.02 ± 0.005[2]	18.48 ± 0.002 [2]
Total Alkaloids	2.26 mg ± 0.381/50 g	1.52 mg ± 0.419/50 g

Values are performed in triplicate and represented as mean±SD.[1]mg GAE/g extract, [2]QE/g of flavonoids in dry sample.

4.1.1 Total Phenolics (TP) Content

Referring to Table 4.2, the evaluation of the total phenolic (TP) content is expressed using the standard calibration curve of Gallic Acid (GAE) a standard phenolic acid (Chandra et al., 2014). All data were collected in triplicate and represented as mean ± SD. The presence of phenolics compound in the plant extract suggests the ability of the *E. americana* plant extract to act as a potent antioxidant and free radical scavenger. This allows the plant to have the ability to stabilize lipids against peroxidation and inhibit numerous types of oxidizing enzymes (Laughton, 1991). In this assay, the bulb segment of the plant exhibits higher content of total phenolic (TP) compared to the root part. As shown in the table, the total phenolic content in bulb segments was recorded at 151.13 ± 0.027 mg GAE/g plant extract and the total phenolic content in the root segment was recorded at only 73.31 ± 0.002 mg GAE/g plant extract.

Very limited research was found on the total phenolic compound of *E. americana* plant extract. In a recent study by Kamarudin et al., (2020), the total phenolic recorded for the bulb segment was 80.52 ± 1.30 mg GAE/g plant extract. The possibility of the differences in the total phenolic data could be attributed to the medium/soil composition where the plant was cultivated (Chandra et al., 2014), the age at which the plant was harvested (Kuntorini et al., 2016), and the method used for plant extraction (Zhang et al., 2011).

4.1.2 Total Flavonoids Content

The total flavonoid content (TF), is expressed using Quecertin's (QE) standard calibration curve graph (Bag et al., 2015). The result of TF comparing the bulb and root segments is shown in Table 4.2, at 22.02 ± 0.005 QE/g, the amount of (TF) in bulb segments is significantly higher compared to the root part of *E. americana* which is recorded at 18.48 ± 0.002 QE/g. All data were collected in triplicate and represented as mean ± SD. Based on the result of the total flavonoids assay, the bulb segment of the plant possesses higher bioactive compound properties compared to the root segment of *E. americana.* In previous literature, it was shown that flavonoids and phenolics are the major compounds that determine the potency of antioxidant properties of an herbal plant extract (Chandra et al., 2014).

Flavonoids have been recorded to possess important physiological properties towards human (Shi et al., 2019). High flavonoid content in a herbal plant extract is known to prevent oxidative damage to DNA, inhibit tumour cell growth and possess both anti-inflammatory and anti-microbial properties (Fernández et al., 2021). They are also

neuroprotective and possess a cardio-protective effect. Flavonoids can be classified into various types based on their chemical structure. Quercetin is a naturally occurring flavonoid found in common fruits such as oranges, berries, grains and green tea. Induce cell cycle arrest, increasing the apoptosis of cancer cells, regulation of signalling pathway, and inhibition of metastasis and angiogenesis were among the mechanism underlying the chemo-preventive properties of quercetin. In 2019, a study conducted by Shi recorded the TF of bulb of *E. bulbosa* was at 448.24 ± 5.89 mg CE/100 g dry weight, another study showed the total TF in *E. bulbosa* bulb was at 64.02 mg GAE/g dry weight (Kamarudin et al., 2020). The extraction method in the two previous studies was the main factor in the differences in TF content (Zhang et al., 2011). The application of different solvent polarity, solvent concentration, temperature assist extraction and solid-liquid ratio attributed to the difference in data recorded. Hence, the results from previous literature are not comparative as it was reported with different units and using different standard calibration curves.

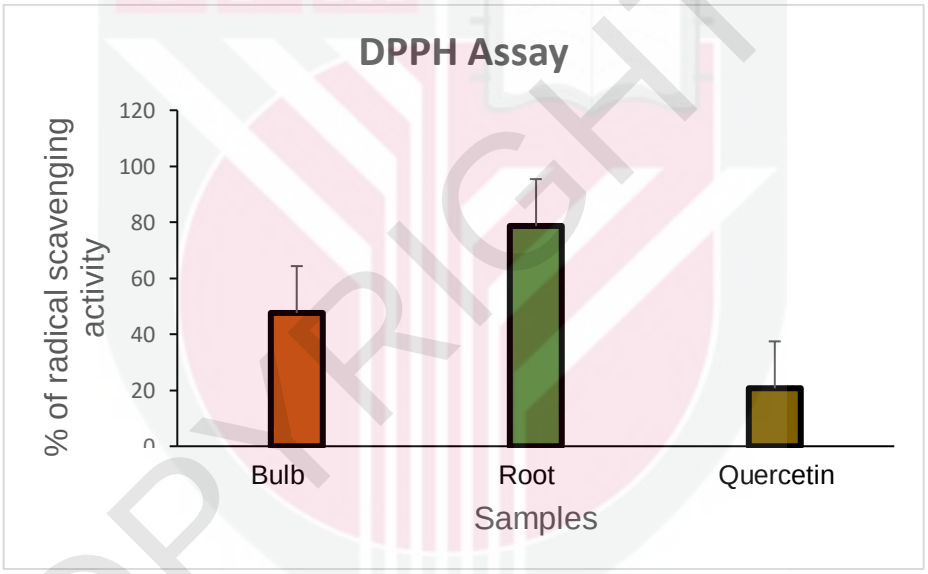
4.1.3 Total Alkaloids Content

In the evaluation of total alkaloids (TA) the TA content of the bulb, segment is at 2.26 mg ± 0.381/50 g of the dry sample and the root segment at 1.52 mg ± 0.419/50 g of the dry sample. Data collected were performed in triplicate and represented as mean ± SD. Alkaloids are defined as nitrogen-containing low molecular weight compounds which suggests it has major bioactive metabolites that have been reported to possess effective pharmacology activities. In the literature, alkaloids in plants have been recorded to have the ability to inhibit the topoisomerase activity (Baikar & Malpathak, 2010). To date, no specific study has been done on the evaluation of the alkaloid content of *E. americana* extract.

Secondary metabolites convey beneficial pharmacological activities that can be significant in the development of cancer chemo-preventive or adjunct cancer treatments (Mehta et al., 2010). A combination of chemo-drugs with natural products has been postulated to display similar effects as conventional chemotherapy drugs but with fewer adverse effects (Lin et al., 2017). Platinum-based chemotherapy drugs such as Cisplatin, Gemcibatine and Docetaxel cause severe unfavourable side effects during therapy such as renal dysfunction, myelosuppression and hearing loss.

4.1.4 *In-vitro* Antioxidant Activity (DPPH Scavenging Activity)

This assay evaluates the ability of the plant's antioxidant property to reduce the radical of DPPH at its IC_{50} (Senguttuvan et al., 2014). The antioxidant ability of plant extract to reduce the DPPH radicals by the IC_{50} is the parameter commonly used to measure the antioxidant activity of the plant (Yusri et al., 2012). The IC_{50} value in DPPH assay is the ability of the plant extract to reduce the concentration of the DPPH initial concentration to 50% reduction. A lower value of the IC_{50} indicates the effectiveness of the plant sample in scavenging the radical activity of DPPH, hence depicting the plant sample as a potent antioxidant. A synthetic antioxidant standard (Quercetin) was used in this evaluation as a comparison of the scavenging ability of the plant sample. The bar chart in Figure 4.1 summarizes the data obtained.



t-tests, $p<0.05$, data presented in mean ± SD: standard deviation

Figure 4.1 : *In-vitro* Antioxidant Activity (DPPH) of *E. americana* Extract. The bar graph shows the comparison between the percentage of DPPH radical scavenging activity in the bulb and root extracts as well as the synthetic standard antioxidant, Quercetin. At 47.60±0.06 µg/mL the bulb shows lower value of radical scavenging activity indicate it's as an effective antioxidant compared to the root segment which at 78.67±0.12 µg/mL. The synthetic standard antioxidant radical scavenging activity value is at 20.72±0.05 µg/mL

The results displayed the mean IC_{50} value of the bulb segment extract was lower compared to the root segment, indicating that the ability of the bulb segment extract of *E. americana* to scavenge the radicals of DPPH (2,2-diphenyl-1-picrylhydrazyl) is more effective compared to the root segment extract. The IC_{50} value was measured at 47.60±0.06 µg/mL compared to the root part at 78.67±0.12 µg/mL of the same plant.

Based on the preliminary qualitative and quantitative phytochemical evaluations performed on the root and bulb segments, it can be established that the bulb segment of *E. americana* recorded the presence of all the secondary metabolites measured; phenolic (TP), flavonoids (TF), alkaloids (TA), terpenoids, glycosides, steroids and the ability to scavenging the radical activity of DPPH. The (TP), (TF), and (TA) values of the bulb segments are significantly higher than the root segment as t-tests were used to determine the difference between means at ($p<0.05$) significant level. As discussed earlier, secondary metabolites have been recorded to have various therapeutic activities and also serve as an indicator of many pharmacological and biological activities. The molecular structure of these compounds enhances their pharmacological benefits such as anticancer activity through their metabolism, absorption, distribution and excretion capacities while minimizing the severe side effects and toxicity (Muhammad Zeeshan et al., 2022). The phytochemical assay results demonstrate the plant extract is potentially beneficial or not to be developed as a health remedy (Gul et al., 2017). Hence, from the data collected, the bulb segment of *E. americana* can be concluded to be the reactive part of the plant and was used for further analysis.

4.2 Cytotoxicity of Ethanol Extract of *E. americana* towards Cell Viability in 2D Cell Culture

Cytotoxicity assays were performed to evaluate the anti-cancer property of *E. americana* ethanolic extract towards both cancer and normal breast cells. In order to optimise the evaluation of the toxicity of *E. americana* extract 3 (three) treatment time frames were used: 24, 48 and 72 hours and 6 (six) different concentrations were applied: 3 µg/mL, 6 µg/mL, 12 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL. Using the Graph Pad Prism 9® software, the IC_{50} value for *E. americana* extract treatment on all cells was calculated. The cytotoxicity assay for this study was constructed based on the finding of the phytochemical evaluation of *E. americana,* since the preliminary phytochemical assay results earlier indicated that the bulb segments of *E. americana* extract yield beneficial phytochemical compounds. Hence, the bulb segment extract was chosen to assess the cytotoxicity of the plant extract. MTT assay was performed on normal cell lines (MRC5 and MCF-10) and on breast cancer cell lines (MCF7 and MDA-MB-231).

At 72 hours of treatment, the ethanol extract of *E. americana* exhibited significant cytotoxic activity on both breast cancer cell lines. The IC_{50} value obtained for the MCF7 cell line was at 25.17 ± 0.392 µg/mL, while the MDA-MB-231 cell line was at 25.23 ± 0.818 µg/mL. The National Cancer Institute (NCI) guideline has set the limit for the cytotoxic activity of the extract to be at IC_{50} < 30 µg/mL at the exposure time of 72 hours (Vijayarathna & Sasidharan, 2012). With this finding, the ethanolic extract of *E. americana* has proven to show cytotoxicity effects towards breast cancer cell lines. Figure 4.2 represents the percentage of cell viability obtained for the breast cancer cell lines.

To further investigate the anti-cancer properties of *E. americana ethanol extract,* a cell viability assay was also applied to normal cell lines; MRC5 and MCF-10A. Treatment of the *E. americana extract* on MRC5 and MCF-10A showed contrasting results compared to the cytotoxicity assay on MCF7 and MDA-MB-231. Remarkably, MRC5 and MCF-10A viability were not affected when treated with all 6 (six) concentrations of *E. americana* extract. For the plant ethanol extract to be classified as non-reactive the IC_{50} value must exceed 70 µg/mL according to NCI standard guidelines (Vijayarathna & Sasidharan, 2012). The IC_{50} values upon treatment on MRC5 and MCF-10A were 84.88 ± 0.411µg/mL and 81.18 ± 0.140 µg/mL, respectively. Hence, *E. americana* is naturally inactive towards healthy normal cell lines as it is generally used as an ingredient in daily food preparation, explained by the common name, "Bawang Hutan". Thus, with the initial findings, *E. americana* is an ideal natural source to be developed as an anti-cancer agent. Figure 4.3 shows the data on the percentage of inhibition in normal cell lines MRC5 and MCF-10A.

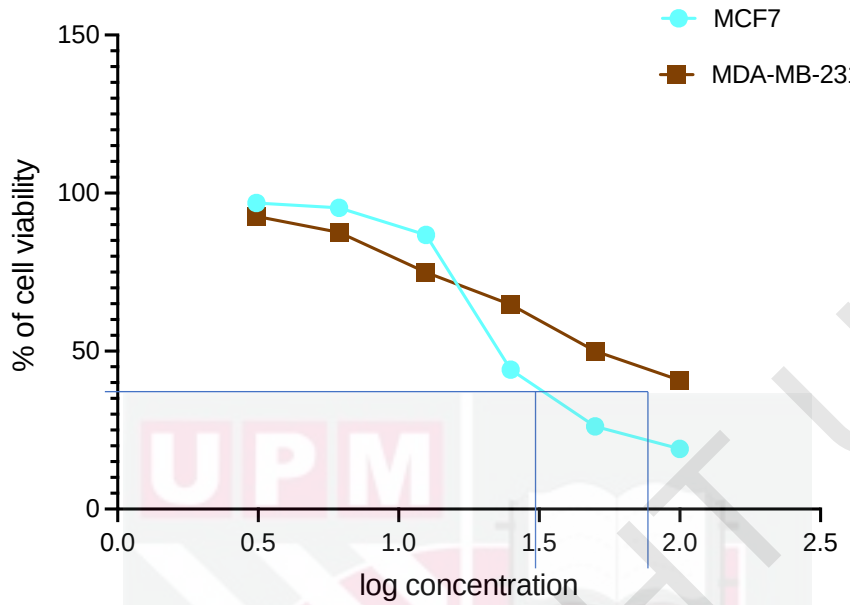


Figure 4.2 : MTT Assay Toxicity effects of *E. americana* ethanol extract against breast cancer cell lines MCF7 and MDA-MB-231 after 72 hours of incubation. Data are reported as mean ± SD

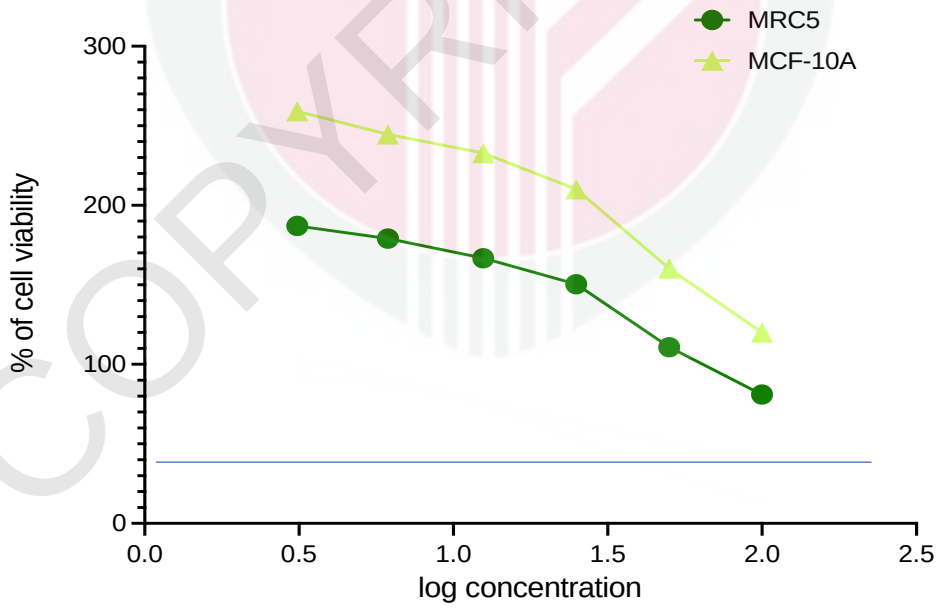


Figure 4.3 : MTT Assay. Toxicity effects of *E. americana* ethanol extract against normal cell lines MRC5 and MCF-10A after 72 hours of incubation. Data are reported as mean ± SD

Previous research publications have shown *E. americana* to have antimicrobial, antiviral, antihypertension, antidiabetic properties and that its antioxidant property is among the potent pharmacological activities of the plant (Insanu et al., 2014). However, there is limited data on its anti-tumour activity on breast cancer and hence, the groundwork of this study was based on the phytochemical/polyphenolic compound of *E. americana* established in the literature which suggests the anti-proliferative potential of *E. americana*. In this preliminary cytotoxic screening of the ethanol extract of *E. americana*, it can be established that the plant exhibits notable pharmacological activity; inhibiting the proliferation of breast cancer cells. Results of the cytotoxicity evaluation against MRC5 and MCF-10A show no significant effects on the proliferation of each cell line based on the IC_{50} values obtained. To further emphasize the difference in the cytotoxicity level of *E. americana* towards normal cell lines and breast cancer cell lines, Figure 4.4 represents merged IC_{50} data of all cell lines treated with the ethanol extract of *E. americana*.

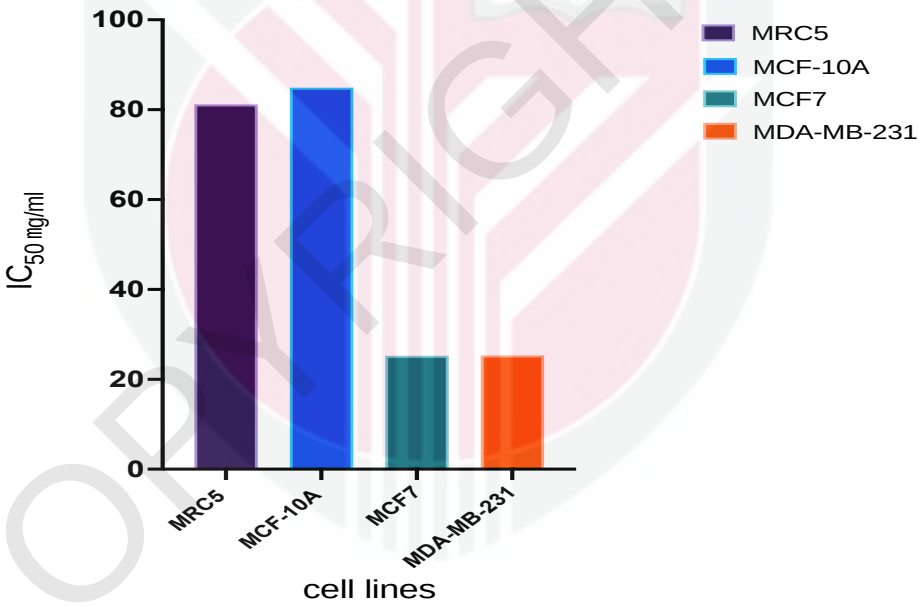


Figure 4.4 : Viability of the Different Cell Lines upon Treatment with *E. americana* Ethanol Extract. The graph shows that the viability of normal cell lines MRC5 and MCF-10A are not affected upon treatment with *E. americana* ethanol extract with IC_{50} values at 84.88 ±0.411 µg/mL for MRC5 and at 81.18 ± 0.140 µg/mL for MCF-10A. The viability of cancer cell lines however was inhibited upon treatment with the plant extract with IC_{50} values at 25.17 ± 0.392 µg/mL for MCF7 and 25.23 ± 0.818 µg/mL for MDA-MB-231. Data is expressed as triplicates; bar ± SD

4.3 Cytotoxicity of Ethanol Extract of *E. americana* towards the Viability of Cell Spheroids/ 3D Spheroids

The IC_{50} results from 2D cell culture model viability led to the testing of spheroids *in vitro* cell viability study. In 2D cell culture, it was determined that the concentration of IC_{50} at 25.5µg/mL produced significant effects on the breast cancer cells. To substantiate the results, the ethanol extract of *E. americana* was tested on 3D cell culture/spheroids. MDA-MB-231 was the breast cancer cell line used for the 3D cell culture viability evaluation. Known as an aggressive phenotype, possessing a high ability to metastasise through interaction with the microenvironment are the factors that led to the application of MDA-MB-231 in the 3D model cell culture (Huang et al., 2020). The viability assays were determined using a double staining assay (PI and Hoechst) for qualitative and Alamar Blue® for quantitative data. The commercially available Alamar Blue® is a redox indicator dye that measures metabolic activity and cellular viability, suitable for measuring spheroids cell culture (Eilenberger et al., 2018).

Two different assays were used in this study, the MTT assay for 2D cell culture viability assay and the Alamar Blue® assay to 3D cell culture cell proliferation assay. Both assays measured different aspects of the cancer cells' condition. The MTT assay was used to measure the number of living cells after treatment with the plant extract (Özkaya & Geyik, 2022). Viable cells catalyse the MTT salt reduction by mitochondrial succinate dehydrogenase, thus it is dependent on the mitochondrial respiration (Chacon et al., 1997). The IC_{50} calculated indicate 50% of cell death due to treatment being applied. The MTT assay was applied as a preliminary evaluation of the cytotoxicity of the plant of interest, *E. americana* towards breast cancer cells. The result of the MTT assay in this study was 25.5 µg/mL, ascertaining the cytotoxicity activity of *E. americana* towards breast cancer cells. The Alamar Blue® assay quantifies the proliferation of breast cancer cells in spheroids form. It is less toxic compared to MTT salt, Alamar Blue® measure multiple metabolic reactions which signifies the adaptability of the assay and it is more suitable to be used on spheroids. The IC_{50} calculated using the Alamar Blue® assay indicates a 50% reduction in cell proliferation due to cell death or inhibition in the proliferation rate of the spheroids (Bonnier et al., 2015).

4.3.1 Cytotoxicity of *E. americana* towards the Viability of Cell Spheroids/ 3D Cell Culture (Monoculture)

The preparation of 3D cell culture is described in Chapter 3; treatment started on the 5th day of culture to generate a uniform size of the suitable spheroids at 300 µm, an optimal size of a healthy ratio between

proliferating cells and necrotic cells due to lack of nutrient and oxygen supply to the centre of the spheroids. As this assay is a continuation of preliminary cytotoxic screening, it was found that the time frame for notable results obtained was at 72 hours of treatment. Hence, the monoculture of MCF7 3D spheroids and MDA-MB-231 3D spheroids treated separately used the same time frame, and the effects of treatment data effects were collected and represented in a bar chart. The percentage of reduction of resazurin in the Alamar Blue® assay was calculated and the IC_{50} for each monoculture spheroid was determined using Graph Pad Prism 9®. Based on previous literature, (Kheirandish-Rostami et al., 2020; Najafzadeh et al., 2022), the negative control also used in these assays was Cisplatin at 20 µg/mL. Figure 4.5 represents the inhibition of spheroid monoculture of breast cancer cells after being treated with four (4) concentrations of *E. americana* extract.

The viability of the spheroids was determined using the Alamar Blue® assay, and the viability was compared to the commercially available cancer drug Cisplatin at 20 µg/mL based on previous literature (Najafzadeh et al., 2022). A lower value of the percentage of resazurin reduction indicates higher cell death or a lower rate of cell proliferation for each treated spheroid (MCF7 and MDA-MB-231). The IC_{50} for each treatment of MCF7 spheroids and MDA-MB-231 spheroids with both *E. americana* and Cisplatin were calculated, and the value obtained was 37.90 ± 4.691 µg/mL for MCF7's spheroids and 48.72 ± 1.848 µg/mL on MDA-MB-231 spheroids. The results showed that the inhibitory concentration values were greater than the 2D culture, indicating the 3D cell culture of each breast cancer cell is more resilient towards the plant extract.

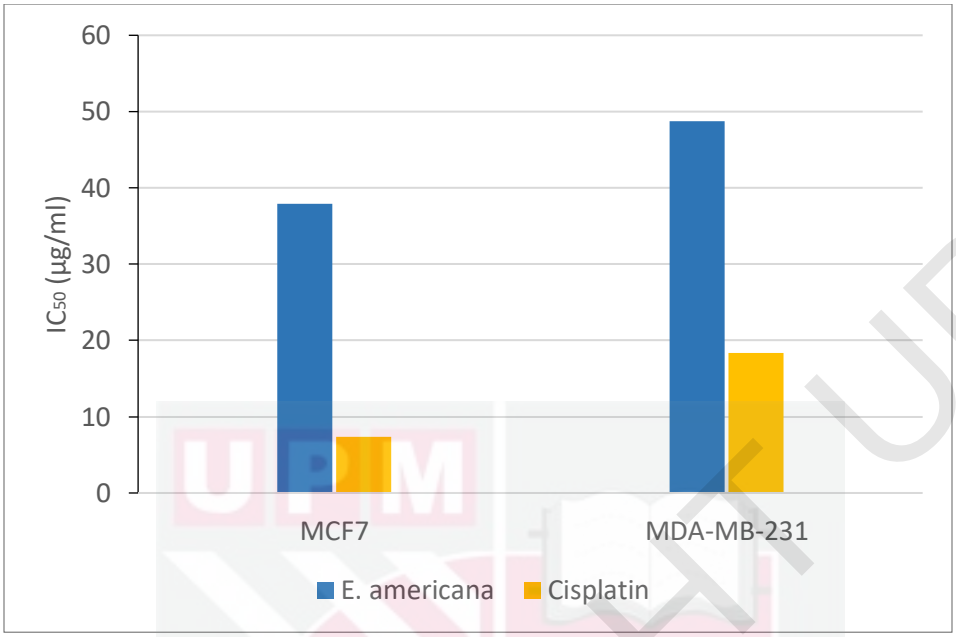


Figure 4.5 : Alamar Blue® Assay on Monoculture Spheroids. The bar graph shows the results of the comparison in IC_{50} values of cancer cell spheroids upon treatment with different concentrations of *E. americana* ethanol extract (25 – 150 ug/mL) and a commercial anti-cancer drug, Cisplatin. The IC_{50} value for MCF7 treated with *E. americana* is 37.90 ± 4.691 µg/mL and 48.72 ± 1.848 µg/mL for MDA-MB-231 treated with *E. americana*. The IC_{50} value for Cisplatin treatment at 20 ug/mL on MCF7 is 7.38 ± 7.309 µg/mL and MDA-MB-231 is 16.77 ± 1.391 µg/mL. Data were expressed as triplicates; bar ± SD

As mentioned earlier, according to the NCI guidelines (Vijayarathna & Sasidharan, 2012), for a compound to be inactive, the minimal inhibitory concentration must exceed 70 µg/mL; therefore, *E. americana* extract can still be categorized as reactive and possess the ability to inhibit the proliferation of breast cancer spheroids.

The 3D cell culture application in this study was employed to further validate the anticancer activity of *E. americana.* As an improved model for the tumour microenvironment, the spheroids developed enhanced cell-to-cell interactions and cell-to-matrix interactions, exhibiting differences in functional behaviour as such differentiation, proliferation, and gene expression (Cui et al., 2017; Huang et al., 2020). As discussed by the authors in the previous study, compared to 2D cell culture, colon cancer HCT-116 cells in spheroids form were discovered to be more resistant to anticancer drugs such as melphalan, fluorouracil, oxaliplatin, and irinotecan3; such chemoresistance has been observed

in vivo as well (Fang & Eglen, 2017). Therefore, 3D cell cultures enable better toxicity and efficacy prediction of plant samples' anticancer capability. In addition, spheroids can easily be cultured with different types of cells to produce co-cultured spheroids.

4.3.2 Cytotoxicity of *E. americana* towards the Viability of Cell Spheroids/ 3D Cell Culture (Co-Culture)

Co-cultured spheroids mimic cell-to-cell interactions between cancer and associated stromal cells; also known as multicellular spheroids. This study has also applied co-cultured spheroids to validate further the anticancer ability and potency of the *E. americana* extract. Fibroblast cells which are abundantly found in the stroma, are commonly used in producing multicellular spheroids. These cells have been documented to assist breast cancer cell progression, invasion, survival and its therapeutic responses (Majety, Pradel, Gies, & Ries, 2015; Sadlonova et al., 2005; Yakavets et al., 2020). Apart from that, fibroblasts associated with breast cancer cells are also involved in the microenvironmental cellular processes including angiogenesis or lymph angiogenesis (Luo, Tu, Liu, & Liu, 2015). The term cancer-associated fibroblasts have been used for the interaction of cancer cells and stroma fibroblasts. Earlier in this study, MRC5 (normal lung fibroblasts) were used to evaluate the cytotoxicity of *E. americana;* no cytotoxic effects were recorded on MRC5. For the production of co-cultured spheroids, luminal breast cancer cell MCF7 and invasive ductal-carcinoma breast cancer cells MDA-MB-231 were co-cultured with MRC5 normal lung fibroblast. Further interaction analysis of the co-culture was also performed to validate the use of co-culture spheroids in this study.

The formation of co-cultured spheroids was explained earlier in Chapter 3. The ratio 1:3 (MCF7; MDA-MB-231:MRC5) is based on the work by Yakavets et al., 2020. Treatment of *E. americana* was applied on day 5 of culture, and results were collected after 72 hours of treatment. Data collected are presented in a bar chart to differentiate the reduction percentage by co-cultured MDA-MB-231 cells and co-cultured MCF7 cells treated with *E. americana* and Cisplatin. Figure 4.6 illustrates the data collected from the assay.

As anticipated, the concentration to inhibit 50% of the co-culture of 3D spheroids were significantly higher compared to the monoculture of 3D spheroids. IC_{50} for co-culture of MCF7 + MRC5 were quantified at 39.22 ± 2.134 µg/mL followed by MDA-MB-231 + MRC5 at 50.01 ± 3.944 µg/mL. The higher value of 50% inhibition of spheroids, depicted by the value of IC_{50} was due to the interaction of cancer cells with their associated fibroblast cells. In an earlier discussion, the association of cancer cells and fibroblast cells exhibit a significant increase in the

survival rate of the spheroids and their ability to resist cytotoxic compounds; in this context, the treatment of *E. americana* extract.

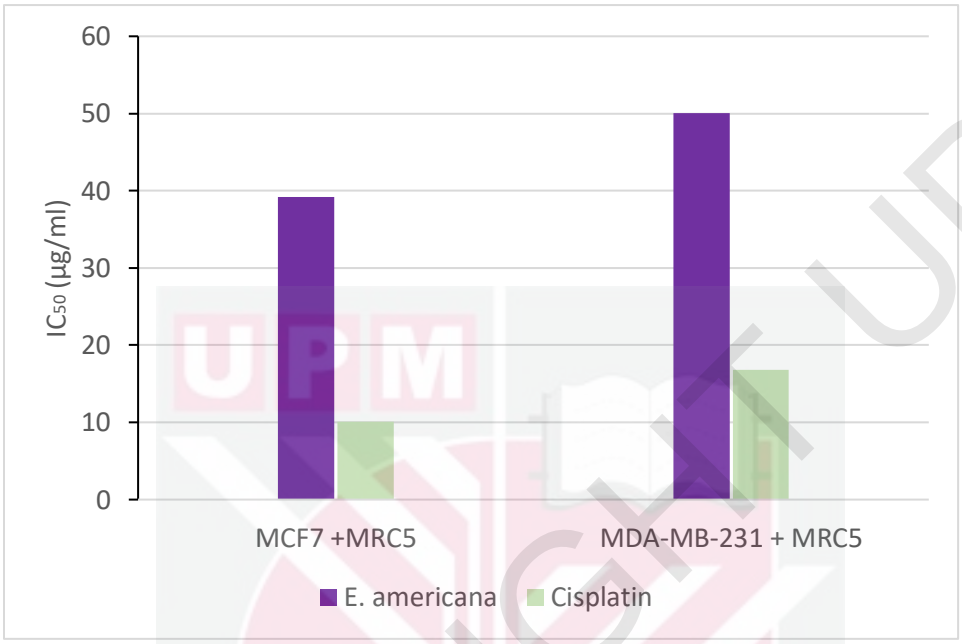


Figure 4.6 : Alamar Blue® Assay on Co-Culture Spheroids. The bar graph shows the results of the comparison in IC_{50} values of mixed culture of cancer cell and normal fibroblast (MRC5) spheroids upon treatment with different concentrations of *E. americana* ethanol extract (25 – 150 ug/mL) and a commercial anti-cancer drug, Cisplatin. The IC_{50} value for co-culture of MCF7 treated with *E. americana* is 39.22 ± 2.139 µg/mL and 50.01 ± 3.944 µg/mL for co-culture of MDA-MB-231 treated with *E. americana.* The IC_{50} value for Cisplatin treatment on MCF7 + MRC5 is 10.08 ± 7.309 µg/ml and MDA-MB-231 + MRC5 is 18.35 ± 2.881 µg/ml. Data are expressed as triplicates; bar ± SD

Treatment with Cisplatin also showed significant differences between the single-cell spheroids and spheroids with fibroblast associates. Data were calculated using two-way ANOVA with a P-value calculated at $p<0.001$, $p<0.05$ is considered to be significant. Table 4.3 summarizes the data collected

Table 4.3 : Alamar Blue® Assay IC_{50} Values Summary

Culture type	Cell Line	Treatment with *E. americana* (µg/mL)	Treatment with Cisplatin (µg/mL)
Monoculture	MCF7	37.90 ± 4.691	7.38 ± 7.309
	MDA-MB-231	48.72 ± 1.848	16.77 ± 1.391
Co-culture (with MRC5)	MCF7	39.22 ± 2.139	10.08 ± 1.362
	MDA-MB-231	50.01 ± 3.944	18.35 ± 2.881

Two-way ANOVA. All values represented with significant value $p<0.05$. Data are presented as mean ± SD; standard deviation.

4.4 Double Staining

Double staining was performed to evaluate the cytotoxicity of *E. americana* in a qualitative approach. This method enables visualization of the shapes and changes in the morphology of spheroids before and after being treated with *E. americana* plant extract. The staining procedure involves the use of Propidium iodide (PI) which stains dead cells red-fluorescent and Hoechst, a blue coloured dye that stains viable cells. Images of the untreated spheroids of both monoculture and co-culture after being treated with plant extract were captured. Interestingly, the images exhibit remarkable changes in shapes and sizes of all spheroids once treated with *E. americana* plant extract. The images were captured using ZEIZZ AxioVert A1 FL-LED inverted fluorescent microscope taken at 20 x magnification and *scale bar*: 100 µm. Figures 4.7(a & b), 4.8(a & b), 4.9(a & b), and 4.10(a & b) demonstrate the findings.

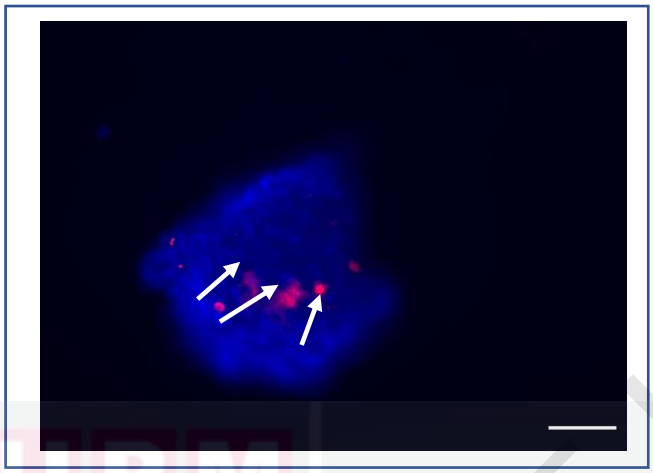


Figure 4.7a : Double Staining of MCF7 Monoculture. (a) The diameter of the untreated spheroid was measured at> 300 µm and the Propidium iodide (PI) fluorescence signal was only localized in the centre of the spheroid (white arrows)

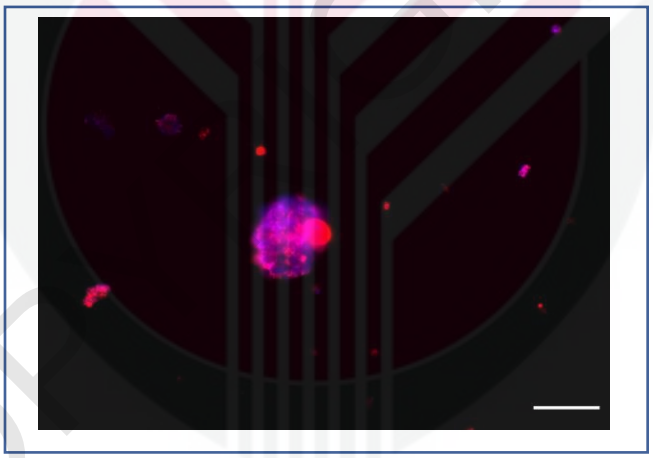


Figure 4.7(b) : MCF7 Spheroid Treated with *E. americana*; Reduction in Size Diameter Observed Compared to Figure 4.7a. The PI signal intensity increased and dispersed throughout the spheroid. *Scale bar*: 100 µm

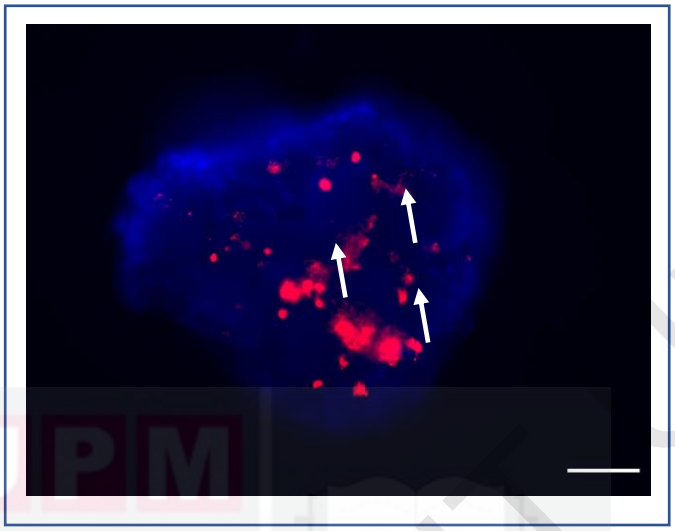


Figure 4.8(a) : Double Staining of MCF7 Co-Culture. (a)Untreated MCF7 co-culture spheroids diameter measured at > 300 µm, low intensity of Propidium iodide (PI) fluorescence signal and localized (white arrows)

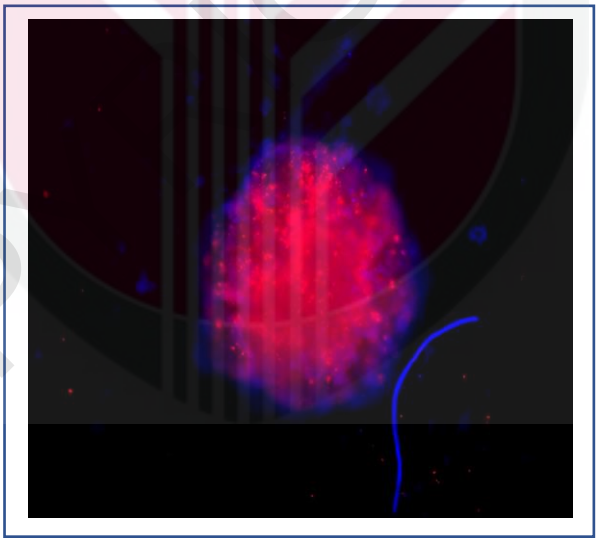


Figure 4.8(b) : Upon Treatment with *E. americana* plant Extract, the PI Fluorescence Signal Intensifies and Dispersed throughout the Spheroid. No Significant Changes in the Diameter of the Spheroids were Observed When Compared to the Figure in 4.8a. *Scale bar*: 100µm

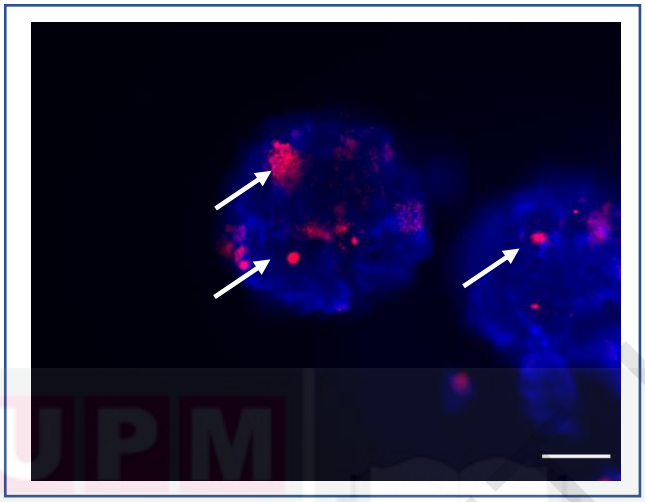


Figure 4.9(a) : Double Staining of MDA-MB-231 Monoculture. Untreated MDA-MB-231 spheroid (a) low intensity of PI fluorescence signal and localized in the centre of the spheroids (white arrows). *Scale bar*: 100 µm

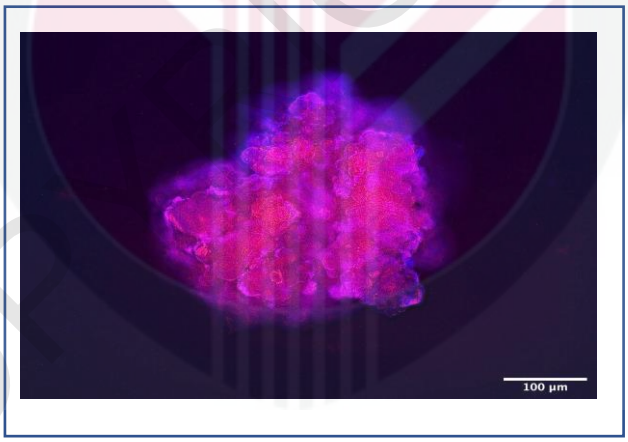


Figure 4.9(b) : Upon Treatment with *E. americana*; Resulted in High Intensity of PI Fluorescence Signal Dispersed throughout the Spheroid. *Scale bars*: 100 µm

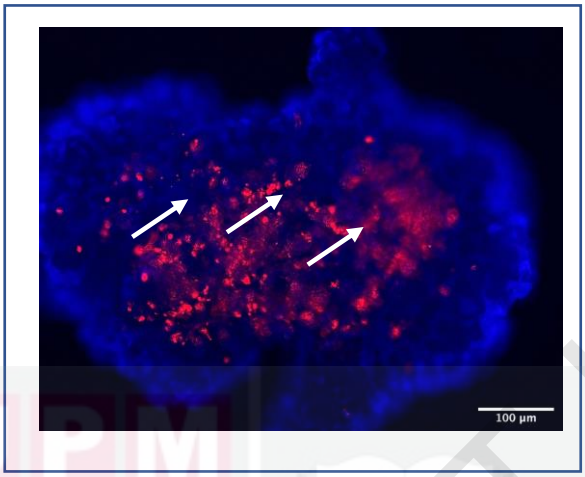


Figure 4.10a : Double Staining of MDA-MB-231 Co-Culture. Untreated MDA-MB-231+MRC5 spheroid (a) PI fluorescence signals were observed localized in the centre of the spheroid (white arrows) *Scale bar:* 100 µm

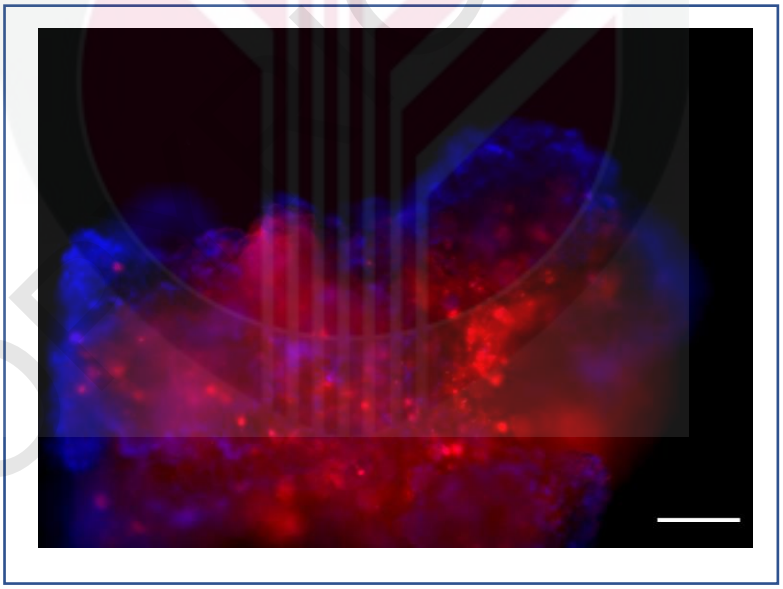


Figure 4.10b : High Intensity of PI Fluorescence Signals Dispersed throughout the Spheroid. No Significant Reduction in Spheroid Size Recorded. Scale bar: 100µm

Double staining assay utilizes 2 DNA intercalators fluorescent dye, the Propidium Iodide (PI) and Hoechst 33342. As Hoechst is known to stain both viable and dead cells in the spheroids, yielding a bright blue signal under the microscope, a counterstain dye is needed to label the dead cells. PI, a red stain, is not permeable to viable cells; therefore, it is used to counterstain the dead cells in the spheroids. All the untreated spheroids (monoculture and co-culture) exude bright blue fluorescence signal, which generally characterises healthy proliferative spheroid. From the image data, after being treated with the plant extract, the staining of dead cells resulted in a co-localized PI red-fluorescent signal and displays a magenta colour. The changes in PI fluorescence intensity indicate cell death possibly induced by treatment with *E. americana* plant extract. Apart from the PI fluorescence intensity signal strength, a reduction in size was also observed on the spheroids treated with *E. americana* plant extract. Table 4.4 summarise the findings of the double staining assay.

Table 4.4 : Double Staining Assay of 3D Spheroids upon Treatment with *E. americana*

Culture type	PI fluorescence signal	Decreased in Size
MDA-MB-231	+++	+++
MDA-MB-231 + MRC5	++	+
MCF7	+++	+++
MCF7 + MRC5	++	+

+; low intensity/changes, ++; moderate intensity/changes, +++; high intensity/changes

Remarkably, the qualitative analysis by double staining methods validates the function of fibroblasts in assisting the response of breast cancer spheroids towards the cytotoxicity treatment. The substantial reduction of diameter in size was only observed in the monoculture of the breast cancer spheroids of MCF7 and MDA-MB-231 as the concentration of *E. americana* plant extract increased. In co-culture spheroids for MCF7 and MDA-MB-231, although the PI fluorescence's signal intensity increased, which signifies necrosis/ apoptosis within the spheroid, the diameter in size of the co-culture spheroids did not reduce upon treatment with *E. americana* plant extract.

4.5 Invasion Assay on 1% Gelatine Coating

In the invasion assay, monoculture and co-culture of MDA-MB-231 were applied based on previous literature on the ability of MDA-MB-231 to display a representation of epithelial to mesenchymal transition (EMT). Breast cancer invasion that leads to metastasis is the main characteristic that increases its mortality rate (Sung et al., 2011). The

aggressiveness of breast cancer invasion can be recognised through certain criteria such as poorly differentiated triple-negative; lack of oestrogen receptors (ER), progesterone receptors (PR) expression and lack amplification of HER2 (human epidermal growth factor receptor 2). The ability to transform from epithelial cells to migratory known (EMT) is associated with the aggressive cancer progression (Huang et al., 2020). MDA-MB-231 cells exhibit all the listed criteria. Cisplatin was used as the negative control at a concentration of 20 µg/mL based on previous literature (Najafzadeh et al., 2022). The invasion area measurement was taken using Zeiss AxioVert A1 FL-LED and analysed using FIJI Image J version 2.1.0. Figures 4.11, 4.12 and 4.13 represent the collected data in image format. The invasion area of each time frame for monoculture spheroids was recorded and represented in the bar chart in Figure 4.14.

4.5.1 MDA-MB-231 Invasion Assay on 1% Gelatine (Monoculture)

Spheroids of MBA-MB-231 monoculture were seeded onto 24 well plates coated with 1% gelatine. Invasion assay utilizes day 5 of spheroids culture. As anticipated, data recorded indicates the significant ability of *E. americana* extract to disrupt the invasion of MDA-MB-231 monoculture spheroids. A larger area of invasion ($p = 0.003$; ANOVA) was recorded by untreated monoculture spheroids, conversely, when a comparison was made with untreated spheroids, a notable reduction in the area of invasion is recorded for spheroids treated with *E. americana* extract at 72 hr of treatment ($p<0.005$; two sample t-test).

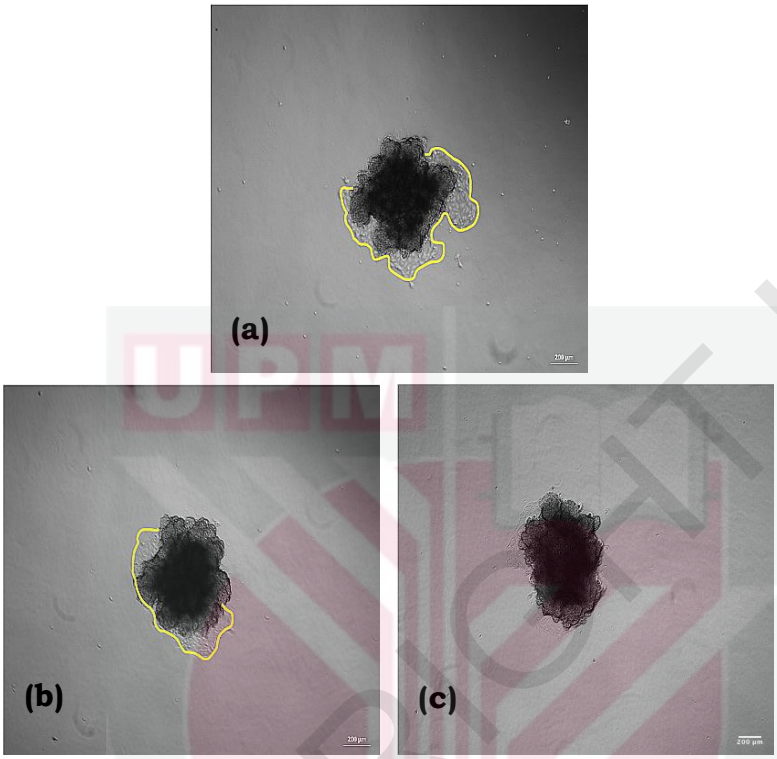


Figure 4.11 : MDA-MB-231 Monoculture Invasion Assay at 24 Hours. The area of the invasion is outlined in yellow line, (a) in untreated monoculture spheroid, area of the invasion can be observed (b) monoculture spheroid treated with 50 µg/mL of *E. americana* plant extract area of invasion (c) No visible area of invasion detected in spheroid treated with Cisplatin. *Scale bar*: 200 µm

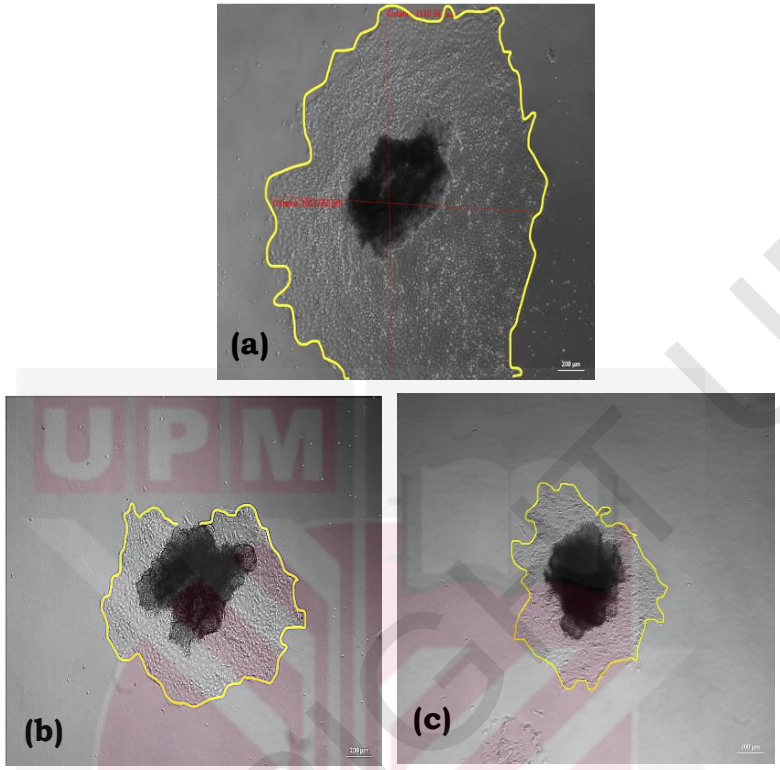


Figure 4.12 : MDA-MB-231 Monoculture Invasion Assay at 48 Hours. The area of the invasion is outlined in the yellow line can be observed in (a) Untreated monoculture spheroid (b) monoculture spheroid treated with 50 µg/mL of *E. americana* plant extract. (c) Spheroid treated with Cisplatin. *Scale bar*: 200 µm

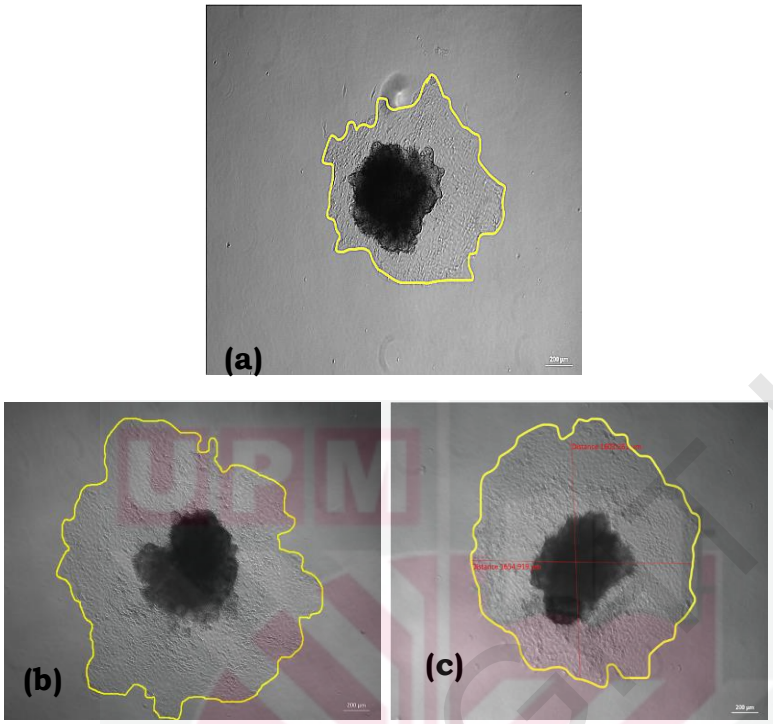


Figure 4.13 : MDA-MB-231 Monoculture Invasion Assay at 72 Hours. The area of the invasion is outlined in the yellow line can be observed in (a) Untreated monoculture spheroid and (b) monoculture spheroid treated with 50 µg/mL of *E. americana* plant extract. (c) Spheroid treated with Cisplatin. *Scale bar*: 200 µm

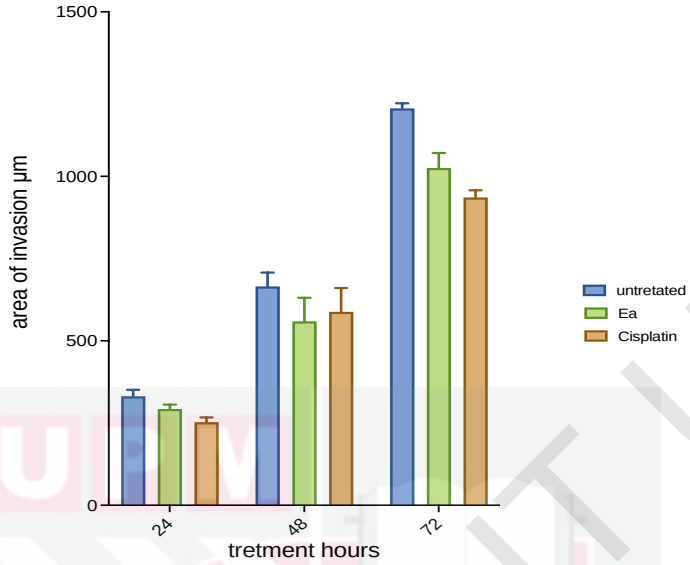


Figure 4.14 : Surface Area of Invasion of Monoculture Spheroids. The bar chart shows the comparison of invasion area between untreated monoculture spheroids, treated monoculture spheroids with *E. americana* and treated monoculture spheroids with Cisplatin, respectively

No significant difference was recorded in the area of reduction between Cisplatin and *E. americana* extract-treated spheroids. Table 4.5 shows the area of invasions at 72 hours of treatment;

Table 4.5 : Surface Area of Invasion (Monoculture)

Treatment	**Surface area of invasion µm**		
	Untreated	*E. americana*	Cisplatin
24 hours	331.6 ± 19.493	292.6 ± 13.398	252.9 ± 14.30
48 hours	666.5 ± 42.174	559.3 ± 71.566	588.1 ± 72.529
72 hours	1206.8 ± 15.083	1025.5 ± 45.355	936.3 ± 21.509

Two-sample t-test. All values are represented with significant $p < 0.005$. Data were presented in mean ± SD; standard deviations.

4.5.2 MDA-MB-231 Invasion Assay on 1% Gelatine (Co-Culture)

The co-cultured spheroids of MDA-MB-231+MRC5 were transferred into 24 well plates on day 5 of the sub-culture. The area of invasion data was taken after 24 hours of transfer. The results are documented as follows in Figures 4.15, 4.16, 4.17, and Figure 4.18 summarise the findings in a bar chart.

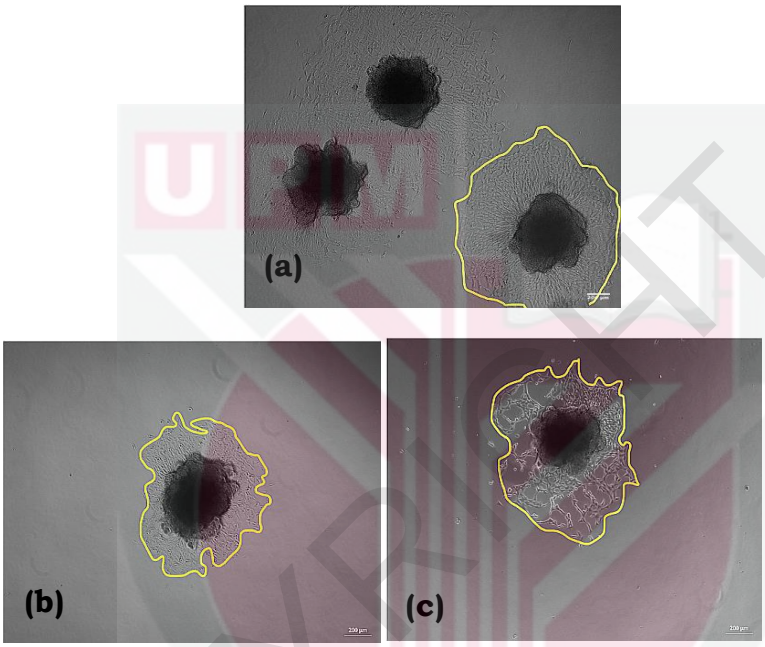


Figure 4.15 : MDA-MB-231 Co-Culture Invasion Assay 24 Hours. The area of the invasion marked is outlined in the yellow line can be observed in (a) Untreated co-culture spheroid (b) co-culture spheroid treated with 50 µg/mL of *E. americana* plant extract. (c). Co-culture spheroid treated with Cisplatin. *Scale bar*: 200 µm

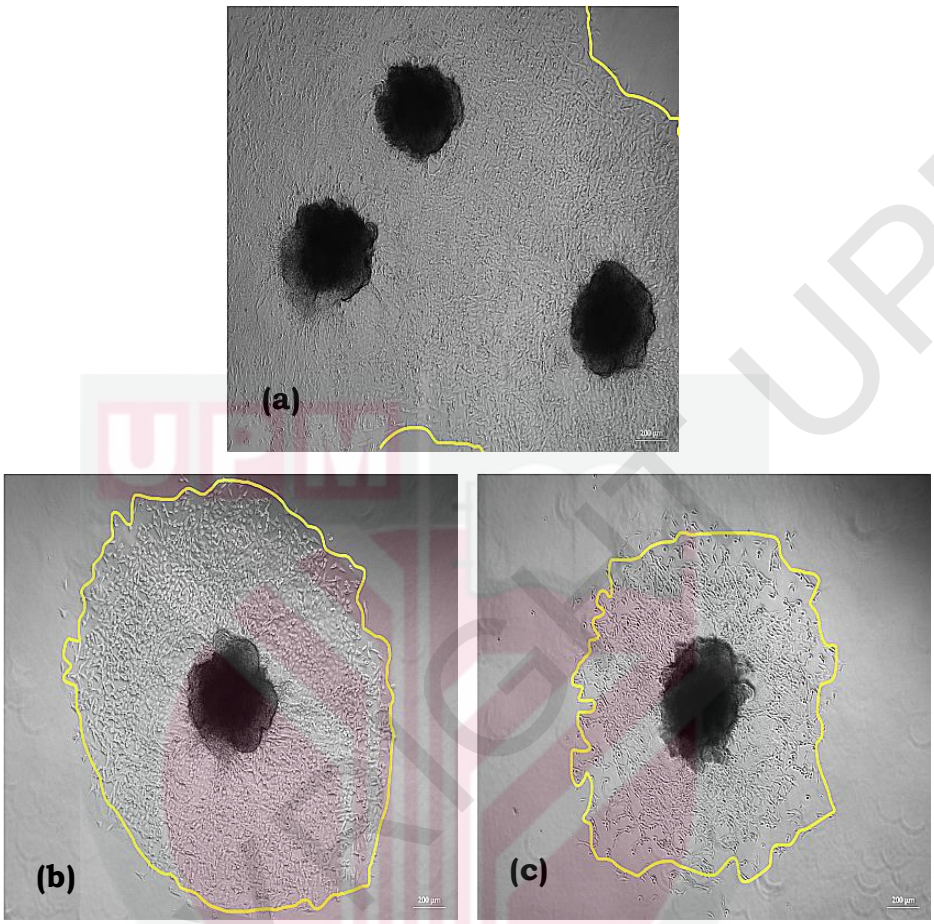


Figure 4.16 : MDA-MB-231 Co-Culture Invasion Assay 48 Hours. The area of the invasion outlined in the yellow line can be observed in (a) Untreated co-culture spheroid (b) co-culture spheroid treated with 50 µg/mL of *E. americana* plant extract. (c) co-culture spheroid treated with Cisplatin. *Scale bar*: 200 µm

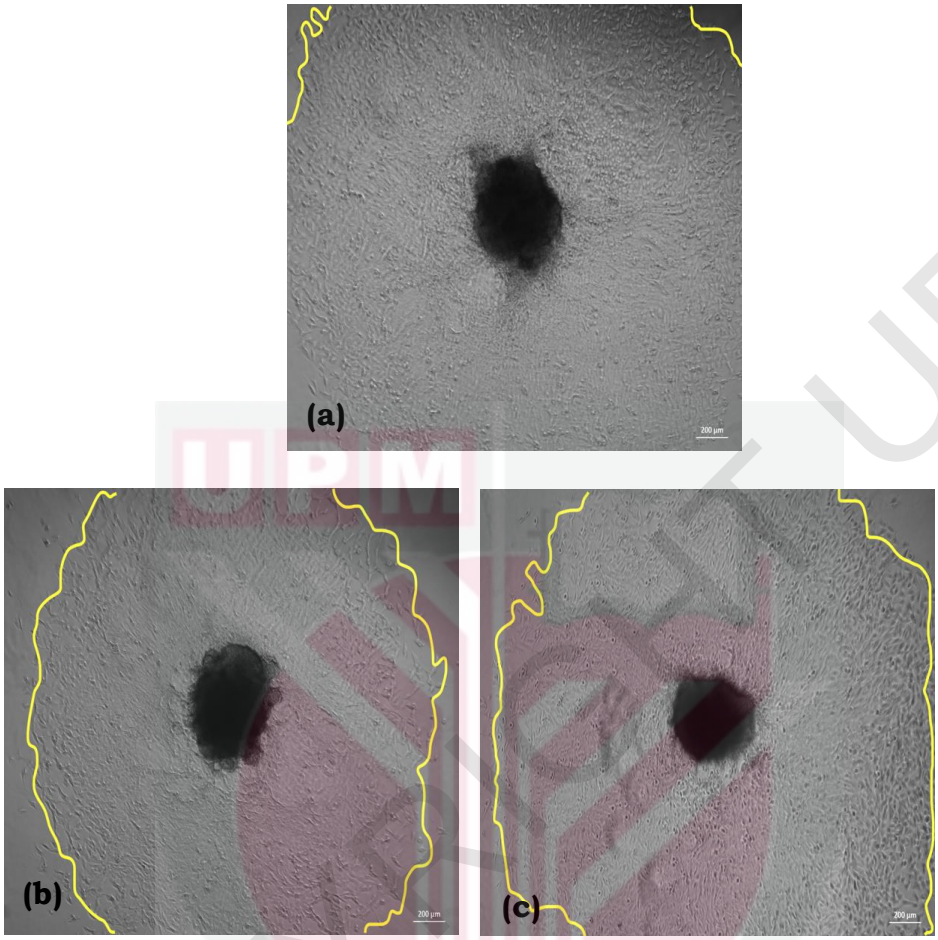


Figure 4.17 : MDA-MB-231 Co-Culture Invasion Assay 72 Hours. The area of the invasion marked is outlined in the yellow line can be observed in (a) Untreated co-culture spheroid (b) co-culture spheroid treated with 50 µg/mL of *E. americana* plant extract. (c) co-culture spheroid treated with Cisplatin. *Scale bar*: 200 µm

The ability of cancer cells to invade is significantly increased by associating with its stromal cells. Fibroblast cells which can be found abundantly in the stroma environment are the perfect candidate for that motive. Increased evidence validates the association of fibroblast with cancer cells particularly cancer-associated fibroblast or also known as CAFs (Luo et al., 2015). The evidence of the association between fibroblast and cancer cells was validated in this invasion assay. The ability of co-culture spheroids to proliferate/ invade the gelatine coating increased significantly compared to monoculture spheroids. At $p<0.005$, t:24; $p<0.000081$, at 48; $p<0.000123$ and at 72; $p<0.00059$ co-culture spheroids exhibit a greater ability to invade/migrate on the gelatine

medium. Analysing the findings from this assay, treatment with *E. americana* extract and Cisplatin exhibit a significant inhibition at 24, 48 and 72 hours. A significant difference between E. *americana* and Cisplatin was recorded at 48 hours and 72 hours. The surface area of invasion for 24 hours, 48 hours and 72 hours are presented in Table 4.6.

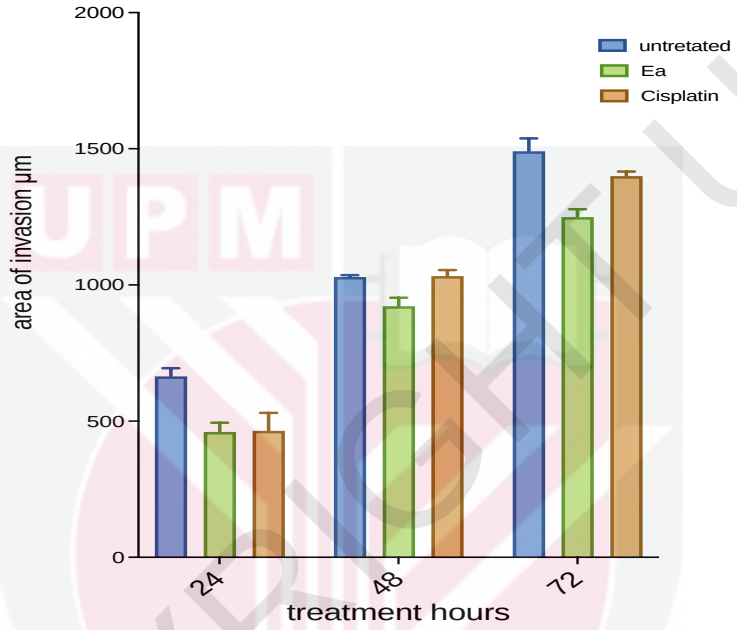


Figure 4.18 : Area of Invasion of Co-Culture Spheroids. Differences in the area of invasion are shown by the bar representative of each untreated monoculture spheroid and treated monoculture spheroid with *E. americana* ethanol extract and treated monoculture spheroid with Cisplatin. Data are expressed as triplicates; bar ± SD

Table 4.6 : Surface Area of Invasion (Co-Culture)

Treatment	**Surface area of invasion µm**		
	Untreated	E. americana	Cisplatin
24 hours	664.6 ± 29.319	460.3 ± 33.704	464.3 ± 66.055
48 hours	1029.2 ± 6.730	922.0 ± 30.723	1032 ± 21.670
72 hours	1490.7 ± 47.361	1249.2 ± 29.114	1399.7 ± 16.390

Two-sample t-test. All values are represented with significant $p < 0.005$. Data were presented in mean ± SD; standard deviation

Through the invasion assay, the cell-to-cell interaction of spheroids generated through co-culture can be validated for this research to recapitulate mini tumours *in vitro*. The significant difference in the invasion area between the untreated monoculture and co-culture demonstrates that the interaction between cancer cells and fibroblast results in a successful aggressive cancer invasion/progression. Figure 4.19 demonstrates the interaction between cancer cells and fibroblasts and the difference in the progression ability between the untreated monoculture versus the untreated co-culture.

According to NCI (Vijayarathna & Sasidharan, 2012), the plant extract concentration used in the invasion assay at 50 µg/mL is considered mild cytotoxic, however, *E. americana* ethanol extract demonstrate the anticancer ability by efficaciously reducing the surface area of invasion of aggressively invasive MDA-MB-231 spheroids. Nonetheless, the fact that it is a crude extract that comprises a synergistic bioactive compound with diverse pharmacological properties, the anticancer effects of the *E. americana* possess the potential as an ideal candidate to be developed as an alternative treatment for breast cancer.

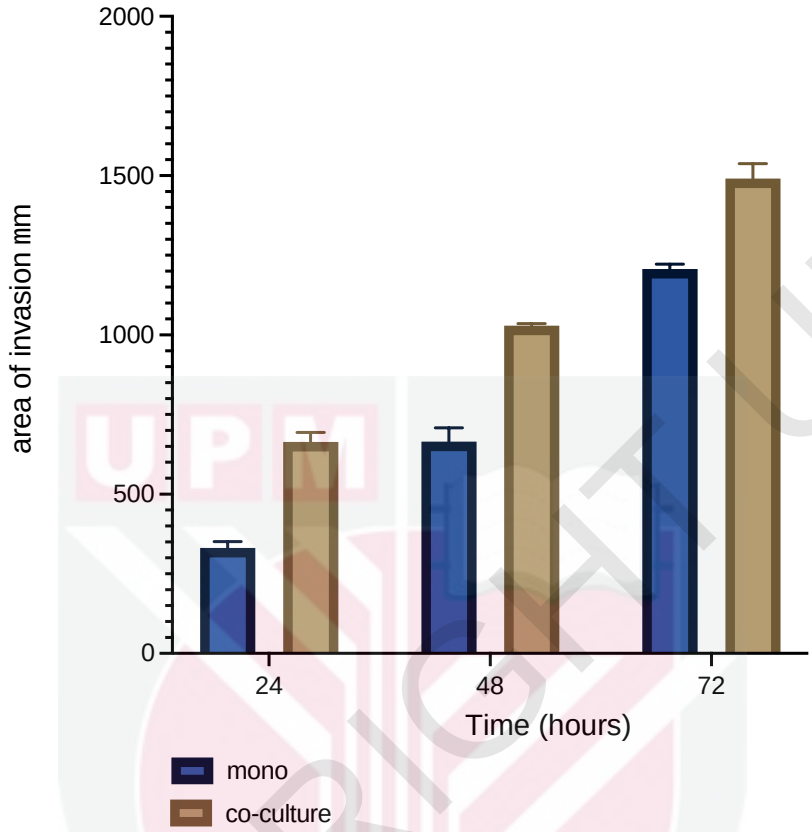


Figure 4.19 : Summary of the Surface Area of Invasion (Monoculture Vs Co-Culture). The difference in bar height signifies the difference in the area of invasion between the untreated monoculture and untreated co-culture spheroids. The invasion area was compared using ANOVA, $p<0.05$ was considered significant, p-value obtained was *$p<0.0001$*. Data is expressed as triplicates; bar ± SD; $P<0.05$; t-test)

4.6 Immunocytochemistry (ICC)

In the invasion assay, it was found that there is a significant difference ($p<0.001$) in the area of invasion between monoculture and co-culture spheroids. It has been suggested that the co-culture of cancer cells with fibroblasts plays a major role in successful spheroids invasion/progression. In the literature, cancer associated-fibroblast (CAFs) tend to proliferate and transform into myofibrils and start producing dysfunctional and excessive ECM to support the cancer progression (Luo et al., 2015; Yakavets et al., 2020). To further investigate the transformation of fibroblasts into CAFs, immunocytochemistry was performed with the application of co-culture

spheroids of MDA-MB-231. Fibroblast transformed to CAFs can be characterised by the expression of alpha-smooth muscle actin (αSMA) (Majety et al., 2015). Figure 4.20 illustrates the findings.

MDA-MB-231 cell lines effectively transformed MRC5 to CAFs. Conditioned media prepared from a 90% MDA-MB-231 confluence flask successfully transformed MRC5 into cancer-associated fibroblast (CAFs). Hence, CAFs can be reproduced *in vitro* in a laboratory setting for further research or evaluation. Henceforth, the spheroids of co-culture used in this study can be confirmed to be associated with CAFs, which explains the aggressive invasion/progression recorded in the invasion assay.

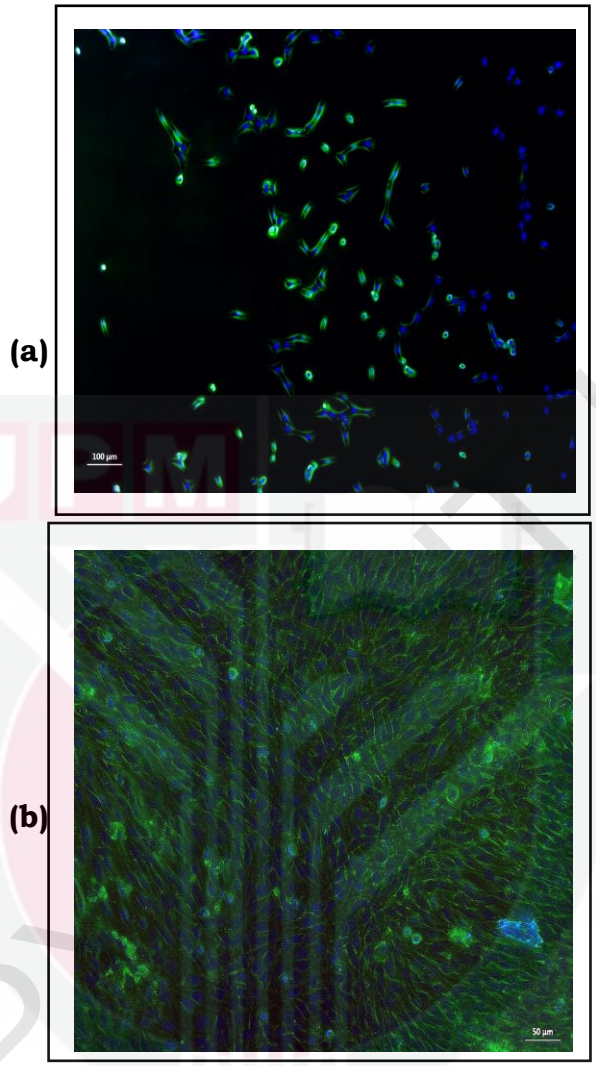


Figure 4.20 : Fibroblast Transformation to CAFs. (a) CAFs exhibit intense green fluorescence. On day 7 (b) a successful transformation of CAFs can be observed, indicated by the green fluorescence signal stained the myofibrils of CAFs. Hoechst was used to stain nuclei with blue fluorescence signal as counterstain

4.7 Histological Staining on Monoculture Spheroids

This procedure aims to examine the cellular structure of untreated MDA-MB-231 spheroids after being treated with *E. americana* extract. The sample preparation for this procedure was devised specifically to accommodate the fragile spheroids generated in this study. The scaffold-

less methods used for spheroids production causes it to disintegrate easily during routine specimen preparation for H&E staining. The agarose cast was then constructed to overcome the setback on the fragile spheroids. Utilising a 3D printer, an agarose cast was made. Figures 4.21 and 4.22 display the agarose cast preparation. The methods developed effectively overcome the setbacks for the H&E histology staining assay. H&E staining was performed, and the data collected is presented in Figures 4.23 and Figures 4.24.

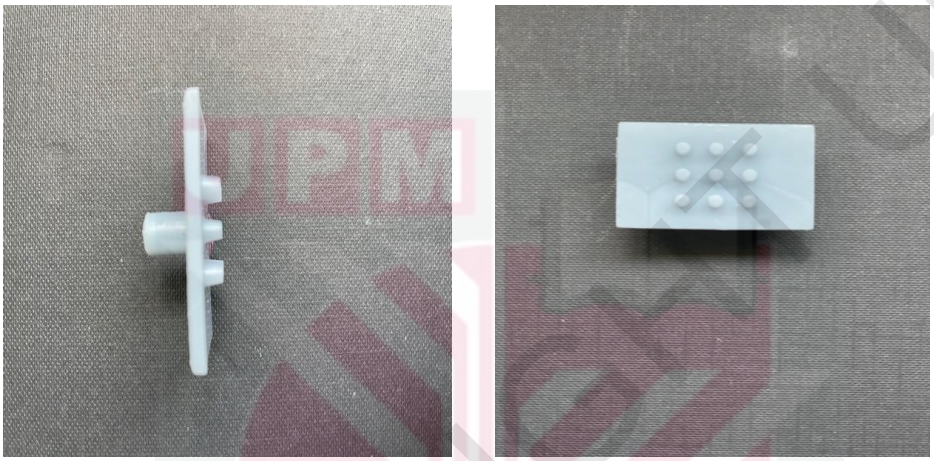


Figure 4.21 : Agarose Cast. The aerial view of the agarose cast. The cast was designed using OpenSCAD software, and the printed material used was ePLA+ in grey

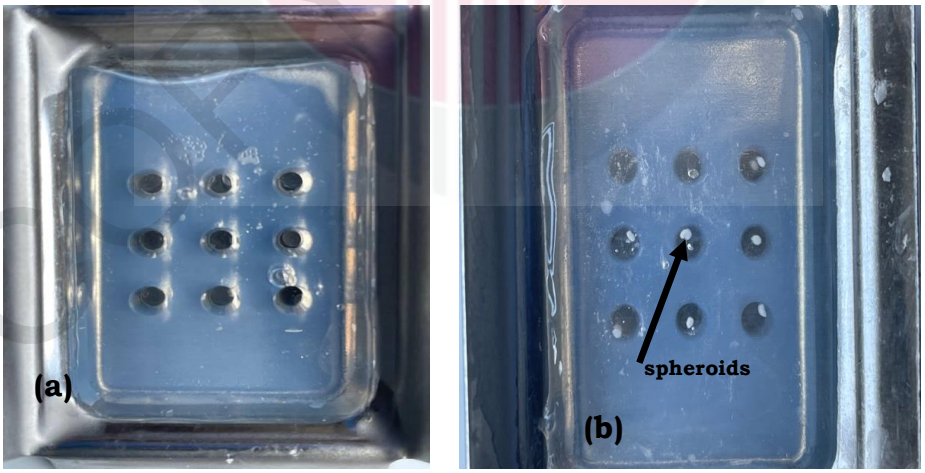


Figure 4.22 : Agarose Array. (a) Solidified Agarose Array (b) Successfully Loaded Spheroids (Arrow) into the Agarose Array

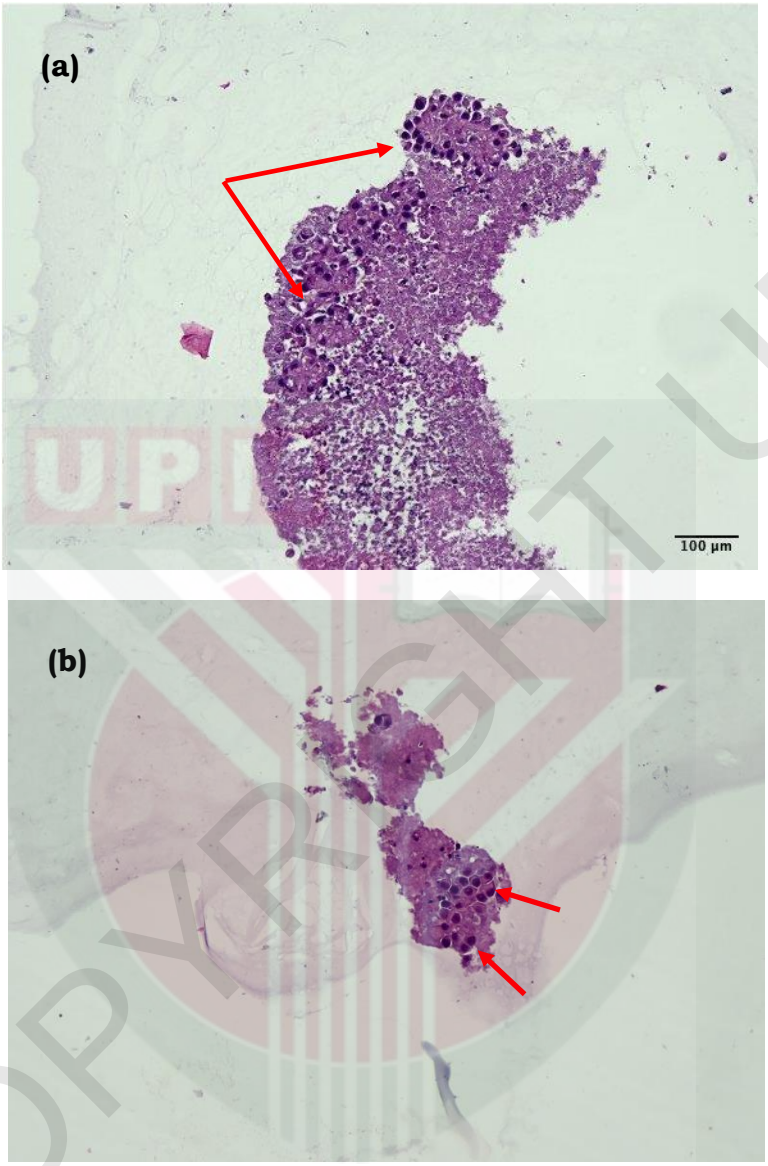


Figure 4.23 : The H&E Staining on the Untreated Spheroid. Haematoxylin stains the nuclei in the spheroids (a & b). The cells in the spheroids (a & b) had organized nuclei and cytoplasm (red arrows). *Scale bar: 100 µm*

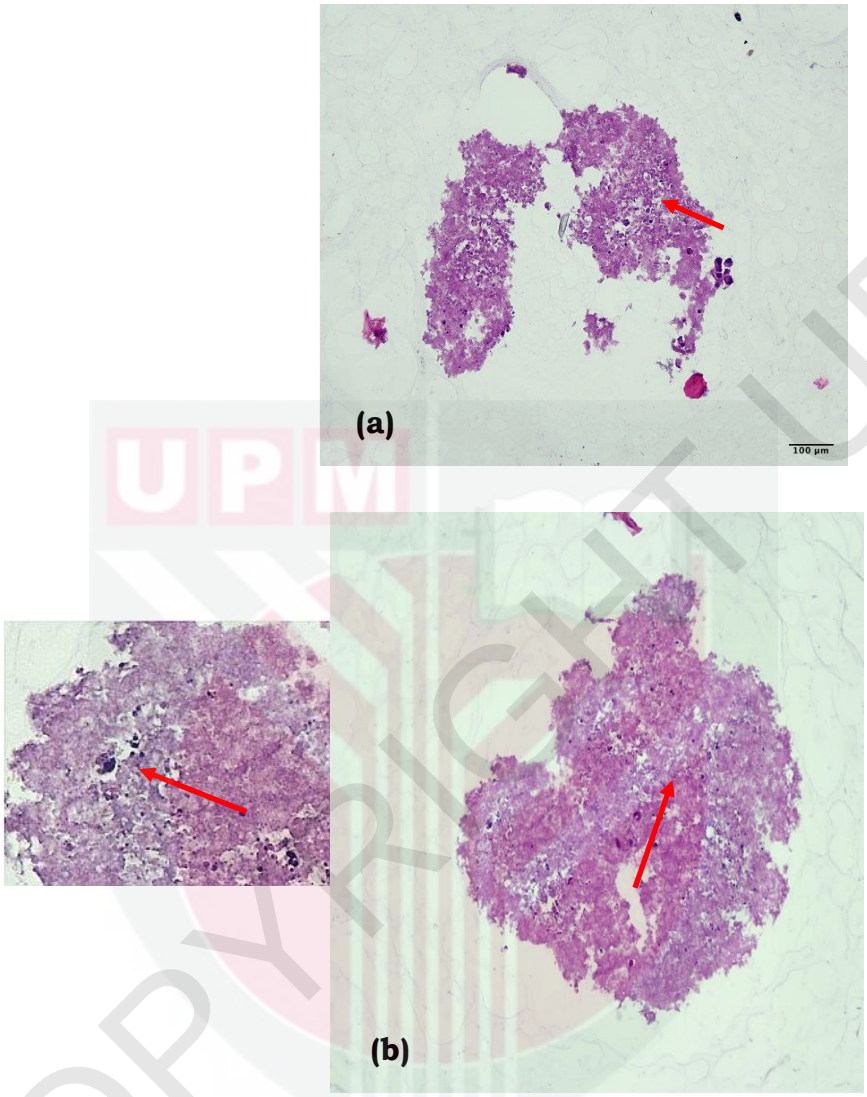


Figure 4.24 : The H&E Staining on Spheroid Treated with *E. americana* Extract. The cells in the spheroids (a & b) disintegrated post-treatment with the ethanol extract of *E. americana* (red arrow). *Scale bar: 100 µm*

In this final analysis, deformation in the cellular morphology were observed as the result of cytotoxicity from the plant extract. From the image data, the untreated spheroids of MDA-MB-231 were seen to maintain the membrane integrity and the haematoxylin stain on the nucleus of the intact cell's images can be seen as noticeably as shown in Figure 4.23. Clustered cells can also be seen on the untreated spheroids images, as depicted in Figure 4.23 (a; i). The data images

obtained from this analysis are comparable to the results described previously by Huang et al., 2020, which focused on characterizing MDA-MB-231 spheroids. The authors demonstrate the structure of MDA-MB-231 spheroids stained with H&E.

In spheroids treated with *E. americana*, the membrane of the cells was completely disintegrated. The debris of the nucleus can be observed within the spheroids. None of the clustered cells can be observed on the treated spheroids, suggesting the treatment of *E. americana* extract on MDA-MB-231 spheroids possess an anti-proliferative action.

These conclusive findings further support the hypothesis that *E. americana* extract anticancer/ antiproliferative activity effectively impedes the proliferation of breast cancer cell lines, breast cancer spheroids, and fibroblast associates.

CHAPTER 5

CONCLUSION

In this study, *E. americana* ethanol extract underwent a series of parameters to validate the pharmacological benefits of the plant. The series started with a review of the phytochemical compounds. From the assays performed, *E. americana* ethanol extract exhibit all the polyphenolic combinations, suggesting that the plant has the potential to be developed as a therapeutic treatment for cancer. The presence of potent phenolics content, flavonoids, and alkaloids in the plant extract substantiates the hypothesis of the anti-cancer property of *E. americana*.

The hypothesis is further proven in 2D cell culture and 3D cell culture/spheroids utilization to assess the *in vitro* anti-cancer properties of the plant. Preliminary screening on 2D cell culture indicates notable cytotoxicity activity as the IC_{50} recorded are at 25.17± 0.140 µg/mL for MCF7 and 25.23 ±0.818 µg/mL for MDA-MB-231. Remarkably, the plant extract does not impose any significant cytotoxicity activity towards MRC5 (lung fibroblast cell line) and MCF-10A (non-tumourigenic epithelial cell line). The study design also included cell culture models that can recapitulate mini tumours *in vitro* which are the cell spheroids, to further investigate the cytotoxicity potential. Treatment of the *E. americana* extract on 3D cell culture also resulted in significant cytotoxic activity. Two types of spheroids were utilized; monoculture and co-culture spheroids. In monoculture spheroids, the IC_{50} value obtained was 37.90±4.691 µg/mL for MCF7's spheroid and 48.72±1.848 µg/mL on MDA-MB-231 spheroid. The IC_{50} for co-culture spheroids was at 39.22 ± 2.134 µg/mL followed by MDA-MB-231+MRC5 at 50.01 ± 3.944 µg/mL. A higher value of IC_{50} obtained in monoculture and co-culture spheroids compared to 2D cell culture proposed that the 3D spheroid culture successfully mimics the *in vivo* cell-to-cell interaction, resulting in a more resilient cell culture and reliable cytotoxic assessment. These methods can be suggested as the standard methods to be used in future drug discovery screening. The cytotoxicity assay was then followed by a double staining assay, an invasion assay, and a histological staining assay. The conclusion of each assay further supports the findings that *E. americana* ethanol extract is shown to possess phytochemical compounds validated as beneficial and potentially as an anticancer agent.

Conclusively, *E. americana* ethanol extract exhibits anticancer property that can be further investigated. Future studies can focus on the mechanism of toxicity of *E. americana* ethanol extract that efficiently inhibits the proliferation of breast cancer cell lines, its spheroids, and spheroids associated with fibroblast *in vitro*.

Future Work

This study has highlighted 2 findings that can be further investigated.
1) The potential of *E. americana* as an anticancer agent. Future works can be done on elucidating and separating the bioactive compound of the plant to obtain a pure bioactive compound. The pure compound can be used for further cytotoxicity study and on the mechanism of action on the proliferation of cancer cells at a molecular level.

2) The transformation of fibroblasts to cancer-associated fibroblasts has been proven possible in a laboratory setting. An in-depth study on how cancer cells can assign normal fibroblasts as their associate might result in finding the mechanism that can reverse the process, and hence impede or even inhibit the progression/metastasis of cancer cells to distal organs.

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APPENDICES

Appendix A

Table 3.1 : List of Cell Culture Types Used in this Research

Cell Type	Description
MCF-10A	Non-tumourigenic epithelial cell line. Display characteristic of a luminal ductal cell but not of myoepithelial. Adherent cells with spindle shape morphology. *Homo sapiens*
MRC-5	Normal lung fibroblast cell. A Diploid cell line and adherent cells with spindle shape morphology. *Homo sapiens*
MCF-7	An adenocarcinoma epithelial cell. Human breast cancer cell line with estrogen, progesterone, and glucocorticoid receptors. Adherent cell with round, oblong, or irregular shape. *Homo sapiens*
MDA-MB-231	A metastatic mammary adenocarcinoma epithelial cell. Human breast cancer cell line with triple-negative characteristics. Adherent cell with round shape morphology. *Homo sapiens*

Table 3.2 : Cell Culture Media Formulation

Cell Type	Media formulation
MCF-10-A	DMEM F-12 + 5% Horse Serum + EGF + Hydrocortisone + Insulin + Pen/Strep.
MRC-5	RPMI + 10% FBS + 5% Pen/Strep + 1% Amp B
MCF-7	RPMI + 10% FBS + 5% Pen/Strep + 1% Amp B
MDA-MB-231	DMEM + 10% FBS + 5% Pen/Strep + 1% Amp B

Appendix B