

**ELUCIDATING ANTICANCER PROPERTIES OF
Bruguiera cylindrica AND *Sonneratia alba* EXTRACTS
AGAINST BREAST CANCER CELLS**

NORAIN BINTI NADZRIN

**MASTER OF SCIENCE
UNIVERSITI MALAYSIA TERENGGANU**

2025

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NORAIN BINTI NADZRIN

**Thesis Submitted in Fulfilment of Requirement for the
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Universiti Malaysia Terengganu**

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My dearest supervisor Assoc. Prof. Dr. Vigneswari A/P Sevakumaran

My respected Co-Supervisor Assoc. Prof. Dr. Noor Wini Mazlan

My beloved family, M.A.A and my friends

For their relentless support, dedication and endless love.

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu in
fulfilment of the requirements for the degree of Master of Science

**ELUCIDATING ANTICANCER PROPERTIES OF *Bruguiera cylindrica* AND
Sonneratia alba EXTRACTS AGAINST BREAST CANCER CELLS**

NORAIN BINTI NADZRIN

2025

Main Supervisor : Associate Professor Dr. Vigneswari A/P Sevakumaran
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Institute : Institute of Climate Adaptation and Marine Biotechnology

Breast cancer remains one of the five most lethal cancers worldwide. However, mortality from breast cancer appears to be increasing beyond disease-related causes. The unnatural semi-synthetic chemotherapies have been shown to contribute to this problem, as they cause adverse side effects and necrosis in healthy cells. Hence, current oncology research has shifted towards discovering anticancer agents derived from natural products that can induce apoptosis. Natural products are considered safer for human consumption as they have a wide range of therapeutic benefits. Plant phytochemicals have long served as important structural templates in the development of cancer treatments. Therefore, the main purpose of this study was to determine the cytotoxicity effects and cellular death pathway exhibited by two native mangrove species, *Sonneratia alba* sp. and *Bruguiera cylindrica* sp., collected from Pengkalan Gelap, Setiu, Terengganu. Six extracts of varying polarities (methanol, ethyl acetate, hexane) were prepared from the twigs of the mentioned mangrove species and tested against breast cancer cells (MCF-7) and normal cells (L6). The MTS assay showed that only three extracts exerted significant cytotoxicity against MCF-7. *S. alba* in hexane extracts (SAH) showed the highest cytotoxicity at an IC₅₀ value of 3.47 µg/mL, followed by *B. cylindrica* in hexane extract (BCH) and *S. alba* in ethyl acetate extract (SAEA) with IC₅₀ values of 7.762 µg/mL and 27.8 µg/mL, respectively. It is worth

noting that all six extracts were weak and non-cytotoxic to L6. The apoptosis assay using Annexin V Apoptosis Detection FITC prevailed that BCH, SAH, and SAEA at the IC₅₀ concentration, induced MCF-7 death via the preferred apoptotic pathway. The trypan blue exclusion assay showed that BCH, SAH, and SAEA extracts reduced MCF-7 viability by less than 50% after 72 hours. In an effort to identify anticancer agents with synergistic effects between the potent BCH, SAH, and SAEA, only SAEA was found to be a strong antioxidant agent with an IC₅₀ value of 0.125 mg/mL. The extracts contained major phytochemicals classes such as coumarins, phenols, alkaloids, flavonoids, terpenoids, and their derivatives, implying that *S. alba* and *B. cylindrica* hold a great promise as supplemental chemotherapy in the treatment of breast cancer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu
sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

**ELUSIDASI SIFAT ANTIKANSER EKSTRAK *Bruguiera cylindrica* dan
Sonneratia alba TERHADAP SEL KANSER PAYUDARA**

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2025

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Kanser payudara kekal sebagai salah satu daripada lima kanser penyebab kematian di seluruh dunia. Namun, kematian akibat kanser payudara nampaknya semakin meningkat yang tidak berkaitan penyakit. Kemoterapi semi-sintetik yang tidak semula jadi telah terbukti menyumbang kepada masalah ini, kerana ia menyebabkan kesan sampingan yang tidak diingini dan nekrosis dalam sel-sel yang sihat. Oleh itu, penyelidikan onkologi semasa telah beralih kepada penemuan agen antikanser yang berasas dari produk semula jadi yang boleh menginduksi apoptosis. Produk semula jadi dianggap lebih selamat untuk penggunaan manusia kerana mereka mempunyai pelbagai manfaat terapeutik. Fitokimia tumbuhan telah lama berfungsi sebagai templat struktur penting dalam pengembangan rawatan kanser. Oleh itu, tujuan utama kajian ini adalah untuk menentukan kesan sitotoksik dan mekanisme kematian sel yang ditunjukkan oleh dua spesis asal bakau tempatan, *Sonneratia alba* dan *Bruguiera cylindrica*, yang diambil dari Pengkalan Gelap, Setiu, Terengganu. Enam ekstrak dengan polariti yang berbeza (metanol, etil asetat, heksana) dihasilkan daripada hujung ranting muda spesis bakau yang disebutkan dan diuji terhadap sel kanser payudara (MCF-7) dan sel normal (L6). Ujian MTS menunjukkan bahawa hanya tiga ekstrak

mempunyai kesan sitotoksiti yang signifikan terhadap MCF-7. *S. alba* dalam ekstrak heksana (SAH) menunjukkan sitotoksiti tertinggi dengan nilai IC_{50} 3.47 $\mu\text{g/mL}$, diikuti oleh *B. cylindrica* dalam ekstrak heksana (BCH) dan *S. alba* dalam ekstrak asetat etil (SAEA) dengan nilai IC_{50} masing-masing 7.762 $\mu\text{g/mL}$ dan 27.8 $\mu\text{g/mL}$. Menariknya, keenam-enam ekstrak menunjukkan sifat lemah dan tidak sitotoksik kepada L6. Asai apoptosis menggunakan Annexin V Apoptosis Detection FITC menunjukkan bahawa BCH, SAH, dan SAEA pada dos IC_{50} , menginduksi kematian MCF-7 melalui mekanisme apoptosis yang diinginkan. Ujian pengecualian tripan biru menunjukkan bahawa ekstrak BCH, SAH, dan SAEA dapat mengurangkan populasi sel MCF-7 yang hidup kurang daripada 50% selepas 72 jam. Dalam usaha untuk mengenal pasti agen antikanser dengan kesan sinergistik antara BCH, SAH, dan SAEA, hanya SAEA menunjukkan kesan antioksidan yang kuat dengan nilai IC_{50} 0.125 mg/mL. Ekstrak- ekstrak ini didapati mengandungi kelas-kelas fitokimia utama seperti kumarin, fenol, alkaloid, flavonoid, terpenoid, dan derivatifnya, menunjukkan bahawa *S. alba* dan *B. cylindrica* mempunyai potensi besar sebagai kemoterapi tambahan dalam rawatan kanser payudara.

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In all honesty, this journey means more than academic achievement to me. Finishing this research got me into mixed feeling of happiness and sad, as completing my master is the last promise I made with my late Abah before he unexpectedly passed away during Semester 1. Even he is no longer here to witness this, I dedicated every each of good deed that may come from this study for my Abah, Nadzrin bin Abu Bakar and may Allah keep him safe and ease his path to Jannah. I also dedicated this thesis to my dearest mother, Tuan Sharifah Nor Rihan and my siblings, who endlessly motivate me to complete my study. I also want to thank my other half, *M.A.A* for always taking care of me until I found the strength to stand again.

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APPROVAL

I certify that an Examination Committee has met on 2nd July 2025 to conduct the final examination of Norain binti Nadzrin, on her Master of Science thesis entitled “Elucidating Anticancer Properties of *Bruguiera Cylindrica* and *Sonneratia Alba* Extracts Against Breast Cancer Cells” in accordance with the regulations approved by the Senate of Universiti Malaysia Terengganu. The Committee recommends that the candidate be awarded the relevant degree. The members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UMT or other institutions.



NORAIN BINTI NADZRIN

Date: 31st July 2025

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LIST OF ABBREVIATIONS

°C	Celcius
µg	Microgram
µL	Microlitre
µM	Micromolar
Akt	Ak strain transforming
ANOVA	Analysis of variance
BCEA	<i>Bruguiera cylindrica</i> ethyl acetate extract
BCH	<i>Bruguiera cylindrica</i> hexane extract
Bcl2	B-cell lymphoma 2 protein
BCM	<i>Bruguiera cylindrica</i> methanol extract
BMI	Body mass index
BRCA	Breast cancer gene
Caspase	Cysteine-dependent aspartate-directed proteases
CCND1	Cyclin D1
CDH1	Cadherin 1
CHEK2	Checkpoint kinase 2
CIPN	Chemotherapy induced peripheral neuropathy
CO ₂	Carbon dioxide
DCIS	Ductal carcinoma in situ
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER+	Oestrogen receptor positive
FADD	Fas-associated protein with death domain
FBS	Fetal bovine serum
FITC	Fluorescein Isothiocyanate
g	Gram
GLOBOCAN	Global Cancer Observatory
h	Hour
HER2	Human epidermal growth factor receptor 2
HR+	Hormone receptor positive
IC ₅₀	Half-maximal inhibitory concentration
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
L6	Mouse myoblast skeletal muscle cell
LCIS	Lobular carcinoma in situ
LCMS	Liquid Chromatography- Mass Spectrometry
MCF7	Michigan Cancer Foundation – 7
mg	Milligram
mL	Millilitre
mTOR	Mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MYC	MYC proto-oncogene, bHLH transcription factor
NCD	Non-communicable disease
p53	Tumour protein 53
PALB2	Partner and localizer of BRCA2
PBS	Phosphate-buffered saline
Pen-Strep	Penicillin-streptomycin
PI3K	Phosphoinositide 3-kinase
PR+	Progesterone receptor positive
PTEN	Phosphatase and Tensin homolog
PTFE	poly(1,1,2,2-tetrafluoroethylene)
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute 1640 Medium
SAEA	<i>Sonneratia alba</i> ethyl acetate extract
SAH	<i>Sonneratia alba</i> hexane extract
SAM	<i>Sonneratia alba</i> methanol extract
TNBC	Triple negative breast cancer
TNFR1	Tumour necrosis factor receptor 1
VS	Vincristine sulphate
v/v	Volume per volume
WHO	World health Organisation

CHAPTER 1

INTRODUCTION

1.1 Research Background

Oncology remains a great challenge as cancer is a non-communicable disease (NCD) that adaptively evolve and require complex individualised therapy (Singh et al., 2024). Even with long history in healthcare system, cancer is still a significant global burden accounted for mortality rate of 9.7 millions and 20 millions of new incidences across the globe (International Agency for Research on Cancer, 2022). The top five global surges in cancer cases includes lung cancer (12.4%), female breast cancer (11.6%), colorectal cancer (9.6%), prostate cancer (7.3%), and stomach cancer (4.9%) (Bray et al., 2024). Breast and lung cancer were held accounted for majority of fatalities for both men and women (Bray et al., 2024).

Breast cancer was found to be the main causal of women-related cancer death, and charted second as most common cancer concerning both genders globally (Arnold et al., 2022; World Cancer Research Fund, 2022). In Malaysia, the Global Cancer Observatory (GLOBOCAN) reported in 2022 that breast cancer was the most lethal cancer for both sexes, accounted of 8,371 incidences and 3,526 mortalities. Breast cancer demonstrates a more rapid invasiveness in women compared to men, as the primary risk factor for susceptibility, being female gender (Giaquinto et al., 2024). Worthy to note, women are more often impacted by breast cancer due to the presence

of several additional risk factors, inclusively hormone regulation, ageing, and distress, which hastened metastasis and leading to poorer survival rates (Lee et al., 2011; Park et al., 2022).

Depending on breast cancer stages, a singular or combination of treatments involving chemotherapy, radiotherapy, hormonal therapy, and surgical intervention are prescribed. Surgical methods such as mastectomy, lymphadenectomy, and lumpectomy serve as the initial procedure in reducing the localised tumour bulk. However, surgical resection poses significant limitations, as it potentially accelerates metastasis while leading to permanent loss of organ and nerve functions (Jiao and Lv, 2023; Tohme et al., 2017). Post-surgery, radiotherapy is usually paired to reduce the chances of cancer relapses. Prolonged exposure to high radiation levels not only eradicates residual malignant cells but also jeopardise healthy cells. Thus, systemic therapies inclusively hormonal therapy and chemotherapy are gaining preference and signify the future trajectory of cancer medications (Anand et al., 2022).

Hormonal therapy suppresses the production of cell proliferation hormones, and has been shown to be effective against hormone-receptor-positive breast cancer only (Lee et al., 2023). On the other hand, chemotherapy demonstrates vast efficacy in cases of metastatic breast cancer (Larsson et al., 2024). Given that chemotherapeutic drugs are frequently administered intravenously, orally, or intrathecally, the drug inhibits malignant cells in distant area as it circulates through the bloodstream (Dewhirst and Secomb, 2017). Henceforth, chemotherapy has rightly earned its reputation as the most commonly prescribed treatment globally. Regardless, prolonged treatment period with synthetic chemotherapeutic medications has shown adverse side effects such as hepatotoxicity, hair loss, infertility, extreme exhaustion, and myelosuppression (Amjad et al., 2023; Anand et al., 2022).

Patients suffer with greater discomfort as synthetic chemotherapy prescribed such as docetaxel, doxorubicin, and epirubicin carries the potential to exert unnatural, necrosis pathways (Christidi and Brunham, 2021; Franks et al., 2023; Moyle et al., 2021). Acute neuropathy complications might occur as necrosis induces the release of inflammatory mediators, leading to tissue inflammation and ulceration. In addition,

synthetic analogue-drugs are found to be uncertain in specifically targeting malignant cells, resulting in acute recurrence and ultimately deteriorating overall survivor health (Amjad et al., 2023). Considering that existing anticancer drugs induce adverse side effects, there is an urgent need to discover and formulate efficient therapeutic strategies against this lethal disease. Thereby, oncology research is currently focused on the development of natural-based chemotherapeutic drugs that can deliver targeted and apoptosis-inducing therapy (Carneiro and El-Deiry, 2021).

Natural products and their derivatives are the primary ingredients for most remarkable pharmaceutical medications today (Nasim et al., 2022). They are widely recognised to possess the ability to treat a variety of human diseases, including cancer, immunological disorders, cardiovascular complications, and infectious diseases (Atanasov et al., 2021). Approximately 35,000 plants were examined for anticancer properties, and 3000 plants showed consistent cytotoxicity towards cancer (Desai et al., 2008). In the application of natural products as cancer treatments, about 60% of approved drugs in clinical sectors are natural origins and their semi-synthetics (Chunarkar-Patil et al., 2024; Demain and Vaishnav, 2011). Screening and research on natural product are currently underway to enhance existing therapy modalities.

Malaysia is blessed with one of the largest extensions of natural-occurring mangrove forests, with 70 species from 28 families (Omar et al., 2020). Markedly, this mangrove forest has not yet been thoroughly researched and investigated for its therapeutic properties. Mangroves are trees and shrubs that thrive along shorelines in tropical and subtropical regions. Growing in stressful environments such as high salinity and fluctuating tides, mangroves synthesise bioactive substances to enhance their survivability (Sudhir et al., 2022). Past literature has identified a variety of isolated compounds from mangrove extracts, including alkaloids, flavonoids, tannins, saponins, phenolics, triterpenes, and (Mitra et al., 2021). Abundant in these major phytochemicals and bioactive compounds, mangroves are well-known for their diverse biological activities including antibacterial (Doifode et al., 2023), antiviral (Ameer et al., 2024), anti-fungal (Sulaiman et al., 2022), anti-inflammatory (Jiko et al., 2024), anticancer (Cerri et al., 2022), antioxidant (Hidayati et al., 2023) and insecticidal (Rahman et al., 2024).

Research is currently taking place to further ascertain whether mangroves may possess potential as a chemotherapy agent via favoured apoptotic mechanisms. Apoptosis, or programmed cell death, is the mechanism of eradicating infected, damage or mutated cell through intrinsic or extrinsic pathways. In contrast to pain-inducing necrotic tissue, apoptotic tissue generally does not elicit pain, as it does not trigger inflammatory responses during disintegration of injured cells (Bock and Riley, 2023). Thus, targeting apoptosis and understanding the pathway in drug discovery is the main strategy in chemotherapy production. Regulated apoptosis prevents the release of damaged cellular components to neighbouring cells, making it very quick and the least harmful to surrounding tissues (Fuchs, 2019). Hence, this approach might mitigate the overall harmful side effects of cancer medication.

Also, mangroves are renowned for their antioxidant properties. Moderate to potent antioxidant capabilities could reduce aggressive cancer progression by neutralising free radicals that induce DNA damage (Didier et al., 2023). The synergistic approach of anticancer and antioxidant properties may enhance the sensitivity of malignant cells to therapies while protecting healthy cells during and after post-treatment period. Since anticancer and antioxidant properties are correlated, this finding will pave the way in enhancing better chemotherapeutic drug production for a better recovery process. Significantly, identification of potential chemotherapeutic agents from mangroves might be a potential breakthrough as a promising supplementation for breast cancer medication.

1.2 Problem Statement

Breast cancer treatment necessitates a comprehensive evaluation by medical professionals, as the tendency of a cancerous cell to metastasise does not consistently adhere to the same timeframe or risk factors. This complicates the battle against breast cancer as a slight variation in progenitor cells mutations results in diverse subtypes. The prior statement thoroughly elucidates the prolonged medication period each therapy regimen to hinder tumorigenesis invasion to other bodily regions.

However, prolonged treatment with synthetic chemical-based therapy regimens induces persistent pain in patients, exacerbating adverse side effects and hindering the total recovery of cancer patients. Numerous clinical reports indicate that synthetic-chemical-based chemotherapy results in a broad spectrum of adverse effects, including nausea, vomiting, headache, fast palpitations, hair loss, severe chest pain, acute fatigue, memory disturbances, and numbness due to chemotherapy induced peripheral neuropathy (CIPN) (Katta et al., 2023). Furthermore, a study indicated that prolonged chemotherapy can adversely affect cardiac health, leading to significant chest pain, pericarditis, and acute cardiotoxicity (Tyebally et al., 2022).

The occurrence of these adverse side effects post-treatments appears to be associated with synthetic chemical-based chemotherapy, which frequently results in necrosis (Zhang et al., 2014). Significant neuropathic impairment occurs as necrosis damages nerves, disrupts normal cell signalling processes, and eliminates healthy cells in the effort to manage malignancies. Therefore, the primary focus of oncological research is the identification of natural resources containing bioactive compounds that can induce targeted apoptosis, as evidenced by screenings of various natural products against cancerous cells. The closure of endearing mangroves represents an important breakthrough, as these natural resources are abundant in phytochemicals and pose fewer safety concerns for consumption.

1.3 Significance of Study

Meeting the current trend of a safer natural therapeutic drug with multi-dependable metabolites, mangrove forests have inspired more researchers and pharmacologists to direct screening studies. Apparently, continuous exposure to extreme conditions such as fluctuating tides, high salinity, and complex waves gives mangroves the capability to adapt well through their unique features of complex root systems and salt filtration abilities. Also, to maintain survivability, these plants are commonly reported consist of with four key classes of secondary metabolites, including phenolic compounds, terpenoids, alkaloids, and sulphur-containing chemicals, which mediated self-protection against other biotic constraints (Guerriero et al., 2018).

These major classes of active metabolites are one of the main factors that ensure the resiliency of mangroves to adapt well and survive in harsh climates (MacDonald et al., 2025). Previous studies revealed that phenolics, flavonoids, and terpenoids present in mangrove extracts are carcinogenic against cancer cells (Saroyo and Saputri, 2021). Thus, being rich in major phytochemicals, mangroves have the ability to induce antiproliferation and halt metastasis of invasive cancer (Lu et al., 2012). Apparently, after analysis, mangrove sp. was found to inhibit the viability and migration of cancer in the same manner as current plant-based chemotherapeutic drugs which both indicated cell cycle arrest and DNA impairment.

Therefore, in this study, several assays were carried out to determine the cytotoxicity effects of selected mangrove species on breast cancer cell line (MCF-7), and normal cell line (L6). It is hoped that bioactive compounds present in the mangrove extracts could exhibit significant anticancer effects through apoptosis without harming normal cell line. Thus, this finding might add an additional value of mangroves as a promising supplemental chemotherapeutic-agents, align with the current scope of endearing natural resources as raw material for chemotherapeutic drugs.

1.4 Objectives

The general objective of this study is to screen the anticancer potential of *Bruguiera cylindrica* and *Sonneratia alba* extracts as a breast cancer inhibitor in the induction of desirable apoptosis pathways.

Specific objectives:

1. To extract active compounds from *Bruguiera cylindrica* and *Sonneratia alba* prior further analysis.
2. To analyse the cytotoxicity effects of *Bruguiera cylindrica* and *Sonneratia alba* extracts against MCF-7 cell line proliferation.
3. To evaluate the phytochemicals of *Bruguiera cylindrica* and *Sonneratia alba* extracts and correlates the anticancer and antioxidant bio-activities.

CHAPTER 2

LITERATURE REVIEW

2.1 Insight on Human Breast Cancer

Cancer continues to pose a great challenge to humanity today, even though the earliest documented case of human cancer can be traced back to Edwin Smith's Papyrus around 3000 BC. According to the World Health Organisation (WHO), cancer caused approximately 10 million deaths in 2020, accounting for one in every six deaths worldwide. The global incidence of cancer is expected to increase over the next two decades, impeding social and economic development due to rising treatment costs and loss of human productivity (Wu et al., 2024). National Cancer Institute stipulated that the projected increase in global cancer incidence to 19.9 million cases with 15.3 million deaths by 2040 is even more concerning. Basically, cancer is defined as an uncontrolled cell proliferation disease that can metastasise to other parts of the body. This disease is commonly associated with cellular mutations and can be classified as carcinoma, sarcoma, lymphoma, or leukaemia.

Carcinoma accounts for 80% to 90% of all cancer diagnoses, due to mutations in epithelial cells that largely line both the inside and outside of the body (Gotzmann et al., 2004; Liang et al., 2022). One of the most common types of carcinomas is breast cancer. Breast cancer can affect both sexes; however, statistics show that breast cancer is more likely to affect women (99%), with only 0.5% - 1% of cases involving male breast cancer (Ionescu et al., 2022). According to WHO, breast cancer, which is the

most commonly diagnosed in 157 out of 185 countries, led to the deaths of 670,000 women in 2022. This disease represents a significant challenge in women's health, accounting for one-quarter of all cancer cases combined, found occurring in every part of the world (Arnold et al., 2022). It is very concerning that breast cancer accounts for more than one in every ten new diagnoses and is the second leading cause of women's declining mortality worldwide (Menon et al., 2024).

Malaysia has high incidences of breast cancer. This disease is the most common types of cancer in Malaysia, with one in every five people suffering from breast cancer of any gender (Malaysia Ministry of Health, 2019). Furthermore, Malaysia's National Cancer Registry department reported that breast cancer is the most common cancer in women, with one in every nineteen women developing breast cancer at least once in their lifetime (Lee et al., 2019). When compared to other ethnicities, Chinese women have the highest cancer survival rate (48.2%), followed by Indian women (47.2%), and Malay women have the lowest survival rate (39.7%). This indicates the complexity of breast cancer as a disease, as it is widely reported to require personalised treatment.

Breast cancer occurs with unregulated proliferation of mammary cells into a tumour mass that can invade another part of the body through the bloodstream and lymphatic system. Commonly, carcinogenesis of breast cancer occurs in the cell lining of the milk ducts or lobules of the breast. Lobules are the milk-producing glands; meanwhile, milk ducts (lactiferous ducts) are the thin, tube-like structures that transport the milk from the lobule to the nipples. Breast cancer can be divided into two major types, in which ductal carcinoma is a condition where a tumour is initiated in the milk duct, and lobular carcinoma is when the tumour starts in the milk gland. Apparently, breast cancer can be invasive and non-invasive. Non-invasive Ductal Carcinoma In-Situ (DCIS) and Lobular Carcinoma In-Situ (LCIS) are when the cancerous cells are localised within the milk duct and lobule only. However, as the tumour started to infiltrate beyond the milk duct and lobule, they were known as Invasive Ductal Carcinoma (IDC) and Invasive Lobular Carcinoma (ILC). Further description of breast cancer progression in each stage is listed in Table 2.1 below

Table 2.1 Stages of breast cancer development and progression.

Cancer stage	Description	Reference
Stage 0	Early form of cancer	(National Cancer Institute, n.d.)
	Occur due to abnormal proliferation of breast cell	
	Pre or non-invasive	
	Diagnosed as carcinoma in-situ	
	Precursor for invasive carcinoma	
Stage I, II, III	Cancer size gradually grows larger	
	Invasion past the basement membrane	
	Cancer spread to neighbouring tissue	
	Invasive cancer	
	Affected many lymph nodes	
Stage IV	Metastatic cancer	
	Spread beyond neighbouring lymph nodes	
	Cancer invades to other part of the body	
	Invasive cancer	
	Final stage cancer	

Early detection of stage 0 through mammography allows removal of tumour bulk through surgical procedure. Nonetheless, DCIS and LCIS exhibit a heterogeneous nature and are considered precursors to invasive cancer. Therefore, if stage 0 breast cancer is left untreated, they can further progress into invasive cancer, IDC and ILC (Buerger et al., 2000; Wang et al., 2024). IDC and ILC are distinct in morphology and have different manifestation patterns, where IDC is more prone to spread towards the brain, bone, liver, and lung; meanwhile, ILC tends to invade the urinary tract, reproductive tract, leptomeninges, gastrointestinal tract, retroperitoneum, and abdominal lining (Borst and Ingold, 1993; Fernández et al., 2011; Leone and Leone, 2015; Rashid et al., 2021). Although ILC is the second most common diagnosis, it is more associated with a poor prognosis scenario than IDC. Patients diagnosed with ILC exhibit poorer survival outcomes due to several factors, including late detection due to

single-file growth patterns, larger tumour size, a higher rate of distant metastases, and reduced sensitivity to chemotherapy (Pereslucha et al., 2023).

Furthermore, the prognosis and severity of breast cancer are determined by hormone sensitivity. Not all breast cancers are hormone sensitive, but 70% of cases show that oestrogen and progesterone influence breast cancer cell proliferation (Al-Shami et al., 2023). This type of cancer is known as hormone receptor-positive (HR+), and it can be further divided into oestrogen receptor-positive (ER+) and progesterone receptor-positive (PR+) breast cancers. Similarly, both PR+ and ER+ types amplified cancer cell development and proliferation by the binding of hormones to receptors found in the nucleus or on the membrane of breast cancer cells (Zattarin et al., 2020). ER+ breast cancer represents the predominant form of hormone receptor-positive breast cancer, constituting 80% of cases of invasive breast cancer (Wei, 2023). Even though HR+ breast cancer has a favourable prognosis, this subtype still highly exposes patients to potential metastatic bone disease (Bae et al., 2015; Lee et al., 2011). There are several other breast cancer sub-types, including inflammatory breast cancer, Paget's disease of the nipples/breast, and HER-2 positive breast cancer, but currently the most aggressive is the triple-negative breast cancer (Valluri et al., 2025).

Triple negative breast cancer (TNBC) represents one of the most challenging subtypes in present-day breast cancer treatment, as it is reported with the poorer prognosis and inadequate treatment modalities (Sukumar et al., 2021). This condition occurs as TNBC appear to be complex type of breast cancer with high molecular heterogeneity (Asleh et al., 2022). The triple negative stands for the lack of all the three important cancer biomarkers inclusively oestrogen receptors, progesterone receptors and human epidermal growth factor receptor-2 (HER2). This further emphasized the low sensitivity of TNBC towards chemotherapy and hormonal therapy as the presence of aforementioned cancer biomarkers are crucial in allowing targeted therapy to occur. Therefore, TNBC is described as a combination of several breast cancer subtypes that contributed to multi-variety of transcriptomic and genomic factors, leading to different respond and behaviour towards clinical treatment (Marra et al., 2020). Additionally, TNBC is reported to be the major contributor of breast cancer related death even though only represented 20% of all breast cancer subtypes (Rana et al., 2021).

All-inclusive, the presence of various breast cancer subtypes indicates that breast cancer evolves throughout time due to epigenetic changes such as DNA methylation, histone modification and alteration in non-coding RNA (Prabhu et al., 2024; Xiong et al., 2025). This epigenetic change further led to abnormal clonal expansion of mutated cells (Baylin and Jones, 2016). The crosstalk interaction between mutant cells and healthy cells within a tumour microenvironment appears to promote cancer growth, angiogenesis, and invasiveness (Ebeling et al., 2023; Naito et al., 2022). This interaction further fuelled clonal selection of the mutated cells, thus leading to cancer heterogeneity, where different populations of cancer cells exist within a tumour site exerted distinct and variant phenotypes (Mierke, 2024).

Cancer heterogeneity is the core problem in clinical management of breast cancer, as it is found to enhance cancer survivability, thus hindering diagnosis and prognosis of breast cancer. Not only does heterogeneity allow the formation of several cancer cell lines, but this variability further influences treatment efficacy in clinical settings, as the same type of cancer is found to jeopardise individuals differently (MacDonald et al., 2025). Given that statement, before cancer progresses into a complex heterogeneous mass, medical practitioners address breast cancer risk factors to reduce progressive cancer risk (Marino et al., 2024).

2.2 Breast Cancer Risk Factor

Substantially discussed by oncologists, the main cause of breast cancer is still unknown. However, this disease shows the affiliation of multiple contributing factors such as heritable genes, environmental exposure, nutritional intake, hormone production, and genetic disruptions in accommodating tumour formation to cancerous invasion (Admoun and Mayrovitz, 2022). These contributing factors are known as cancer risk factors. Apparently, these risk factors are important in current breast cancer treatment, as they provide researchers and health sectors with adequate information, thus allowing better implementation of preventive strategies in arresting cancer from becoming more aggressive.

Cancer risk factors remain relevant in improving primary breast cancer prognostics, as they are found to influence overall patient survival and cancer recurrence rate (Abubakar et al., 2021). Considering cancer risk factors gives significant advantage in managing this disease, as controlling the risk factors has been shown to reduce breast cancer development and progression (Menon et al., 2024). Breast cancer risk factors are divided into two categories, inclusively modifiable and non-modifiable (Ho et al., 2020). Figure 2.2 provides examples of modifiable and non-modifiable breast cancer risk factors.

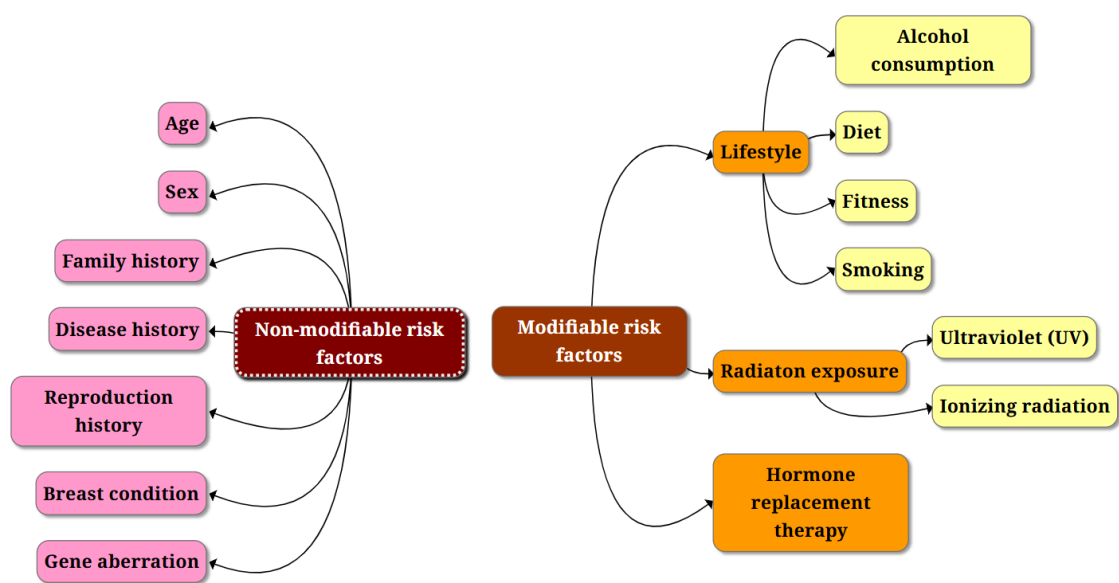


Figure 2.2 The common risk factors associated with breast cancer (Created using Canva).

Non-modifiable risk factors are unchangeable traits that individuals are born with (Boehme et al., 2017; Sloan and Gelband, 2007; Weeden et al., 2023). Apparently, women are impacted greatly by breast cancer compared to men because women are more prone to hormonal imbalances (Admoun and Mayrovitz, 2022). Breast cancer-related mortality is greater in women over the age of 50, and they were found to experience worse diagnosis and prognosis (Li et al., 2024). Women who have first-degree relatives (sister, mother, and daughter) with a history of breast cancer are 2-3 times more likely to be threatened (Liu et al., 2021; Menon et al., 2024). Furthermore, a person with a personal history of primary cancer is more likely to get a recurrence in either breast (Menon et al., 2024). Breast cancer rates are greater in women who do not have children and have late first-time pregnancies because they are exposed to

more oestrogen throughout their lives (Londero et al., 2024). Women are particularly vulnerable because they have complex breast structures with dense breast and glandular tissue, increasing the probability of gene mutation (Feng et al., 2018).

Meanwhile, modifiable risk factors are often acquired through lifestyle choices and environmental exposure (Ding et al., 2022). Alcohol consumption is highly linked to cancer angiogenesis, with drinkers having a 21% higher chance of developing breast cancer than non-drinkers (Chen et al., 2024; Feng et al., 2018). Smoking undoubtedly contributes to breast cancer mortality where death rates are greater among current smokers (28%) than among nonsmokers (Sollie and Bille, 2017). Diet and exercise are two variables that contribute to body mass index (BMI), where obesity in postmenopausal women can lead to elevated oestrogen levels, thus increasing the risk of breast cancer (Picon-Ruiz et al., 2017). Radiation exposure is undoubtedly a significant component of cancer development, since it may cause DNA damage and consequent growth of mutated cells. Breast cancer rates are also observed to be increased with extended hormone replacement treatment that included oestrogen and synthetic progestin (Micha et al., 2022).

According to Loftsrød et al. (2020), avoiding unfavourable lifestyle habits such as binge drinking, smoking, and eating an unhealthy diet might delay cancer progression and reduce death in breast cancer patients. As a result, health organisations capitalise on this modifiable risk factor and use it to establish standards for community awareness of breast cancer. Delving deeper in this context, non-modifiable and modifiable risk factors projected two distinct types of breast cancer: sporadic and hereditary. Hereditary breast cancer is caused by non-modifiable breast cancer risk factors. In contrast, sporadic cancer cases are linked with modifiable risk factors. According to statistics, 90% - 95% of breast cancer incidences are sporadic, while hereditary cancer accounts for just 5% - 10% (Apostolou and Fostira, 2013; Menon et al., 2024).

Although sporadic cancer contributed to the majority of the breast cancer cases, this type of cancer is less to almost non-heritable (Lu et al., 2014). On the other hand, familial cancer is highly heritable, as a faulty gene can be potentially passed down

through generations (Hemminki et al., 2021). Since sporadic cancer arose gradually due to external risk factors, this type of cancer usually required time to initiate mutations in germline cells and manifest symptoms in middle to late adulthood (Ferrell et al., 2020). In contrast, individuals with a family history of cancer face a greater danger of developing aggressive malignancies at a young age due to the presence of a dominant mutant gene since birth (Rahner and Steinke, 2008). Therefore, as revealed by several studies, hereditary cancer poses a significantly higher risk of death (Wu et al., 2018). However, it is interesting to note that both sporadic and hereditary breast cancer incidences started with genetic mutation risk factor.

As evidenced through several research and clinical reports, cancer is a genetic disorder that is highly dependent on metabolic pathways for growth and development (Gyamfi et al., 2022; Huang et al., 2025). Genetic instability leads to DNA mutation, characterised by chromosome rearrangement, point mutations and disruption of DNA repair mechanisms. Coupled with the modification and destabilisation of histones acetyl and chromatin damages, the breast cancer gene will be rapidly amplified, leading to cancer stemness and metastases (Audia and Campbell, 2016). Sporadic cancer is associated with the myelocytomatosis oncogene (MYC), Cyclin D1 protein (CCND1) and human epidermal growth factor receptor 2 (HER2). Meanwhile, hereditary cancer is commonly mediated by breast cancer genes (BRCA1/2), partner and localiser of BRCA2 (PALB2), checkpoint kinase 2 (CHEK2), cadherin 1 (CDH1), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and tumour protein 53 (TP53). Further descriptions of common genes associated with breast cancer and their carcinogenic mechanisms are listed in Table 2.2 below.

Table 2.2 Gene associated with breast cancer development and their mechanism of actions.

Gene	Mechanism of Action	Reference
MYC	MYC overexpression leads to inhibition of pro-apoptotic gene such as p21 and p53, preventing DNA damage response in cancer cell.	(Dhanasekaran et al., 2022)
CCND1	Accelerate cell cycle phase from G ₁ to S phase by activation of cyclin-dependant kinases (CDK4/6).	(Wang et al., 2012, 2018)

HER2	High level of HER2 activated P13K/AKT/mTOR pathway, enhance migration and survival capability of cancer cells. (Carpenter and Lo, 2013)
BRCA1/2	Nonsense and frameshift mutations of BRCA prevent DNA damage responses thus promoting accumulation of mutated gene. (Fu et al., 2022)
PALB2	Mutation of PALB2 hinder the repair of homologous recombination in DNA damage responses. (Nikkilä et al., 2013)
CHEK2	Mutated CHEK2 prevent apoptosis of cancer cell by altering cell cycle phase at G ₁ checkpoint, allow cancer cell to further enter S phase and further replicate the damage cellular content. (Luo et al., 2018)
CDH1	Mutated CDH1 causes low expression of E-cadherin protein that will trigger epithelial-mesenchymal transition (EMT) and further support cancer migration activity. (Zhou et al., 2024)
PTEN	Loss functioning PTEN due to gene mutations promote PI3K/AKT pathway, thus increasing cancer survival and carcinogenesis rate. (Tufail, 2025)
TP53	Mutation in TP53 inhibits the usual stress response due to DNA damage; thus, apoptosis is not activated, allowing the cancerous cell division cycle to occur. (Marei et al., 2021)

Although genetic mutation plays a significant role in cancer development, constant carcinogenesis would be impossible without the involvement of another risk factor. Precisely, modifiable and non-modifiable risk variables obviously influence one another in the creation of an optimal tumour microenvironment to facilitate cancer invasiveness. Identifying cancer risk factors is significant in general because it may give improved understanding of the cancer molecular pathway, enabling the development of a reliable and effective therapy strategy. This stage also enables researchers to optimise current treatment options in both local and systemic therapies,

all of which are presently used in clinical settings. Improving these therapy options will improve patients' quality of life throughout the neoadjuvant period, as well as in post-treatment and palliative care.

2.3 Current Breast Cancer Therapy Intervention

The strategies for combating aggressive metastases in neoadjuvant and adjuvant cancer cases are expanding. Cancer therapy can be divided into two categories, inclusively localised treatment and systemic treatment. Localised treatment is recommended in early cancer stages when a cancer mass in a specific body part can still be completely removed. Meanwhile, systemic treatments are used in metastatic cases, where cancer has spread to a distant part of the body. Interestingly, a combination of local and systemic therapy has been shown to improve cancer prognosis (Ji et al., 2024; Liang et al., 2023).

As a result, the cornerstone of cancer treatment is to prescribe a combination of two or more mediated therapies to achieve a synergistic effect. Supporting the previous claim, a study clearly emphasises that scientists' perspectives on cancer have shifted as the advent of combination medicines has the potential to activate multiple pathways simultaneously, resulting in a more potent anticancer effect (Ayoub, 2021). Figure 2.3 below depicts the available breast cancer therapies and their examples in the medical sectors.

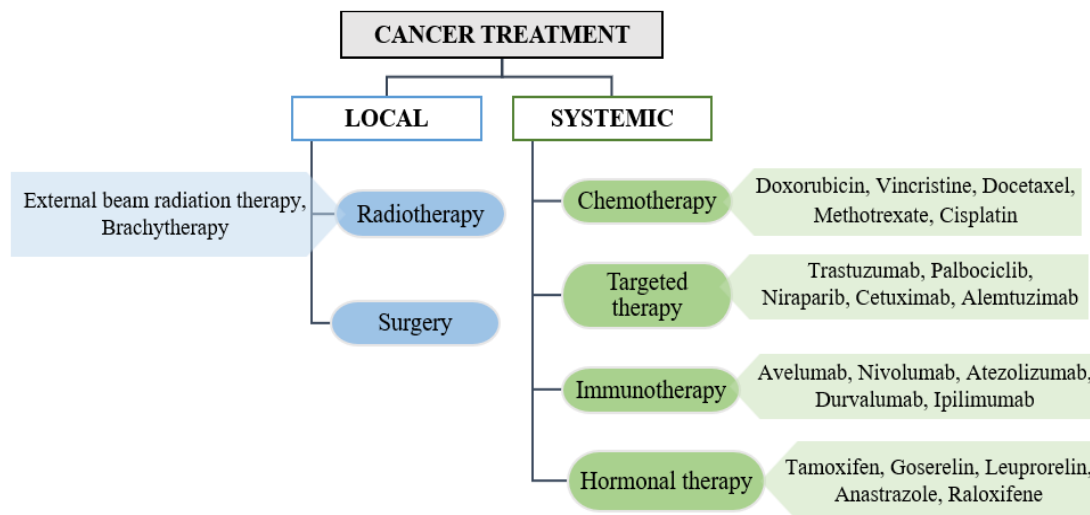


Figure 2.3 Common examples of current breast cancer treatment (Created using Canva) (Breast Cancer Now, 2022; Goulart and Shankaran, 2019; Maani and Maani, 2023; Qin et al., 2022; Shuel, 2022; Veazy, 2021).

With multi-variety options of treatment prescribed according to patient condition, further assessment of patients' life quality post-treatment is the vital endpoint of every cancer clinical trial to deduce the efficacy of medical parameters utilised (Paraskevi, 2012). While contemporary medication greatly enhanced the survival rates of cancer patients, survivors nevertheless face unpleasant treatment effects and consequences (Odle, 2014). Even cases of residual chronic pain post-therapy are frequently underreported; it is undisputed that present cancer treatment may degrade healthy tissue and have negative side effects (Reynolds, 2022). Apparently current cancer therapies are associated with adverse side effects, from the most common to severe, and cause death (Amjad et al., 2023; Domschikowski et al., 2021).

Cancer treatment's most common effects include lethargy, nausea and vomiting. Long-term medication may lead to several significant adverse effects, including appetite loss, hair loss, taste changes, diarrhoea, limb numbness, skin problems, flu-like symptoms, allergies, injuries of the mucosal line, memory disturbance, hearing failure, and kidney problems (Altun and Sonkaya, 2018). Moreover, the recovery from cancer is substantially self-control dependent. This further explains that women who are more emotionally vulnerable seem to bear more

pain going through systemic chemotherapy, radiation, and surgical procedures, as these mentioned methods exclude mixed emotions of fear, paradoxical anxiety, and paramount psychosocial distress (Paraskevi 2012). Not only that, patients are reported to have experienced even more acute pain with follow-up therapy (Lustberg et al., 2023). Further adverse side effects of each cancer treatment, including surgery, radiotherapy, chemotherapy, targeted therapy, immunotherapy and hormonal therapy, are explained below.

2.3.1 Chemotherapy

Although chemotherapy is accredited as the world's most utilized cancer therapy, undeniably this therapy procedure has its own adverse effects (Anand et al., 2022). Chemotherapy drugs that either originated from synthetic chemical or plant-based can be further subdivided into 5 main classes including alkylating agents, antimetabolites, mitotic spindle inhibitors, topoisomerase I/II inhibitors, and other derivatives (enzyme, antibiotics, proteasome inhibitors, tyrosine kinase inhibitors) (Bukowski et al., 2020).

The administration of certain chemo-drugs from classes topoisomerase I/ II inhibitors, vinca alkaloids, and platinum-based can be interlinked to severe high incidences of post-chemotherapy affecting a total of 44.5% out of 1000 patients (Ingrand et al., 2020). In addition, according to the study conducted by Katta et al. in 2023 to compare the differences between the statistic of clinal and patient's self-reported chemotherapy adverse effects, the clinical reports are founded to be rather generalized.

2.3.2 Immunotherapy

Immunotherapy can be classified into five major classes which are adoptive cell therapy, treatment vaccines, monoclonal antibodies, immune checkpoints inhibitors (ICI), and immune system modulators, which mainly kill cancer cells by activating immune responses in the patient's body (Kichloo et al., 2021). Similar to other treatment regimens, adverse effects of immunotherapy are directly proportional to the stage of cancer, body condition, duration of the treatment, and dosage of immunotherapy drugs taken (National Cancer Institute, n.d.).

2.3.3 Hormonal Therapy

The mechanism of hormonal therapy such as Tamoxifen, Exemastene, Letrozole, and Anastrozole hinders hormone receptor signals, leading to suppression of hormones that initiate metastasis (Ibrar et al., 2022). Cancer Research UK stated in 2022 that this suppression of signals could either block or lower the hormonal content in the body. Therefore, this therapy may cause unwanted side effects after treatment due to the loss of control, and imbalances of required hormones for regular bodily functions, perhaps leading to autoimmune illnesses. (Cancer Research UK, 2022).

2.3.4 Radiotherapy

Radiation toxicity can be observed as early as a few weeks after treatment (Majeed and Gupta, 2023) and, the most common side effects of radiation therapy experienced by the patients are skin changes, fatigue, limb weakness, and mucosa (American Cancer Society, 2022). Prolonged exposure to ionizing radiations can lead to chronic radiation-induced skin reactions (RISR) such as secondary skin cancers, telangiectasias, fibrosis, radiation-induced keratoses, acute ulcerations, and severe wounding (Wei et al., 2018). Other examples of RISR include erythema, dry desquamation, moist desquamation, atrophy, and nerve pain (Chan et al., 2014).

2.3.5 Surgery

Surgical procedure is the first prescribed treatment for breast cancer, however its efficacy is limited with localized cancer only. The results of cancer surgery are closely associated with elements such as body temperature, anaesthesia administration during surgery, blood transfusion, and the patient's post-operative status (Tohme et al., 2017). A study by Horowitz et al. in 2015 pinpointed that certain anaesthetics could impart an appropriate microenvironment for tumour growth. As evidence, quoted a study that worked with opioids, the reported outcomes stated that suppression of immunity and activation of stress responses promoted metastases occurrence (Horowitz et al., 2015).

2.4 Cancerous Cell Death Mechanism

To date, most of the cancer therapies are recorded to exhibit unnecessary pain-causing side effects that could lead to poor cancer prognosis. Several research studies have shown that this pain is subjected to the mechanism of cancerous cell death in response to treatment. The current therapy regimen carries the potential to kill the healthy cells causing side effects, as it is widely reported to cause necrosis in killing cancer cells (Peng et al., 2022; Zhang et al., 2014). Necrosis is an unnatural cell death pathway that usually occurs as cells experience irreversible injury, causing the cell to swell and release the degradative enzyme and cytokines to neighbouring cells (Khalid and Azimpouran, 2025; Ruffolo, 1964).

For example, synthetic chemotherapy and radiotherapy could induce necrosis in dense cancerous bulk (Hsu et al., 2018). Chemotherapy is designed to target and alter the genomic content of the cells; however, synthetic chemotherapies are found to have ascertain characteristics that might cause extensive and uncontrolled damage to cancerous cells. In the same manner, radiation therapy was also found to exert necrosis in cancerous cells, as excessive thermal and radiation exposure could lead to cell injuries and mediate the generation of reactive oxygen species (ROS) that could amplify

mutation. This condition of tissue injury further activated inflammatory responses, which are found to be the main pain-causing factor in this issue.

Necrosis is pain causing due to several factors. As necrosis activated the inflammatory responses, this cellular death pathway involved the release of inflammatory cytokines such as chemokines, interleukin and tumour necrosis factor, which will increase the sensitivity of pain receptors such as nociceptors (Glaubitz et al., 2023; Zhang et al., 2011). Other than that, necrosis is found to cause nerve damage as pressure from neighbouring swelling tissue presses on the nerve (Wallace and Perera, 2023). Furthermore, as the necrotising cell ruptures, the release of damaged intracellular content will surely subject the neighbouring tissue to unnecessary injuries and irritations. This emphasises the need to manage therapy-induced necrosis, as it is found to be the underlying condition that hinders patient recovery and survival.

Therefore, the current oncology research focus shifted towards finding anticancer agents with apoptosis potential. Apoptosis is a natural cellular death pathway that involves a regulated self-destructive mechanism. Basically, apoptosis is a highly controlled death mechanism that maintains cellular homeostasis by balancing dead and alive cells (He et al., 2024). Metabolically and morphologically, apoptosis is significantly different from necrosis, as it is an energy-dependent response that rapidly removes the damaged cell without alarming the inflammatory reaction (Guo et al., 2024). Morphologically, cells that undergo apoptosis appear to shrink with condensed chromatin and blebbing in the membrane before fragmenting into small vascular structures with protective membranes, called apoptotic bodies (Mustafa et al., 2024). The apoptotic bodies will then be engulfed by phagocyte cells.

Metabolically, apoptosis involved multiple signalling pathways that can be activated with various stimuli, such as internally, externally, therapeutically and even from signals of body developments. As apoptosis is found to be highly sensitive towards cellular stress, it is beneficial as the goal of therapeutic exposure, such likely with the usage of chemotherapy (Carneiro and El-Deiry, 2021). Apoptosis is deemed efficient in a targeted approach, as it is reported to successfully kill cancerous cells while minimising toxicity towards neighbouring tissue. Mainly apoptosis is the key

component in cancer treatment, as it shows a significant balance by allowing targeted treatment optimisation while being low hazardous to surrounding tissue as cancerous cells die. There are several chemotherapies known to induce apoptotic death in cancerous cells, such as paclitaxel, etoposide, cisplatin, and vincristine.

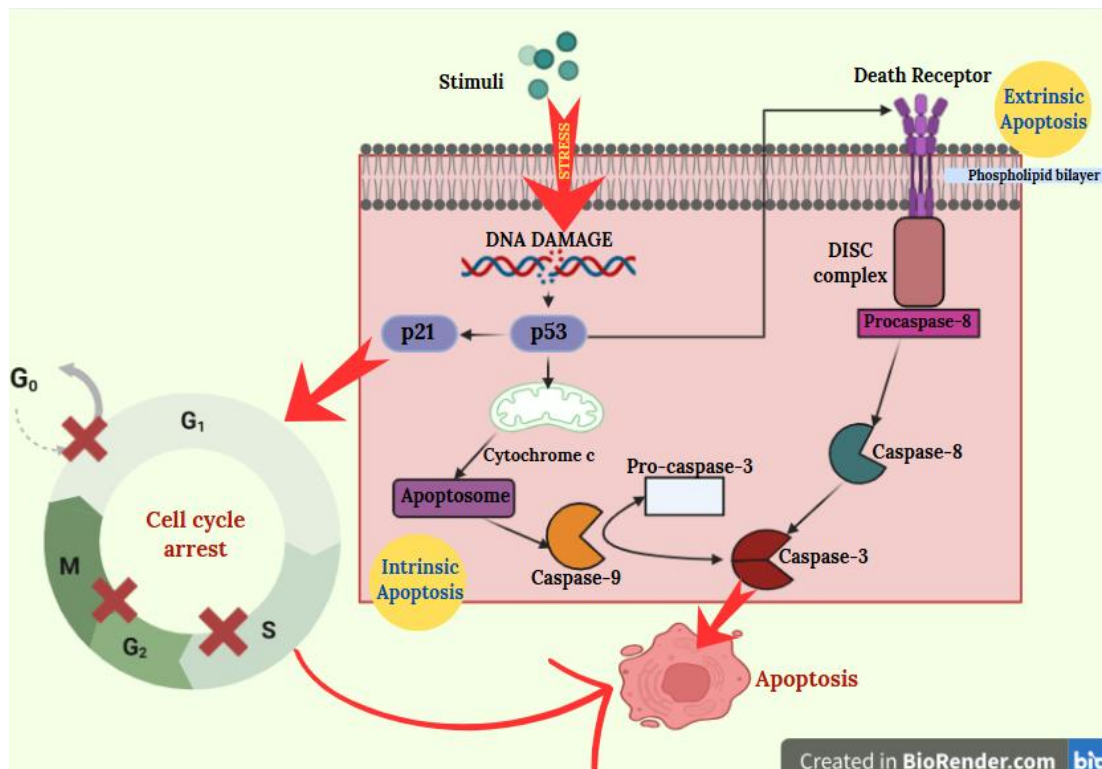


Figure 2.4 Schematic illustrations of apoptosis pathway in respond to stimuli (created using BioRender).

In the context of chemotherapy-induced apoptosis, there are several way apoptosis is activated in cancerous cell. First and foremost, through intrinsic apoptosis pathway which is a mitochondrial dependant pathway. Through this pathway, the administered chemo-drugs will cause generation of ROS that could lead cellular stress and damage DNA of cancerous cell, thus halting cell cycle phase (Yang et al., 2018). Next, the stress condition in intercellular environment will lead to activation of pro-apoptotic gene from the Bcl2 family, inclusively Bax and Bak to disrupt mitochondrial membrane integrity, leading to membrane permeabilization and release of cytochrome c (Cosentino et al., 2022). Cytochrome c will then bind to apoptotic protease activating-1 protein and form apoptosome (Yuan and Akey, 2013). Without further a due, apoptosome will activate procaspase-9 into caspase-9 which will then activates

caspase-3 and caspase-7 (Brentnall et al., 2013). The activation of caspase-3 indicated apoptosis occurrence where cellular component, DNA and structural protein of cancer cell will be degraded before engulfed as apoptotic bodies.

Other than that, chemotherapy such as paclitaxel, doxorubicin, and etoposide could induce apoptosis in cancerous cell through extrinsic pathway. This pathway involved the activation of death receptors on plasma membrane such as Fas (CD95) and tumour necrosis factor receptor 1 (TNFR1) through stimulation of death ligands tumour necrosis factor (TNF) and Fas ligand (FasL) (Green, 2022). Death inducing signalling complex (DISC) will be formed when death ligands attach to their receptors and recruit adapter protein Fas-associated death domain (FADD) (Scott et al., 2009, p. 200). After successful activation of DISC, procaspase- 8 is transformed into caspase-8 which will mediate executioner caspase-3/6/7 activations (McIlwain et al., 2013). The effector caspase will then disintegrate mutated cell damage structural protein, DNA and cellular structure in a regulated cell death manner (Lopez and Bouchier-Hayes, 2022).

Additionally, cancer is also described as a disease with insufficient apoptosis as cancerous cell have the ability to evade apoptosis by altering cellular genomic content. Targeting apoptosis through chemotherapy might alleviated this problem as downregulation of PI3K/mTOR pathway and upregulation of p53 can reduce unregular cell proliferation rate while increasing apoptosis rate (Talib et al., 2018, p. 201). Overall current cancer therapy prefers apoptosis from natural products as it directs a more targeted killing without inflammation and restrict the optimization of tumour microenvironment that support cancer development and invasion. Furthermore, apoptosis is set as the major characteristic of a successful chemotherapeutic drugs as it reduces the chance of cancer recurrence. As apoptosis involve rapid cellular death mechanism that ensure the elimination of all mutagenic content without releasing harmful substances that can further cause neighbouring cell mutation, it is found reliable to minimize the risk of cancer recurrence and metastasis.

2.5 Overview on Mangrove Ecosystem

Mangroves are a vital part of the ecosystem that provide various benefits to environmental and social well-being. This ecosystem is a natural sustainer and is known as an earth green belt, as it significantly reduces wave energy and stabilises land from storm surges, soil erosion and tsunamis (van Bijsterveldt et al., 2020). Mangrove forests are known as the largest carbon sinks that absorb most of the carbon dioxide in the atmosphere into their biomass and soil. This is able to mitigate climate changes, as mangroves were found to be very efficient in capturing carbon, about four to five times faster than terrestrial forests (Chatting et al., 2022). Other than that, the presence of mangroves' complex root networking prevents pollution and improves water quality by trapping sediments and pollutants from entering marine ecosystems (Akram et al., 2023). Since long ago, mangroves have supported infrastructure development with timber, fuelwood, medicinal plants, food and fishing spots. This coastal ecosystem even supports economic expansion through tourism and aquaculture industries.

Mangrove ecosystems are unique coastal wetlands that can be found in tropical and subtropical regions (Nagelkerken et al., 2008). This rich and biodiverse ecosystem is situated in the area where land meets the sea, so they can be found along the intertidal zone, riverbanks, and estuaries (Ma et al., 2020). This intertidal ecosystem provides shelter to numerous halophiles, shrubs, trees and animals. Interestingly, mangroves are exposed to extreme living conditions such as regular flooding, high salinity, extreme temperature, nutrient scarcity and low oxygen levels throughout their life cycle. However, they maintained their reproduction and longevity through this challenging environmental stress with remarkable adaptations (Madhavan et al., 2024; Sudhir et al., 2022). To deal with the fluctuating tides, they have specialised root systems such as stilt roots to anchor them in waterlogged soils, while pneumatophores absorb more oxygen from the surroundings. Mangroves can also excrete the excessive salt from the ocean through a specialised salt gland in their leaves or by keeping the salt as a storage. To deal with poor nutritional availability in swamp soil, mangroves have complex root systems to trap sediments. Not only that, but mangroves' leaves leathery and thick surface protects them from evaporation.

Metabolically, producing bioactive compounds or phytochemicals abundantly is part of the mangrove resilience strategy in coping with harsh environmental factors. Apparently, phytochemicals such as tannins, alkaloids and flavonoids provide defence mechanisms for mangroves against ecological constrictions, pathogens and deterring herbivores (Mitra et al., 2023, p. 202). Other than that, phytochemicals such as alkaloids, phenolic acids, saponins, abscisic acid, polyphenols, betaines, proline and flavonoids protect mangroves from high salinity by maintaining osmoregulation, stabilising cells by detoxifying excess ions, regulating cellular stress responses and reducing permeability of cell walls towards salt (Nizam et al., 2021). Other than that, phytochemicals such as phenols and flavonoids are responsible for neutralising ROS (Singh et al., 2025). In addition, mangrove is known as insecticidal, as it produces phenols to repel harmful insects (Sudhir et al., 2022).

Interestingly, not only are these phytochemicals responsible for protecting mangroves in one of the most stressful ecosystems on earth, but they also confer great health benefits to humans. Mangroves have been practised in traditional medicine by our ancestors for centuries, as they offer cures and health benefits (Awuku-Sowah et al., 2022). Mangrove leaves and bark are used as antiseptics for wounds, as they are found to hasten the wound healing process while preventing infection (Abeyasinghe, 2010). Other than that, mangrove extracts are used in treating burns, eczema and rashes, as they can prevent inflammation (Arora et al., 2022). Furthermore, mangrove decoction from bark, leaves or roots is consumed to relieve ulcers, skin disease, dysentery and stomach aches (Bibi Sadeer et al., 2021). Furthermore, red mangroves are known to have the ability to treat severe fevers such as malaria (Karthi et al., 2020). In treating respiratory diseases such as asthma and cough, mangrove leaves are usually boiled into drinkable tea or used as inhalations (Awuku-Sowah et al., 2022). Last but not least, traditionally mangroves are used as a treatment for joint and muscle inflammation, as they have the ability to reduce swelling and pain (Shilpi et al., 2012).

With known efficacy in treating multiple human illnesses, mangrove is gaining significant attention in current medical applications for its wide therapeutic value. Up to date, mangroves are utilized in research and drug discovery for infection, chronic disease, cancer, diabetes and wound healing. Currently, mangroves are involved

directly in ongoing studies in search of significant anticancer, antimicrobial, antioxidant, and anti-inflammatory natural-based agents. Focusing on cancer research, mangroves are utilised as they are found to induce unique targeting ability in cancerous cells without harming normal cells. Other than that, as mangroves are chosen to be further delved into, documentation of the anticancer potential of mangroves is still lacking, even though they could induce significant synergistic effects that can enhance the efficacy of current cancer treatment.

Major parts of the mangrove plants such as the leaves, roots, and fruits are the most studied due to their well-documented pharmacological properties. In contrast, secondary part such as the twigs have received comparatively little scientific attention, despite their potential to serve as a valuable source of bioactive compounds. Twigs, functioning as intermediary structures between roots and leaves, are involved in both nutrient transport and defence, which may lead to the accumulation of distinct secondary metabolites with possible therapeutic relevance. Therefore, research on mangrove twigs could provide novel insights and contribute to the discovery of new therapeutic potentials, thereby expanding the existing literature for mangrove in oncology research.

2.5.1 *Bruguiera cylindrica*



Figure 2.5.1 *Bruguiera cylindrica* (NParks, n.d.)

Bruguiera cylindrica is a mangrove species from the family Rhizophoraceae. They are native species in South Asia, Southeast Asia, Australasia and East Asia, where they are widely distributed in countries such as Malaysia, Indonesia, Sri Lanka, India and northern Australia. This mangrove species predominantly habituates where seawater meets freshwater, so they can be found thriving along the coastline, sheltered bays, river tides and estuaries. This plant grows ideally in the mid to upper intertidal zone, as it is not highly salt tolerant and prefers brackish water. Commonly *B. cylindrica* is known as “white-flowered mangrove” and “reflexed orange mangrove”. In Malaysia they are commonly known as “Bakau Putih” and can be found in Terengganu, Kedah, Johor, Penang and Perak (MyBis, 2025).

B. cylindrica can be distinguished from other mangrove species through their unique botanical characteristics as shown in Figure 2.5.1. *B. cylindrica* has pendulous and small white petal flowers with green sepals and small bristles, where the flowers can usually be found in the junction of the stem and leaves. The flowers can be grouped in a cluster of 2-5 flowers or borne singularly. Other than that, this species has small elliptical leaves with pointed tips that appear glossy dark green on the upper side,

lighter green on the underside and yellowish when unmaturred. One of the key characteristics in identifying *B. cylindrica* is the cylindrical and elongated propagules that are slightly curved and hanging on the branches, with a green-brown or reddish hue. This species has smooth, greyish bark that will develop corky patches with age. Similarly with other mangroves, *B. cylindrica* has knee-like pneumatophores roots with lenticels that allow oxygen and nutritional intake during temporary tidal flooding. This aerial root provides anchorage for *B. cylindrica* in soft and muddy soils from erosion of currents and tides. *B. cylindrica* have viviparous fruit that appear to be small in elongated oval shape, typically brown when matured and green when immature.

2.5.2 *Sonneratia alba*



Figure 2.5.2 *Sonneratia alba* (NParks, n.d.)

Sonneratia alba (Figure 2.5.2) from family Lythraceae is a native species in tropical and subtropical regions such as Southeast Asia, East Asia, East Africa, the Indian Subcontinent, Australasia and the western Pacific. In Southeast Asia, this plant is commonly distributed in Malaysia, Thailand, Indonesia, Myanmar, Vietnam, Singapore and the Philippines. *S. alba* is known as a pioneer species with strong vitality against strong tidal waves and high salinity. This species can be found thriving in the low intertidal zone where this plant has the ability to withstand longer submerged periods and brief low tides. *S. alba* can be found growing in mangrove forests, estuaries and mudflats. This species is commonly known as “mangrove apple”. In Malaysia, *S. alba* is known as “Perepat” or “Pedada” and commonly distributed in Terengganu, Selangor, Johor, Sabah and Sarawak (MyBIS, 2025).

S. alba has large, showy and nocturnal flowers with numerous stamens of pinkish bases and white in colour. The flowers are borne in small clusters or singly with small petals and long stamens. The leaves of *S. alba* are obovate with narrow bases and rounded tips, where they grow oppositely along the branches. The leaves appear with thick, waxy, leathery and smooth surfaces with glossy green colour on the upper side and pale green on the underside. *S. alba* can be directly distinguished by

their pencil-like pneumatophores that are elongated, vertical and grow above the soil surface. Not only do these pneumatophores provide oxygen during prolonged submerged periods, but they also aid the stabilisation of *S. alba* against the high tides. Unlike other mangrove species, *S. alba* does not produce elongated propagules, but they develop round, buoyant fruit for water dispersal purposes. These fruits appear green and unripe and have a yellow-brown hue as they fully ripen.

2.5.3 Therapeutic Potential of Mangrove

Majority of the marine natural resources including mangroves reportedly possessed therapeutic values including antibacterial, anti-fungal, immunomodulator, antimicrobial, neuroprotective, analgesic, anti-malarial, and even anticancer (Malve, 2016). Ethnopharmacology is widely practiced in treating many illnesses as plant shows significant therapeutic derivatives while being low hazardous for consumptions (Opara and Wokocha, 2008). Hence, within the current trend of endearing a safer natural therapeutic drug with multi- dependable metabolites, mangrove forests have inspired more researchers and pharmacologists to direct screening studies.

Greatly influenced by abiotic and biotic changes such as location of growth and aging, different parts of mangroves plants show vast array of healing promises (Khan and Aljahdali, 2022; Mitra et al., 2021). Henceforth, mangroves are considered essential in drug discovery studies as they are found rich in different types of phytochemicals classes including alkaloids, flavonoids, tannins, amino acids, saponins, phenolics, carotenoids, aliphatic alcohols, hydrocarbon, fatty acids, terpenes, carbohydrate and other more related compounds (Bandaranayake, 2002). Previous studies revealed that phenolics, flavonoids, and terpenoids present in mangrove extracts to be carcinogenic against cancer cells (Saroyo and Saputri, 2021).

Thus, being rich in phytochemicals such as alkaloids, mangroves have the ability to induce antiproliferation and halt metastasis of invasive cancer (Lu et al., 2012). Hence, tumour bulk emergence through oxidation can be suppressed well (Illian

et al., 2018). So, moving back to the perilous base that current treatment regimen for cancer is still flawed due to the adverse effects of synthetic chemical-based drugs, utilizing mangroves in a sustainable manner meets the criteria for generating a potentially-safer chemotherapeutic drugs (Abotaleb et al., 2020; Khan et al., 2022). Figure 2.5.3 below show several bioactive compounds from several native mangrove species in Malaysia.

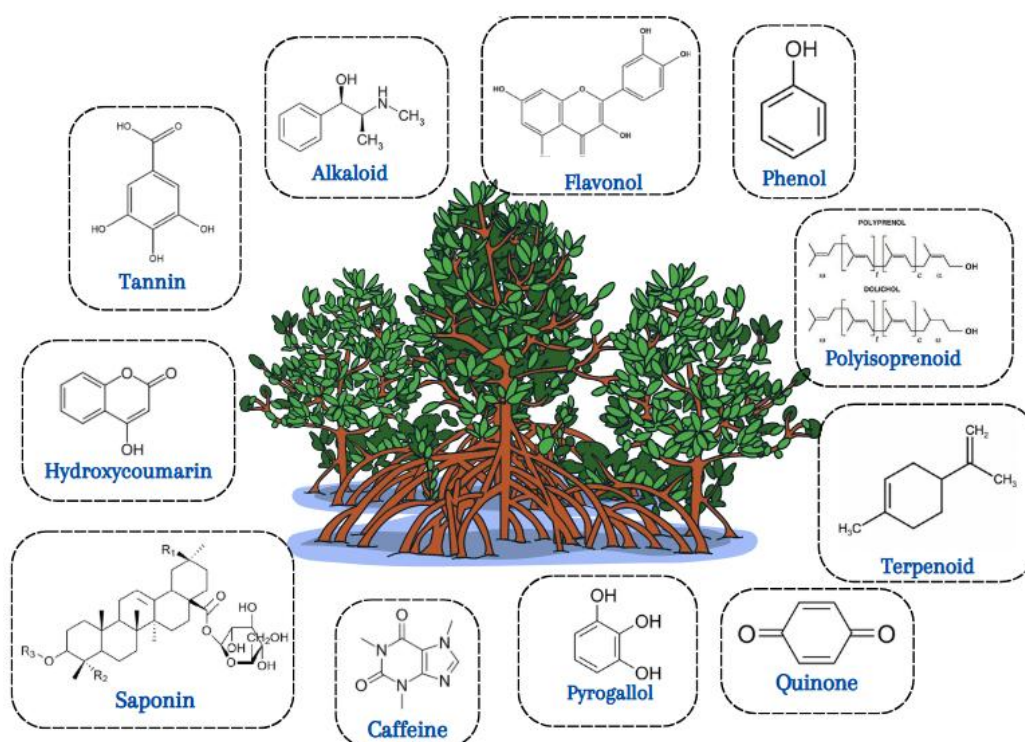


Figure 2.5.3 Common examples of bioactive compounds from several native mangrove species (created using Canva).

Mangrove plants have been subjected as potential agent in cancer research. Interestingly, past research prevailed that phytochemical classes extracted from mangrove have promising ability to be cytotoxic against cancerous cell line proliferations. Table 2.5.3 below listed mangrove species from past research that have been screen for their anticancer properties.

Table 2.5.3 Common examples of mangrove phytochemicals and their anticancer mechanism.

Mangrove sp.	Anticancer Agent	Anticancer Mechanism	Reference
<i>Aegiceras corniculatum</i> (Twigs)	Alkylated benzoquinones	Modulation of the pro-apoptotic p53 pathway as a high level of ROS induces oxidative stress	(Faizan et al., 2024)
<i>Avicennia marina</i> (Leaves)	Hymecromones	G ₀ /G ₁ arrest in HCT-116—activation of cyclin D/E, CDK2, or CDK6 to the R point hinders cell progression. S-phase arrest in MCF-7 and HepG2—p53 activation leading to ubiquitination and apoptosis	(Albinhassan et al., 2021)
<i>Avicennia marina</i> (Root)	• Phenols • Flavonoids	Increase the expression of pro-apoptotic genes such as p53.	(Merlin et al., 2021; Tanjung et al., 2022)
<i>Avicennia lanata</i> (Leaves) <i>Avicennia alba</i> (Leaves)	Polyisoprenoids	Inhibit proliferation by detaining the cell cycle at G ₀ /G ₁ phase through p53 and Bcl2 pathways	(Qurrahman et al., 2020)
<i>Avicennia alba</i> Blume. (Leaves)	Polyisoprenoids	Inhibition of Cox-2 induces downregulation of PGE prostaglandin, leading to the suppression of the Bcl-2 gene and the initiation of early apoptosis (G ₀ /G ₁ phase)	(Illian et al., 2019)
<i>Bruguiera gymnorrhiza</i> (Leaves)	• Alkaloid • Phenol • Terpenoid	Apoptosis occurs by the means of caspase-3 and caspase-9 activations	(Chaudhry et al., 2020)
<i>Exoecaria agallocha</i> L. (Leaves)	Bergenin	G ₂ /M phase cell cycle arrest occurs owing to reduced expression of cyclin A and B1, along with the amplification of p21 and p53 genes	(Sultana et al., 2022)
<i>Lumnitzera racemosa</i> (Leaves)	• Pyrogallol • Caffeine	Increase the expression of tumour suppressor protein while causing cell arrest at S-phase due to reduction of anti-apoptotic genes such as	(Dokunmu et al., 2023; Ayu, 2022; Maheshwari

		P13K/p-AKT, S-phase kinase-associated protein, and CDKs while activating p53 upstream to Bax activation	and Sharma, 2023)
<i>Rhizophora apiculata</i> (Leaves)	• Caffeine • Oxalic acid • Luteolin	Induction of Bax and Bcl-2 family genes, which impaired the membrane of mitochondria, producing cytochrome c, leading to programmed cell death	(Dokunmu et al., 2023; Maulana, 2021; Prasher et al., 2022)
<i>Rhizophora mucronata</i> Lam. (Leaves)	• Quinoline alkaloid • Pyridine alkaloid	Cell cycle arrest by downregulating both Bcl-2 and mitotic cyclin	(Youssef et al., 2023)
<i>Ipomoea tuba</i> (Leaves)	• Docosanoic • Octadecatrienoic • Cis-9-octadecanoic acids • Triterpenoids γ-sitosterol • Terpene alcohol	Inhibit anti-apoptotic genes such as Bcl-2	(Venkateswarulu et al., 2022)
<i>Xylocarpus granatum</i> (Leaves)	• Fumaric acid	Mediation of cellular stress condition and inhibition of NF-kB pathway	(Assidqi et al., 2024; Xia et al., 2018)
<i>Xylocarpus moluccensis</i> (Leaves)	• Flavonoid • Steroid • Terpenoid	Extrinsic apoptotic death through activation of caspase-3 and 8	(Chaudhry et al., 2020)
<i>Xylocarpus moluccensis</i> (Bark)	• Phenol • Tannin • Glycoside • Saponin		

It is worth noting that mangrove phytochemicals prompt both apoptosis and mitotic cell arrest. The presence of these bioactive compounds in mangroves is shown to have the ability to induce an ideal apoptosis cell death mechanism, as apoptosis occurs around 20 times faster than cancer cell division (Meng et al., 2021). Generally, this could reduce the progression of neoplasia into the manifestation of metastatic cancer. Basically, a high accumulation of ROS leads to alteration of organelles, membranes, lipids, protein, and nucleic acid (Redza-Dutordoir and Averill-Bates, 2016). This leads to the intrinsic apoptosis pathway by upregulation of Bak and Bax proteins on the outer layer of the mitochondrial membrane with simultaneous

downregulation of Bcl2/XL complexes in order for cytochrome c to be released into the cytosol and engaged with apoptotic protease activating factor-1 (APAF1) (Dewson and Kluck, 2009). Henceforth, the formation of the apoptosome activated caspase 9 to transmit the signals from procaspase-3 to caspase-3, leading to the apoptosis of cancer cells (Nakamura and Takada, 2021).

Overall, from past research, mangrove showed potential as promising anticancer agents. Therefore, screening of mangrove *in-vitro* and *in-vivo* must be done to prevailed therapeutic effects of local mangrove species in search of reliable natural product which appear to arrest cancer progression through desirable apoptosis pathway.

2.6 Conservation of Natural Resources

In efforts to accommodate the rapidly increasing globalization and urbanization, many natural resources are severely threatened by erratically expanding harvesting, and critical biodiversity degradation issues. A recent article stipulated that the pace of extraction worldwide approximated to rise by 60% by the year 2060 due to the current emerging prospect of utilizing raw materials from Mother Earth (Neslen, 2024). Rightly, natural resources become the centrepiece of economic growth elements as the demand for applications increases in the industrial field (Mondal and Palit, 2022). Trajectory, this strategy has been largely held accountable for nature's diminishing capacity to further contribute widely as a mankind life support system (Brauman et al., 2020). Hence, in order to ensure that neither renewable nor non-renewable resources will be depleted in the foreseeable future, sustainable development is pivotal for conserving the ecosystem and all of its constituents.

Hence, there are few steps taken by the policy makers and scientist to further conserve and ensure the well-being of nature while the global plat-derived drug market demand is expected to hit 5 trillion US dollar by 2050 (Negi et al., 2018). So straightly focusing on employing natural resources in research, one of the steps that can be taken

is by sustainable harvesting during sample collection. This aforementioned statement can be further deployed by using different part of the plants in research to such likely using the root, shoot, leaves, barks/ stem which possessed more availability (Manohar, 2012). Additionally, researchers could benefit natural resources of interest by taking only the mature part of the plant and left the others to fully grow in their habitats (Rabgyal and Pelden, 2021).

Aside from that, to improve plant productions with the necessity for adequate rotating harvesting, practitioners should harvest non-endangered species and only collect a minimal amount of the plants' parts (Semenya et al., 2020). Additionally Given the rapid demise of wild plants, remote sensing of the ecosystem of interest is required to ensure the spread of a habitat or species within its range (Schwager and Berg, 2021). By carrying out this step, researchers could strategically plan sample collection and even plan for replantation if the species number was significantly threatened by the outgoing urbanization. Last but not least, as some plant samples cannot be stored for long periods of time due to protein degradation, replantation and species rehabilitation could be done on a greater scale to ensure the conservation of natural resources at the intersection of human needs and, undoubtedly, to make sure natural resources could maintain their optimal role in regulating the environment (Chen et al., 2016).

CHAPTER 3

METHODOLOGY

The experimental procedures were executed in accordance with the research design outlined in Figure 3.0 below to meet the objectives of this study.

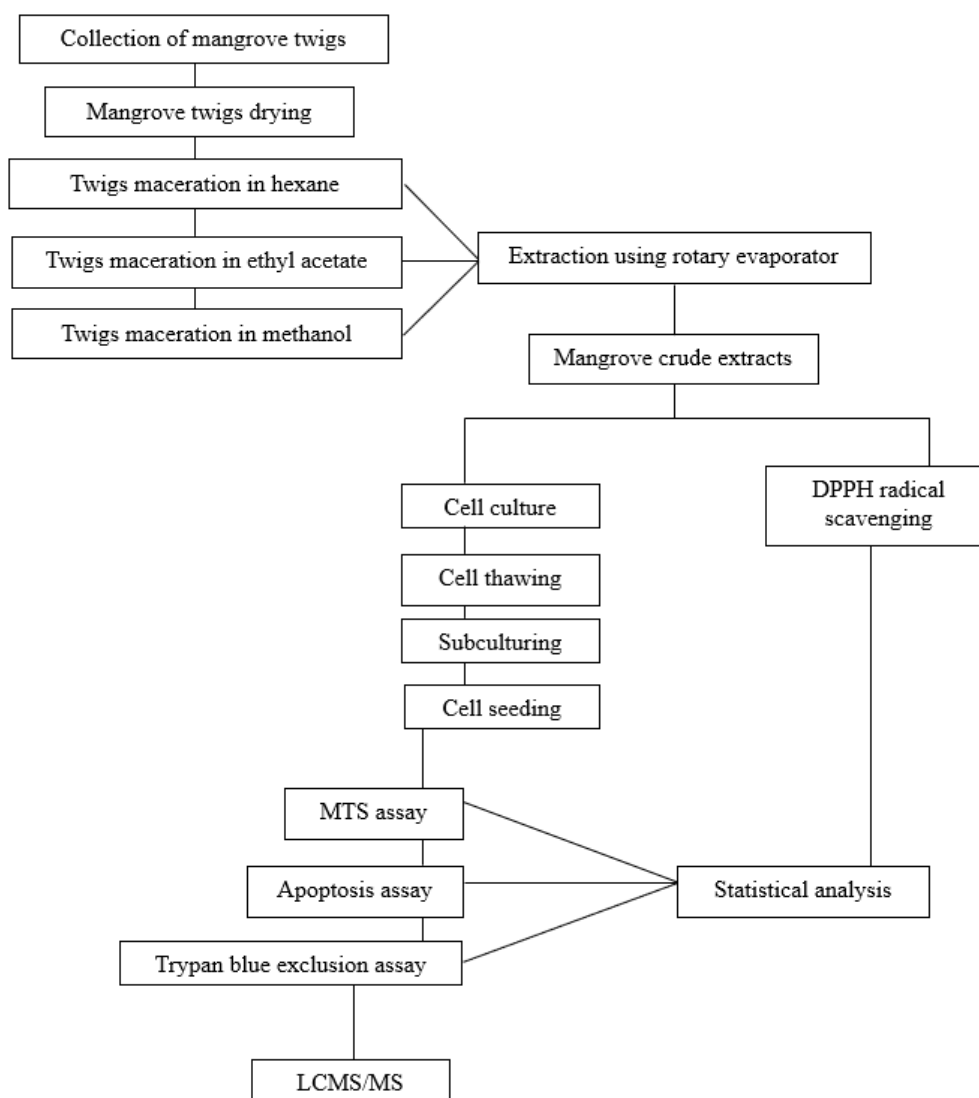


Figure 3.0 Flowchart of the methodology.

3.1 Collection of Mangrove Plant

For this study the twigs of two mangrove species, *Bruguiera cylindrica* and *Sonneratia alba* were selected for anticancer properties screening. The identification of selected mangrove species used in this research was conducted by botanist Encik Muhamad Razali bin Salam from Faculty of Science and Marine Environment, Universiti Malaysia Terengganu. The description on these two mangrove species, family and coordinate of location collected were tabulated in Table 3.1, and the picture of twigs from both species and their powder form can be referred in Figure 3.1 below.

Table 3.1 Species, family and locations of collected mangrove samples

Species	Family	Location of Collection
<i>Bruguiera cylindrica</i>	Rhizophoraceae	Pengkalan Gelap, Setiu, Terengganu (5°40'07"N 102°43'49"E)
<i>Sonneratia alba</i>	Lythraceae	

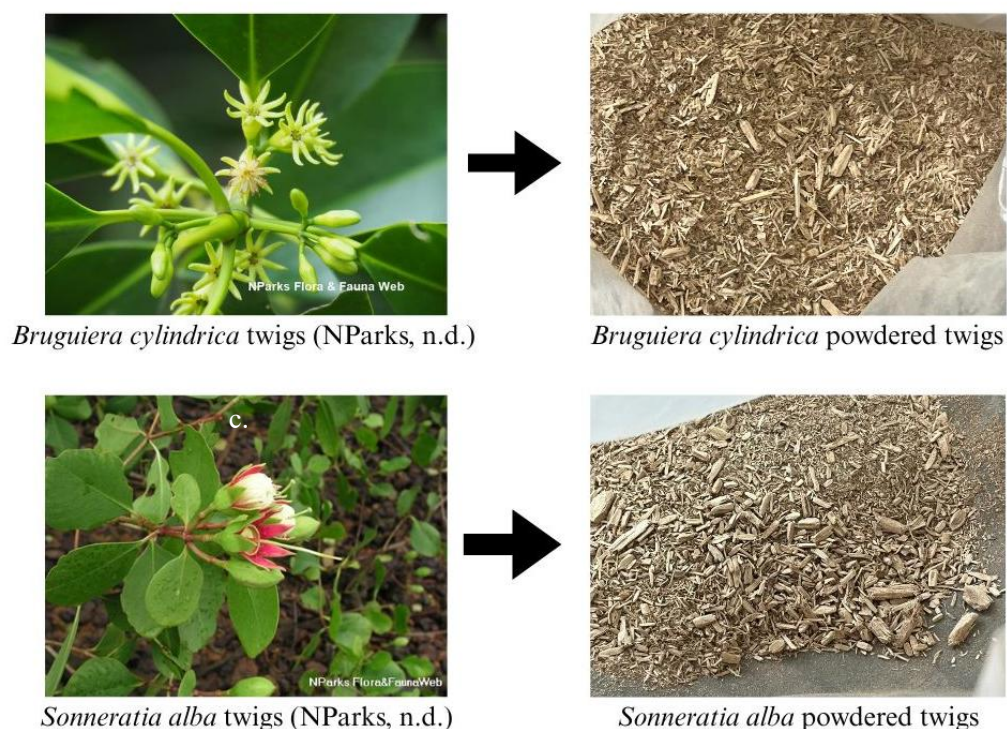


Figure 3.1 Selected mangrove twigs and their powdered form used in this research.

3.2 Preparation of Mangrove Extracts

This method was carried out as reported by Chaudry et al., (2020) with slight changes. The selected twigs of mangroves samples were prepared accordingly prior testing for anticancer properties. Initially, the twigs of both *B. cylindrica* and *S. alba* underwent drying in the oven at a maximum temperature of 35°C. The dried twigs were subsequently ground into a powdered form prior to extraction. In a 1:4 ratio of sample to solvent, 50 g of powdered mangrove species (twigs) were immersed in 200 mL of hexane overnight, with this process being repeated for three consecutive days.

The solvents were filtered into a round bottom flask using Whatman filter paper No. 1, prior to concentration with a rotary evaporator at 40°C.

Following that, serial exhaustive extraction methods were employed by soaking the same twigs in ethyl acetate and methanol, resulting in three distinct types of extracts for each species: hexane extract, ethyl acetate extract, and methanol extract. The extracts obtained were then poured into sample vials and then left to dry in an oven at 40°C. During the drying process, the obtained extracts were continuously weighed until a constant weight of the crude extracts was achieved. The completely dry crude samples were stored in a 4°C chiller.

3.3 Cell Culture

In order to fulfil the objectives of this study, human adenocarcinoma Michigan Cancer Foundation-7 (MCF-7) and mouse skeletal muscle (L6) were cultured to investigate and compare the effects of particular mangrove extracts on both cancerous and normal cell lines. The processes carried out for cell culture encompassed seeding, subculturing, thawing, maintaining, and cryopreserving both cell lines for subsequent applications. Cell culture methods carried out in Section 3.3.1, 3.3.2, 3.3.3, and 3.3.4, as documented by Khoeliqoh et al., (2024) and Oskal et al., (2020).

3.3.1 Phosphate Buffered Saline Preparation

Phosphate Buffered Saline (PBS) was prepared meticulously for the purpose of cell washing prior to commencing cell culture works. Ten PBS tablets were dissolved in 1 L of distilled water in a bluecap bottle until fully diluted. Following autoclaving, the solution was filtered and aliquoted into 50 mL centrifuge tubes using Minisart 0.2 UM, 28 MM FM cellulose acetate sterile Luer lock syringe filters from Sartorius.

3.3.2 Thawing Cryopreserved Cells

MCF-7 and L6 cell lines were bought from American Type Culture Collection (ATCC). The cryopreserved MCF-7 cells (1 mL) were carefully transferred into a 15 ml centrifuge tube containing 3 ml of fresh Rosewell Park Memorial Institute medium, RPMI-1640 (Gibco), and centrifuged for 5 minutes at 1000 rpm and 20°C. Following centrifugation, the cell pellets obtained were carefully re-suspended in 1 ml of fresh RPMI medium before being transferred into new T25 flasks containing 5 ml of RPMI-1640 media supplemented with 10% Foetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep). The flasks were subsequently incubated at 37°C in a 5% (v/v) CO₂ incubator. These procedures were then repeated for thawing L6 cells; however, L6 cells were thawed into a different fresh medium, Dulbecco's Modified Eagle Medium (D-MEM, Gibco), with 10% FBS and 1 % Pen-strep.

3.3.3 Cells Sub-culturing

As the flask reached 80% confluency, the cells were sub-cultured to ensure their nutritional requirements were met. First, the media was removed, and then the cells were washed twice with 3 ml of PBS to guarantee the elimination of all toxins and dead cells. 1 mL of Trypsin-EDTA solution (0.25% Trypsin, 1MM EDTA) was added to the T25 flask and incubated (5% (v/v) CO₂, 37°C) for 5-10 minutes until complete detachment of the cells from the flask surface. After the cells were fully detached, 3 mL of fresh medium was added. The contents of the flask were subsequently transferred into a 15 mL centrifuge tube and subjected to centrifugation at 1000 rpm for a duration of 5 minutes at a temperature of 20 °C. The supernatant was removed, and the cells were re-suspended in 1 mL of fresh medium prior to being transferred to new T25 flasks filled with complete media as described in section 3.3.2. The flasks were then placed in a CO₂ incubator at 37°C for incubation. MCF-7 and L6 at passage 5 were directed into further cytotoxic assay.

3.3.4 Cryopreservation of Cells

The freezing media consisted of FBS and Dimethyl sulfoxide (DMSO). To cryopreserve the cells, the old media was discarded, and the cells were washed twice with 3 mL PBS before being trypsinised with 1 mL Trypsin-EDTA. The cells were then incubated in a 5% (v/v) CO₂ incubator at 37°C until all cells detached.

The cells were then transferred to a 15 ml centrifuge tube containing 3 mL of fresh medium and centrifuged at 1000 rpm, 20 °C for 5 minutes. The acquired cell pellets were suspended in 900 µL of FBS and 100 µL of DMSO. The cells were subsequently placed into 1.5 mL cryovial and maintained at -20 °C for 30 minutes to 1 hour before being transferred to either a -80 °C freezer or a liquid nitrogen tank.

3.4 *In-Vitro* Anticancer Assay

Cytotoxicity testing of mangrove extract was performed on both MCF-7 and L6 cells to investigate its effects on breast cancer cells (MCF-7) and normal cells (L6) using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS assay. MTS was utilized to figure out the necessary extract dosage to achieve half-maximal inhibition of the cell population (IC₅₀) in both cell lines, hence evaluating the toxicity of the selected mangrove species on cell proliferation. For additional evaluation, samples exhibiting active IC₅₀ values were analysed in an apoptosis assay to clarify the death mechanism induced by the extracts. Additionally, a trypan blue exclusion assay was performed to assess the daily decline in breast cancer cell count. Anticancer assay carried out in Section 3.4.1, 3.4.2, 3.4.3, 3.4.4, 3.4.5, and 3.4.6 as documented by Khoeliqoh et al. (2024) and Oskal et al. (2020).

3.4.1 Cells Seeding in 96-Well Plate

Before commencing the assay, the cell confluency in the T25 flask must attain 80%. Subsequent to washing the cells with PBS, they were trypsinised using Trypsin-EDTA and resuspended in 1 ml of complete media consisting of medium, 10% FBS, and 1% Pen-Strep. 10 μ L cells were then loaded into the automated cell counter to quantify the viable cells for the experiment. The cell density for MCF-7 was 2x10⁴ cells per well, while for L6 it was 2x10⁵ cells per well. The cells were subsequently suspended uniformly in complete media within a pipetting reservoir before being seeded at 100 μ L per well into a 96-well plate. The well plates were incubated for 24 hours in a 5% (v/v) CO₂ incubator at 37°C prior to treatment.

3.4.2 Two-Fold Dilution of Crude Mangrove Extracts

An amount of 10 mg of six distinct types of crude mangrove extracts were diluted in 1 mL of DMSO within a 1.5 mL microcentrifuge tube, respectively. Two-fold dilutions were subsequently conducted on all selected extracts with complete media. Nine distinct concentrations of mangrove samples were acquired, ranging from the highest at 100 μ g/mL, 50 μ g/mL, 25 μ g/mL, 12.5 μ g/mL, 6.25 μ g/mL, 3.125 μ g/mL, 1.5625 μ g/mL, 0.78125 μ g/mL and the lowest at 0.390625 μ g/mL.

3.4.3 Treatment of Cells with Mangrove Extracts

The media in the 96-well plate were entirely discarded following a 24 h incubation period. Complete media serving as a negative control were added to well 10. Subsequently, wells 1 to 9 of the well plate were filled with 100 μ L of two-fold diluted extracts in accordance with the concentration sequence according to section 3.4.2. For positive control of anticancer activities, vincristine sulphate, a well-known anticancer drug, was diluted with DMSO to a concentration of 1 mg/mL, followed by

two-fold dilutions, 100 µL treatment was dispensed into the wells. The well plate was subsequently incubated in a 5% (v/v) CO₂ incubator at 37°C, with observations conducted at 24, 48, and 72 h intervals.

3.4.4 MTS Assay

Both MCF-7 and L6 cells viabilities were detected using CellTiter 96® AQueous One Solution Proliferation Assay (MTS Assay Promega, USA). After the 72 h treatment incubation period, 20 µL of MTS reagent were pipetted into each well and incubated for additional 1 ½ hour. Subsequently, the viability was assessed at 490 nm absorbance for both cells using microplate reader. The inhibitions of cells were calculated using the formula below:

$$Inhibition (\%) = \left(1 - \frac{OD_{test} - OD_{blank}}{OD_{control} - OD_{blank}} \right) \times 100$$

OD_{test} = Treated cell absorbance

OD_{blank} = Complete media + MTS absorbance

OD_{control} = Untreated cells in complete media absorbance

The reading of control samples were taken from untreated cells. Percentage of inhibition vs (log₁₀) sample concentration (µg/mL) was plotted using GraphPad Prism. From the graph, IC₅₀ value was measured based on sigmoidal-dose response curve.

3.4.5 Apoptosis Assay

Apoptotic activity in MCF-7 cells were assessed by utilising the Annexin V Apoptosis Detection Kit, FITC, from eBioscience. 2 x 10⁴ MCF-7 cells were seeded

into a 96-well plate. Following a 24 h incubation period, cells were then treated with the IC₅₀ dosage obtained from the preceding MTS assay. The apoptosis kit was supplied with two distinct dyes: Annexin V FITC which stained the phosphatidylserine of apoptotic membrane and Propidium Iodide stained the apoptotic nuclei.

Before each observation, the media were withdrawn, and the cells were rinsed with 100 µL of PBS. Afterward the cells were washed and resuspended in 1X binding buffer, 100 µL each step. 5 µL of Annexin V FITC dye staining were conducted and the cells were incubated for 15 minutes.

Subsequently, the binding buffer was withdrawn again prior to washing the cells and resuspending them in 100 µL of 1x Binding Buffer. Stained MCF-7 were then additionally stained with 5 µL of Propidium Iodide. Analysis of apoptotic activity in MCF-7 cells was conducted under a fluorescence microscope in 12 h, at every 4 h interval.

Quantification of apoptotic cells were made ImageJ (Fiji, NIH, USA). Total of apoptotic cells from merged fluorescence microscopy images (RGB) were converted to 8-bit grayscale. Thresholding was applied to highlight all the fluorescent cells. The images were then binarized to create binary mask of the cell regions. Noise reduction was performed when necessary to improve signal clarity with “Despeckle”. Cells count were obtained by “Analyze particle” with circularity adjusted to 0.1 – 0.3, to include apoptotic MCF-7 only.

3.4.6 Trypan Blue Exclusion Assay

100 µL (2×10^4 cells per well) of MCF-7 were seeded into 96-well plate and incubated for 24 hours prior to treatment. Samples having positive anticancer activity, acquired from the MTS assay, were diluted based on the determined IC₅₀ value and administered as treatment for 24 h, 48 h and 72 h. Following the incubation period, the cells were washed and eventually trypsinised using 10 µL of trypsin (Gibco), then

incubated until complete detachment of the cells was observed. Subsequently, trypan blue staining was performed by combining trypan blue dye with trypsin in a 1:1 ratio. Rapid observations were conducted with the automated cell counter, DeNovix.

3.5 DPPH Radical Scavenging assay

The antioxidant properties of the sample were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, as documented by Blois (1958) and Gulcin and Alwasel (2023). To prepare a 6×10^5 M DPPH solution, 2.37 mg of DPPH powder was dissolved in 100 mL of methanol. 10 mg/mL of the selected crude sample was prepared, and 1 mg/mL of quercetin served as the positive control. Samples and quercetin were initially pipetted into the 96-well plate following the two-fold dilution procedure depicted in the figure below, with treatment dosages ranging from 0.156 mg/mL to 10 mg/mL, and 0 mg/mL designated as the blank control.

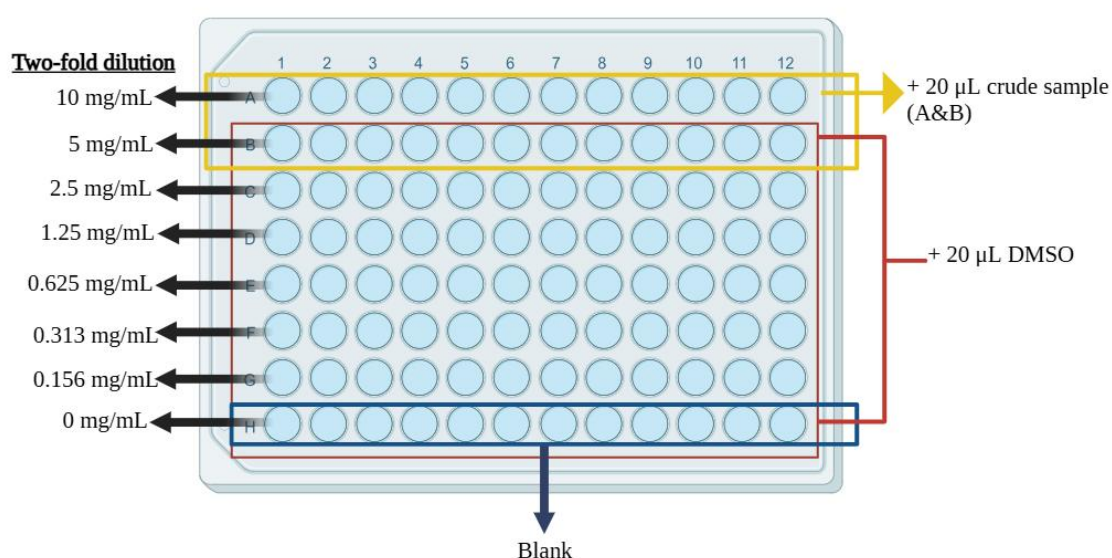


Figure 3.5 DPPH radical scavenging assay experimental design

Finally, 200 µL of DPPH in MeOH was added to each well and incubated in the dark at room temperature for 30 minutes. The absorbance measurements were

measured at 517 nm via a multiplate reader. The percentage of DPPH radical scavenging activity was calculated using the formula below:

$$DPPH \text{ scavenge activity (\%)} = \frac{A_o - A}{A_o} \times 100\%$$

A_o = Control absorbance

A = Sample absorbance

3.6 Liquid Chromatography - Mass Spectrometry (LC-MS/MS)

LC-MS/MS analyses were performed to evaluate the phytochemical constituents in extracts that contribute to synergistic anticancer and antioxidant effects. Referring to the methods outlined by Zakaria et al. in 2021, 100 mg of selected samples were diluted in 1 mL of methanol, followed by vortexing for 30 minutes and an additional sonication for 30 minutes.

Then, the samples were filtered through 0.2 μ M PTFE membrane into a vial before metabolomics profiling using Ultra-High Performance Liquid Chromatography- Tandem Mass Spectrometry (UHPLC-MS/MS) equipped with Orbitrap MS/MS through electrospray ionization (HESI) probe heating. The instrument underwent calibration by Pierce LTQ ESI Positive (#88323; Thermo Scientific) and Negative Ion (#88324; Thermo Scientific) Calibration Solutions. Liquid chromatography separation was accomplished using a C18 column (1.7 μ m particle size, 100 mm length, 2.1 mm diameter; Synchronis, #97102-102130; Thermo Scientific).

The liquid chromatography conditions were established as follows: column temperature at 55 °C, autosampler temperature at 10 °C, injection volume at 2 μ L, and flow rate at 0.45 mL/min. Solvent A was water (W6-4; Fischer Scientific) and solvent

B was acetonitrile (A955-4; Fischer Scientific), both containing 0.1% formic acid (A117-50; Fischer Scientific) (v/v). An elution gradient of 22 minutes was established as follows: at 0 minutes, 0.5% B; at 1 minute, 0.5% B; at 16 minutes, 99.5% B; at 20 minutes, 99.5% B; and at 22 minutes, 0.5% B.

The HESI was conducted in both positive and negative modes, independently. The HESI MS source was calibrated as follows: sweep gas flow rate set to 50 arbitrary units (AU), auxiliary gas flow rate at 18 AU, sweep gas flow rate at 0 AU, capillary temperature at 320 °C, S-lens level at 55 AU, auxiliary gas heater temperature at 300 °C, and spray voltage at 3.5 kV for positive mode or 3.0 kV for negative mode.

Mass spectrometry data was obtained using a complete MS scan, succeeded by data-dependent tandem mass spectrometry (ddMS2) utilising Xcalibur 4.2.27 software (Thermo Scientific). A comprehensive MS scan was acquired at a resolution of 60,000 with a mass/charge (m/z) range of 100-1,000, whereas the ddMS2 scan was obtained at a resolution of 15,000 with stepwise normalised collision energies of 20, 40, and 60 AU.

The data generated by LCMS were pre-processed with MS-DIAL (v5.2.240424.3) according to Tsugawa et al., in 2015. The tolerances for MS1 and MS2 were established at 0.01 Da and 0.025 Da, respectively. The minimum peak height was established at 30,000 amplitudes, and the mass slice width was set at 0.05 Da. Metabolite identification relied on the MS2 public database utilising legitimate standards from the MS-DIAL website (version17; <https://systemsomicslab.github.io/compms/msdial/main.html>). Blank features were eliminated based on a sample maximum/sample average signal exhibiting less than a 5-fold change. The annotated characteristics for MS1 peak morphology and MS2 reference peak alignment were carefully examined.

3.7 Statistical Analysis

Cytotoxicity assays were conducted in triplicates, and the results were presented as a percentage of cell growth inhibition. The IC₅₀ values for cell growth inhibition were obtained using a non-linear regression model (curve fit) that was based on sigmoidal dose-response curves (variable) calculated in GraphPad Prism version 10.2.0 software (GraphPad, USA). The mean and standard deviation of the obtained IC₅₀ values were computed using similar software. The significance of differences in growth inhibition between the test concentration and control was assessed using t-test, analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test for post-comparison, calculated with IBM SPSS Software, US. P-values less than 0.05 were regarded as statistically significant.

CHAPTER 4

RESULT & DISCUSSION

4.1 Solvent-Based Variation in Crude Yield of Mangrove Extracts

Past research has identified over 10,000 phytochemical types and structures, making them widely documented and well-known as a source of pharmacologically relevant material (Leitzmann, 2016; Park, 2023). Definitively, phytochemicals are plant-produced bioactive compounds essential for their growth and protection. Plants produce two types of phytochemicals throughout their life cycle, and they can be categorised into primary and secondary metabolites.

Similarly, mangroves' primary metabolites are fundamental for physiological development, including carbohydrates, nucleic acids, fatty acids, proteins, lipids, pyrimidines, and purines (Elshafie et al., 2023). Secondary metabolites such as alkaloids, terpenoids, flavonoids, tannins, saponins, and phenols are responsible for protecting mangroves against biotic and abiotic constrictions (Divekar et al., 2022). Interestingly, these secondary metabolites not only support the defensive mechanisms but also demonstrate promising therapeutic value for humans, such as anticancer, antioxidant, antibacterial, and anti-inflammation (Mitra et al., 2021).

With known efficacy as an alternative treatment, mangroves are assessed to reduce global diseases burden. In every natural product research, phytochemical

extraction is the key component. This initial step facilitated the development of plant-based pharmaceuticals and their semi-synthetic analogues. However, phytochemical functionality is highly sensitive to temperature, light, solvent used, and extraction method utilised (Mungwari et al., 2025). Hence, investigating phytochemical composition necessitates the use of suitable extraction techniques in order to maintain the quality of the extracted compound and its amount of yield.

In this investigation, multilevel maceration was carried out in solvents of increasing polarities (hexane-ethyl acetate-methanol) to extract diverse secondary metabolites, because secondary metabolites can only be dissolved in solvents of similar polarity. Tables 4.1 below listed the crude extract mass (g) in three different solvent and their percentage of yield (%). The percentage of yield (%) was calculated as followed:

$$\% \text{ Yield} = (\text{Weight of Dried Extract} / \text{Weight of Dried Sample}) \times 100$$

Table 4.1 Mangrove crude yield (%) obtained. Each value represented mean $\pm$ S.D. (n=2) (Two independent experiments).

Species	Dry Weight (g)	Yield (%)		
		Methanol	Ethyl acetate	Hexane
<i>Bruguiera cylindrica</i>	50 $\pm$ 0.00	5.84 $\pm$ 0.11	1.53 $\pm$ 0.16	1.02 $\pm$ 0.05
<i>Sonneratia alba</i>	50 $\pm$ 0.00	5.86 $\pm$ 0.07	2.14 $\pm$ 0.12	1.24 $\pm$ 0.07

As indicated in Table 4.1 above, the crude mass and percentage of yield obtained for mangrove sample extraction vary in each solvent. Using methanolic extract, both *Bruguiera cylindrica* (BC) and *Sonneratia alba* (SA) achieved the highest crude yield of 5.84% and 5.86% respectively. In the meantime, the lowest percentage of yield was obtained by hexane extracts with BC (1.02%) and SA (1.24%). The ethyl acetate extracts exhibited intermediate yield percentages with BC (1.53%) and SA (2.14%) among the three solvents. This signifies that the highest polarity methanol extracted more of the phytochemical content in mangroves twigs samples meanwhile

the non-polar hexane solvent extracted the least amount. This finding indicates that both mangrove species exhibited a higher yield tendency with the increase of solvent polarity.

This finding aligned with previous research by Andianto et al. (2024). The most polar ethanolic extracts used in the study dominated the yield from mangrove wooden slabs of several origin, [*B. cylindrica* (5.58%, 5.67%), *R. apiculata* (8.33%), *B. gymnorhiza* (2.81%)] in comparison with lower polarity ethyl acetate [*B. cylindrica* (1.32%, 1.98%), *R. apiculata* (0.94%), *B. gymnorhiza* (0.58%)] and non-polar hexane [*B. cylindrica* (1.07%, 0.82%), *R. apiculata* (0.05%), *B. gymnorhiza* (0.73%)]. Andianto et al. (2024) affirmed that the yield of extractive compounds is significantly impacted by solvent selection and solvent polarity. This signifies the efficiency of multilevel maceration, as it allows a wider range of compound extraction, respectively, to solvents of varying polarities.

The multilevel maceration approach comprises the extraction of samples employing solvents of successive polarity (Gori et al., 2021). This study initiated multilevel maceration by soaking the ground SA and BC twigs in non-polar hexane. This procedure was carried to dissolve non-polar molecules, including lipophilic compounds, pigments, flavonoids, phenols and several alkaloids (Cravotto et al., 2022; Wrona et al., 2019). Table 4.1 demonstrates that hexane extracted the least amounts of phytochemicals from both mangrove species. Nonetheless, the non-polar extraction was performed first to purify the extract and prevent the co-extraction of unnecessary components before the polar solute extraction (Zhang et al., 2023, 2018). Polar solvents are significantly more effective in extracting polar secondary metabolites such as saponins, glycosides, alkaloids, and terpenoids, which are abundantly synthesised by plants (Nawaz et al., 2020). This further explained the higher percentage of yield achieved from SA and BC twigs using ethyl acetate and methanol solvents.

Aside from that, Table 4.1 elucidate differences in crude extract yield between BC and SA species. Malik et al. (2017) reported a similar pattern, with *B. sexangula*, *B. cylindrica*, *R. apiculata*, *A. alba*, and *L. racemosa* showing variation in percentage yield within each species, despite sampling from the same location, Setiu Wetlands,

Terengganu. Other than that, past research from Andianto et al. (2024) proven that extracted yield of mangroves species *B. cylindrica*, *R. apiculata* and *B. gymnorhiza* have different crude extraction yield. Apparently, phytochemical content of natural product differs significantly according to species, genus, plant organ, growing location and climate conditions (Kulkarnii and Manohar, 2024; Mitra et al., 2021). Adaptation to the aforementioned conditions causes changes in mangroves chemical characteristics. This causes variations in phytochemical distributions throughout mangrove organs.

Differences in phytochemical distribution in particular species increase mangrove uniqueness and inspire researchers' interest in conducting more screening assessments. It is worth noting that there is currently no scientific evidence or publications on the potential yield of *Sonneratia alba* and *Bruguiera cylindrica* twig extracts and their effectiveness as breast cancer treatments. Thus, further anticancer and antioxidant assays were performed to assess the extract's activities against breast cancer (MCF-7) and normal cell line (L6) prior to antioxidant and liquid chromatography analysis.

4.2 Screening of Mangroves Extracts Cytotoxicity Effects

According to Łukasiewicz et al. (2021), breast cancer is the primary cause of mortality in women and poses a considerable challenge to health sectors globally. Breast cancer mortality is growing above disease-related fatalities, suggesting that current cancer treatment incompetency may be a significant reason (Juthani et al., 2024). Multiple clinical trials have reported that the most commonly prescribed chemotherapies are associated with high-risk toxicities (Baltussen et al., 2023). Particularly, semi-synthetic chemotherapies and their analogues have been noted to cause detrimental side effects in patients over prolonged treatment periods (Amjad et al., 2023; Katta et al., 2023). Thus, natural products that have long provided structural template for cancer medication development are widely screened for cytotoxicity to improve chemotherapy efficacy (Chaachouay and Zidane, 2024).

This study opted for the MTS assay as 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) is more recently developed tetrazolium salt and have greater advancement compared to 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (Femina et al., 2023). Principally, the MTS assay is a one step, tetrazolium-based method that relies on the reduction of yellow tetrazolium salt by metabolically active cells into soluble, purple formazan crystals. Similar with MTT assay, MTS assay offers absorbance readings of viable cells and has been extensively reliable for evaluating cell viability, cytotoxicity, and proliferation. Nevertheless, it is important to mention that MTS eliminates the need of solubilisation, thereby reducing the error of losing cells. Furthermore, without the washing step, MTS produced a deeper shade of formazan, increasing the accuracy of absorbance readings.

Other than that, MTS assay was conducted to determine half maximal inhibition concentration (IC_{50}). Basically, IC_{50} is essential for evaluating pharmacological effectiveness, conducting comparative studies, and screening for the required medication dosage (Aykul and Martinez-Hackert, 2016). In cancer drug development, IC_{50} is the most commonly used indicator in measuring drug potency, as it signifies the concentration of drugs needed to inhibit cancerous cell growth by 50% (Arokia Femina et al., 2023). A lower IC_{50} value indicated higher drug potency, meaning that drugs are effective at lower concentrations. Meanwhile, higher IC_{50} indicated lower drug potency, requiring high concentration to be effective. Thus, in cases of cancer with prolonged treatment periods, the administration of highly potent therapeutic drugs with low IC_{50} is very important, as this could reduce systemic toxicities (Berrouet et al., 2020).

In this study, the six twigs crude extracts obtained from *B. cylindrica* and *S. alba* were first assessed in MTS assay against breast cancer cell line (MCF-7) and normal cell line (L6). Other than that, clinically known vincristine sulphate (VS) was also subjected to cytotoxicity analysis in determination of semi-synthetic chemotherapy drug effects against breast cancer and normal cell line. The aim was to determine the cytotoxic effects expressed as IC_{50} , via a sigmoidal dose-response curve of cell inhibition percentage against sample concentration, after 72 h incubation

period. Further result on MTS assay of each mangrove extracts in respective solvents in comparison of positive control vincristine sulphate were discussed below.

4.2.1 *Bruguiera cylindrica* Hexane Extract (BCH)

The cytotoxicity study demonstrated that BCH produced a dose-dependent manner after 72 h incubation period. Referring to Table 4.2.1, starting from a low dosage of 12.5 $\mu\text{g/mL}$ up to the highest 100 $\mu\text{g/mL}$, more than 50% of MCF-7 were found inhibited. With an increment of treatment dosage ranging from 25 to 50 $\mu\text{g/mL}$, the percentage of MCF-7 inhibition rose to 56.86% and 60.88%, respectively. Interestingly, the extract produced a potent cytotoxicity after 72 h with an IC_{50} value of 7.76 $\mu\text{g/mL}$ (Figure 4.2.1). It is worth noting that BCH showed a strong IC_{50} value. Thus, the viability of MCF-7 was found to decrease with an increase in treatment concentration. This trend can be further observed in Figure 4.2.2 below.

Table 4.2.1 Percentage of MCF-7 inhibition for *B. cylindrica* hexane extract after 72 h incubation period. The 0 $\mu\text{g/mL}$ refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration ($\mu\text{g/mL}$)	% of MCF-7 Inhibition, n=3 (Mean)
0	0
0.391	21.5
0.781	27.36
1.563	36.98
3.125	38.26
6.25	47.53
12.5	53.50
25	56.86
50	60.88
100	61.62

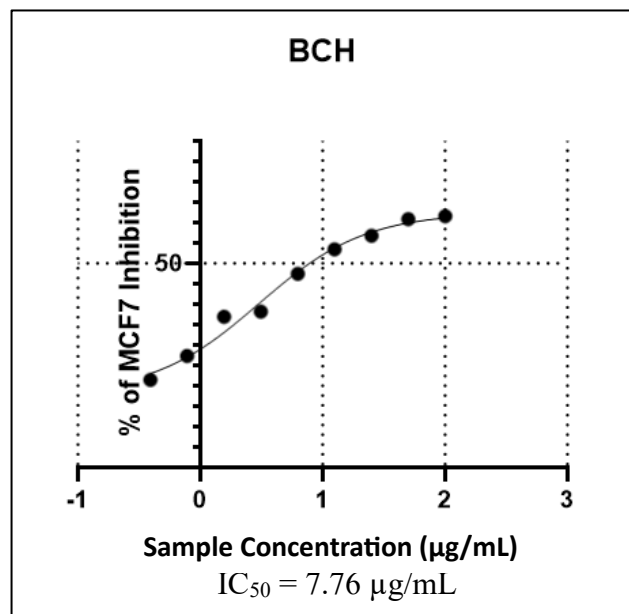


Figure 4.2.1 Log₁₀ sigmoidal-dose responses of *B. cylindrica* hexane extract against MCF-7 cell inhibition after 72 h. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

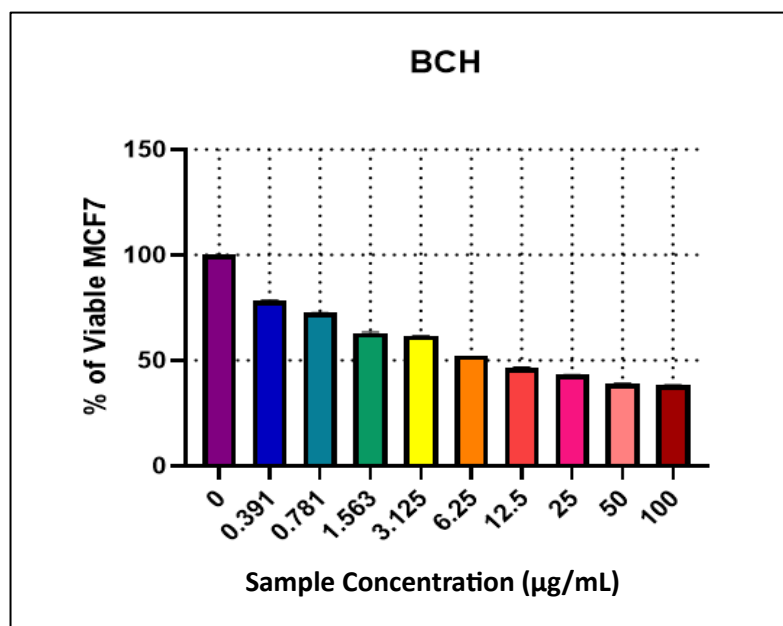


Figure 4.2.2 Percentage of viable MCF-7 against *B. cylindrica* hexane extract after 72 h incubation period. Each value represented Mean $\pm$ S.D (n=3) (three-independent experiments).

Meanwhile, for normal cells (L6), BCH was found to have the ability to inhibit normal cells to half at the highest concentration of 100 µg/mL only, inhibiting 70.74%. Throughout three days of treatment, the second highest treatment concentration, 50

µg/mL, only inhibited 25.99% of normal cells. BCH was also found to exert low toxicity in L6 with a 0-50 µg/mL treatment dosage. In contrast with MCF-7, BCH displayed a weak IC₅₀ against L6 with the value of 75.86 µg/mL as indicated in Figure 4.2.3. As can be referred to in Figure 4.2.4, with the decreased treatment dosage concentrations of 25 µg/mL - 0.391 µg/mL, it is observed that L6 cell viability increased, ranging from 90% to 98% viable.

Table 4.2.2 Percentage of L6 inhibition for *B. cylindrica* hexane extract after 72 h incubation period. The 0 µg/ml refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (µg/mL)	% of L6 Inhibition, n=3 (Mean)
0	0
0.391	1.176
0.781	1.073
1.563	1.092
3.125	3.397
6.25	4.822
12.5	7.271
25	9.735
50	25.99
100	70.74

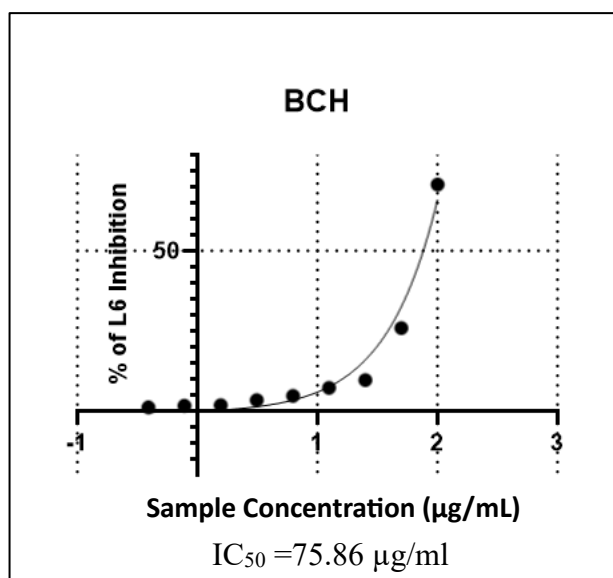


Figure 4.2.3 Log₁₀ sigmoidal-dose responses of *B. cylindrica* hexane extract against L6 cell inhibition after 72 h. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

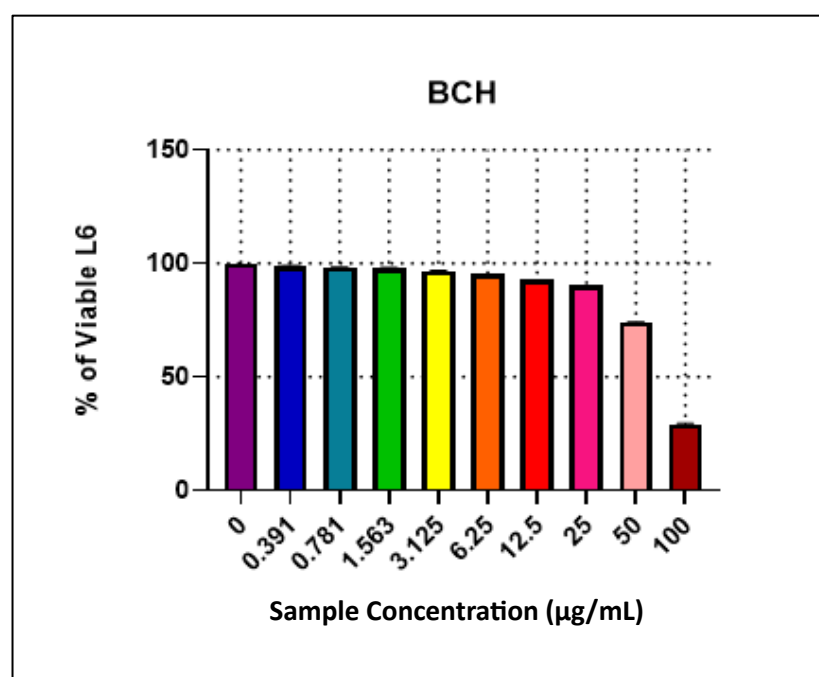


Figure 4.2.4 Percentage of viable L6 against *B. cylindrica* hexane extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

4.2.2 *Bruguiera cylindrica* Ethyl Acetate Extract (BCEA)

According to Table 4.2.3, overall, BCEA shows low inhibition against MCF-7. The lowest concentration of BCEA (0.391 µg/mL) just inhibited about 17.32% of the cells. Tested with concentrations of 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, and 3.125 µg/mL, respectively, the percentage of MCF-7 inhibition increases in the range of 22-25%. BCEA at the highest concentration of 100 µg/ml inhibited only 27.59% of MCF-7. Therefore, referring to graph 4.2.5, BCEA failed to inhibit MCF-7 up to 50%. This finding indicated that BCEA was inactive as an anticancer agent, thus explaining MCF-7 viability remained more than 70% after 72 h incubation period as presented in Table 4.2.6 below.

Table 4.2.3 Percentage of MCF-7 inhibition for *B. cylindrica* ethyl acetate Extract after 72 h incubation period. The 0 µg/ml refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (µg/mL)	% of MCF-7 Inhibition, n=3 (Mean)
0	0
0.391	17.32
0.781	17.95
1.563	18.58
3.125	22.80
6.25	23.17
12.5	23.42
25	23.76
50	24.87
100	27.59

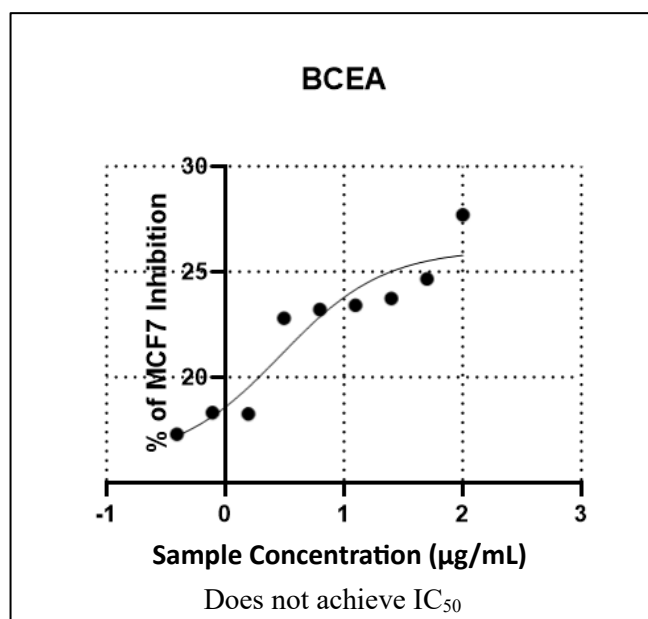


Figure 4.2.5 Log₁₀ sigmoidal-dose responses of *B. cylindrica* ethyl acetate extract against MCF-7 cell inhibition after 72 h. Each value represented mean (n=3) (three-independent experiments).

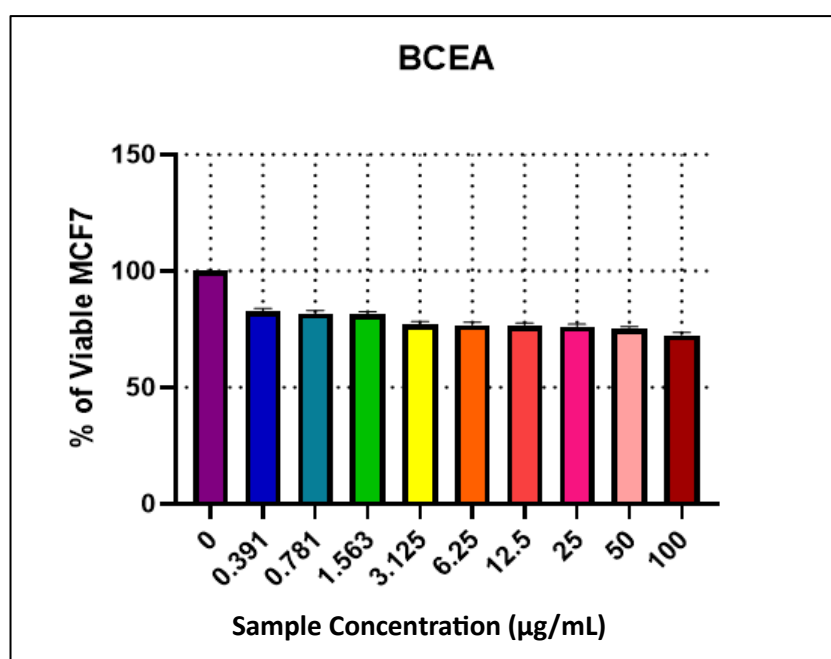


Figure 4.2.6 Percentage of viable MCF-7 for *B. cylindrica* ethyl acetate extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiment)

The same trend was observed with BCEA against L6. At the highest concentration of 100 µg/mL treatment, BCEA just inhibited 42.38% of normal cells. With concentrations of 50 µg/mL, about 31.71% of L6 cells were inhibited. L6 cell viability remained stable nearing 100% with usage of 1.563 µg/mL (90%), 0.781 µg/mL (95.71%), and 0.391 µg/mL (96.17%). Thus, according to Table 4.2.4 below, BCEA failed to inhibit L6 up to 50%, signifying BCEA as non-toxic against normal cell proliferation. Therefore, as can be seen in Figure 4.2.8 below, the viability of normal cells remains more than 57.52% throughout 3 days of treatment.

Table 4.2.4 Percentage of L6 Inhibition for *B. cylindrica* ethyl acetate extract after 72 h incubation period. The 0 µg/ml refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (µg/mL)	% of L6 Inhibition, n=3 (Mean)
0	0
0.391	3.886
0.781	4.490
1.563	9.324
3.125	18.39
6.25	24.67
12.5	27.68
25	28.26
50	31.71
100	42.48

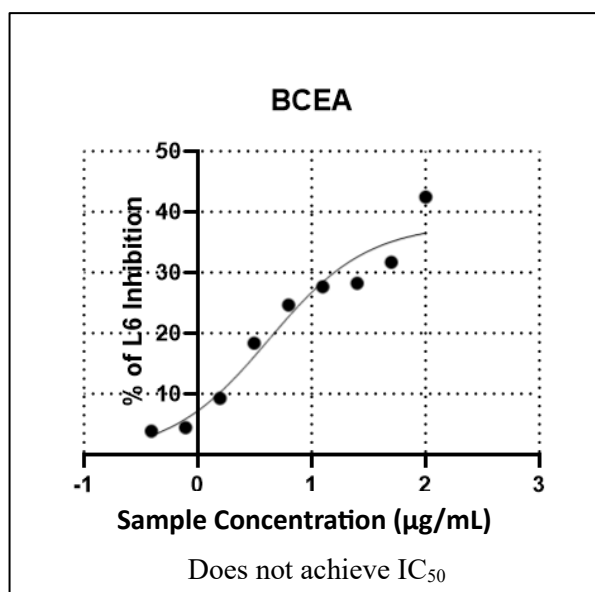


Figure 4.2.7 Log₁₀ sigmoidal-dose responses of *B. cylindrica* ethyl acetate extract against L6 cell inhibition after 72 h. Each value represented mean (n=3) (three-independent experiments).

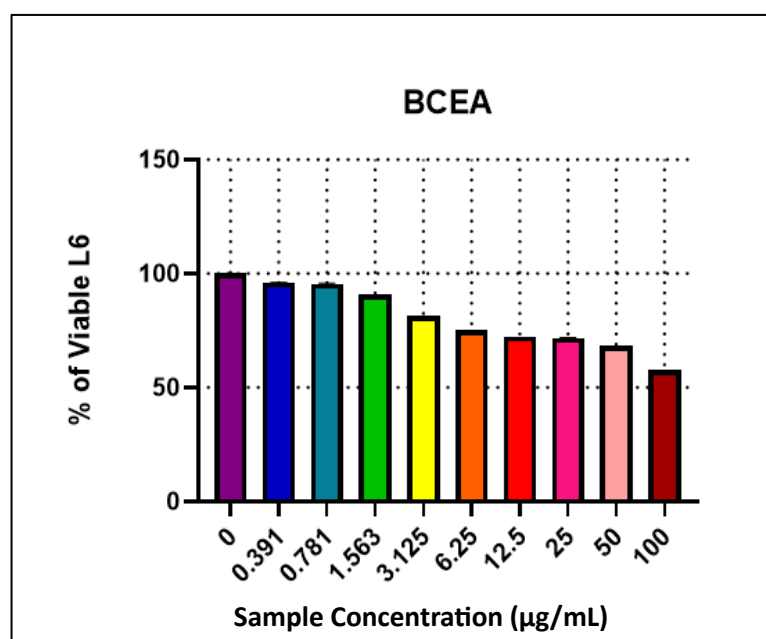


Figure 4.2.8 Percentage of viable L6 for *B. cylindrica* ethyl acetate extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

4.2.3 *Bruguiera cylindrica* Methanol Extract (BCM)

Upon cytotoxicity testing after 72 h treatment, 100 µg/ml of BCM exhibited successful inhibition against MCF-7 cells up to 52.43%. However, referring to Table 4.2.5 below, the percentage of inhibition for other concentrations (0.391 µg/mL–50 µg/mL) did not exceed 30% of inhibition. Therefore, This finding indicated that BCM displayed weak toxicity against breast cancer cells (MCF-7) with the achieved weak IC₅₀ value of 94.5 µg/mL, as can be seen in Figure 4.2.9. According to Figure 4.2.10, majorly, MCF-7 viability still remained more than 50% after 72 h treatment period.

Table 4.2.5 Percentage of MCF-7 inhibition for *B. cylindrica* methanol extract after 72 h incubation period. The 0 µg/ml refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (µg/mL)	% of MCF-7 Inhibition, n=3 (Mean)
0	0
0.391	10.58
0.781	11.72
1.563	12.31
3.125	13.40
6.25	17.31
12.5	19.12
25	26.25
50	28.58
100	52.43

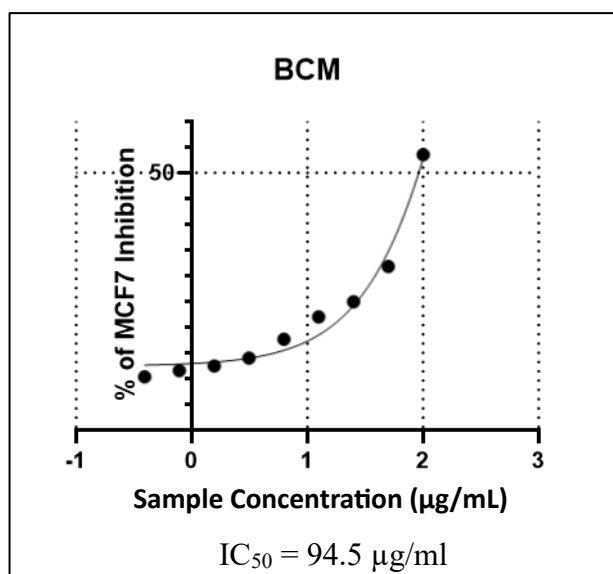


Figure 4.2.9 Log₁₀ Sigmoidal-dose responses of *B. cylindrica* methanol extract against MCF-7 cell inhibition after 72 h. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

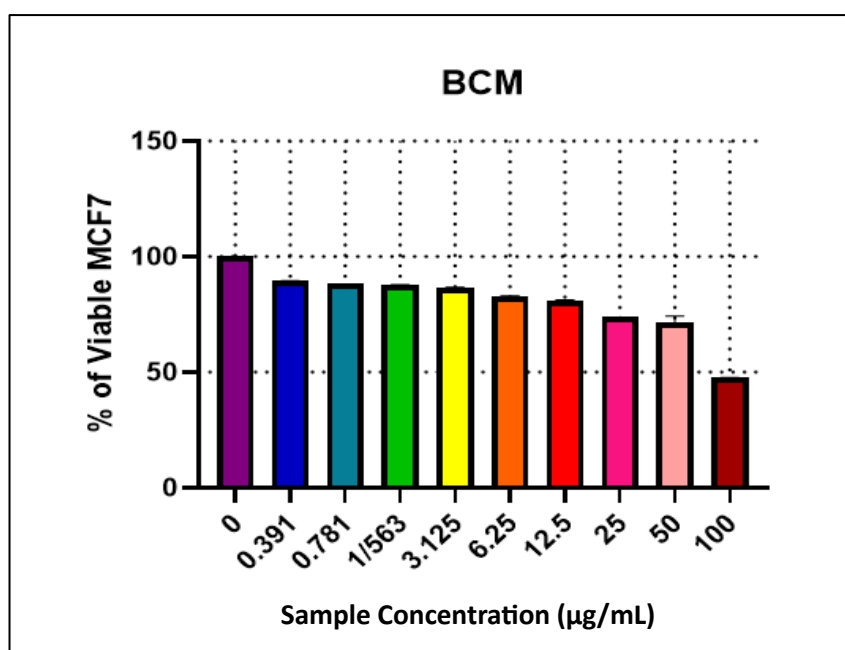


Figure 4.2.10 Percentage of viable MCF-7 for *B. cylindrica* methanol extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

Similarly, BCM was found to display very weak toxicity against L6. Referring to table 4.2.6, the highest inhibition was observed with the usage of 100 µg/mL BCM (46.63%). Therefore, relating to Figure 4.2.11, BCM did not inhibit L6 up to 50%. This indicated BCM as non-toxic against normal cell proliferations. Figure 4.2.12 below presented that lower concentration of BCM implicated a higher percentage of L6 viabilities in the ranges of 73.41% up to 89.71%, nearing 100% viability.

Table 4.2.6 Percentage of L6 inhibition for *B. cylindrica* methanol extract after 72 h incubation period. The 0 µg/ml refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (µg/mL)	% of L6 Inhibition, n=3 (Mean)
0	0
0.391	10.32
0.781	20.41
1.563	22.19
3.125	22.99
6.25	26.61
12.5	32.56
25	32.97
50	42.82
100	46.63

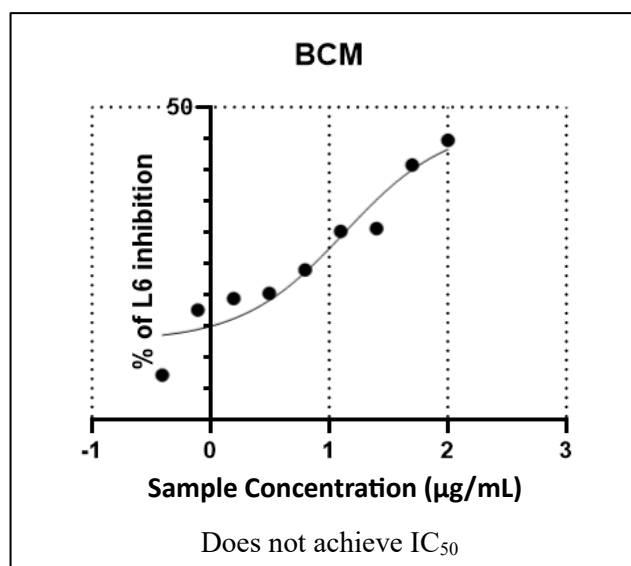


Figure 4.2.11 Log_{10} sigmoidal-dose responses of *B. cylindrica* methanol extract against L6 cell inhibition after 72 h. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

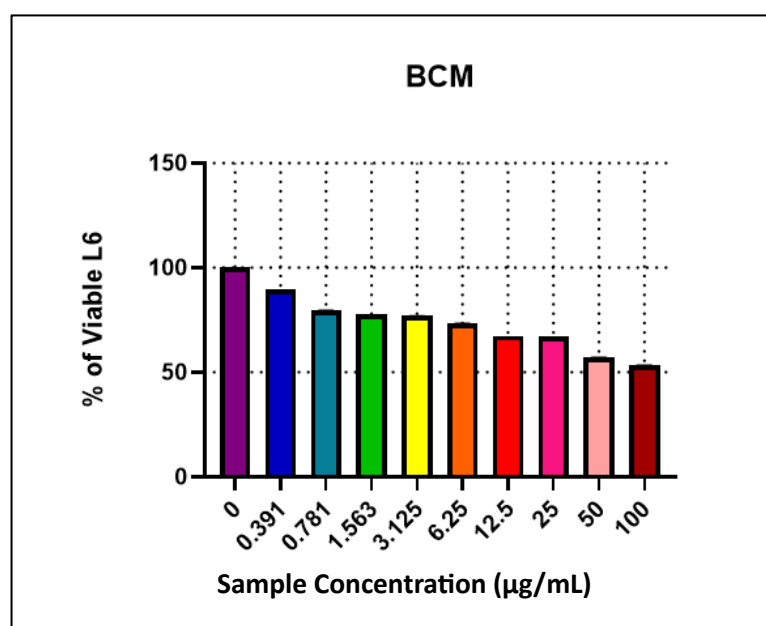


Figure 4.2.12 Percentage of viable L6 for *B. cylindrica* methanol extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

4.2.4 *Sonneratia alba* Hexane Extract (SAH)

The cytotoxicity study demonstrated that SAH implied dependable inhibition against MCF-7. As can be seen in Table 4.2.7, spectacularly, about 70.24% of MCF-7 were found inhibited with 100 µg/mL of SAH dosage after 72 h incubation period. Moreover, starting from the low concentration of 3.125 µg/mL onwards, MCF-7 was found to be inhibited more than 50%. Specifically at the concentrations of 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, and 50 µg/mL, the inhibition rate increased ≥ 50 µg/mL, where the values of inhibitions were 51.32%, 54.20%, 65.95%, and 67.11%, respectively. Thus, referring to Figure 4.2.13, SAH prevailed with a very strong IC₅₀ value of 3.47 µg/ml against the breast cancer cell line. Therefore, after 72 h treatment period, the viability of MCF-7 decreased to 50%-30% (Figure 4.2.14).

Table 4.2.7 Percentage of MCF-7 inhibition for *S. alba* hexane extract after 72 h incubation period. The 0 µg/ml refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (µg/mL)	% of MCF-7 Inhibition, n=3 (Mean)
0	0
0.391	24.30
0.781	41.47
1.563	48.08
3.125	49.54
6.25	51.32
12.5	54.20
25	65.95
50	67.11
100	70.24

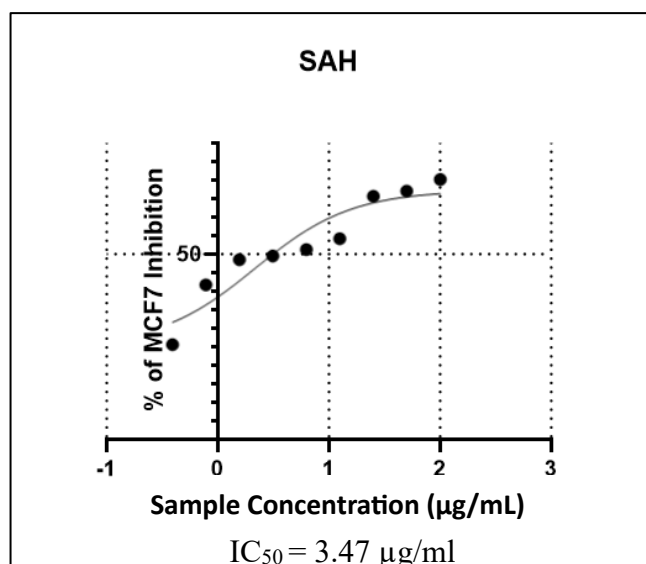


Figure 4.2.13 Log₁₀ sigmoidal-dose responses of *S. alba* hexane extract against MCF-7 cell inhibition after 72 h. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

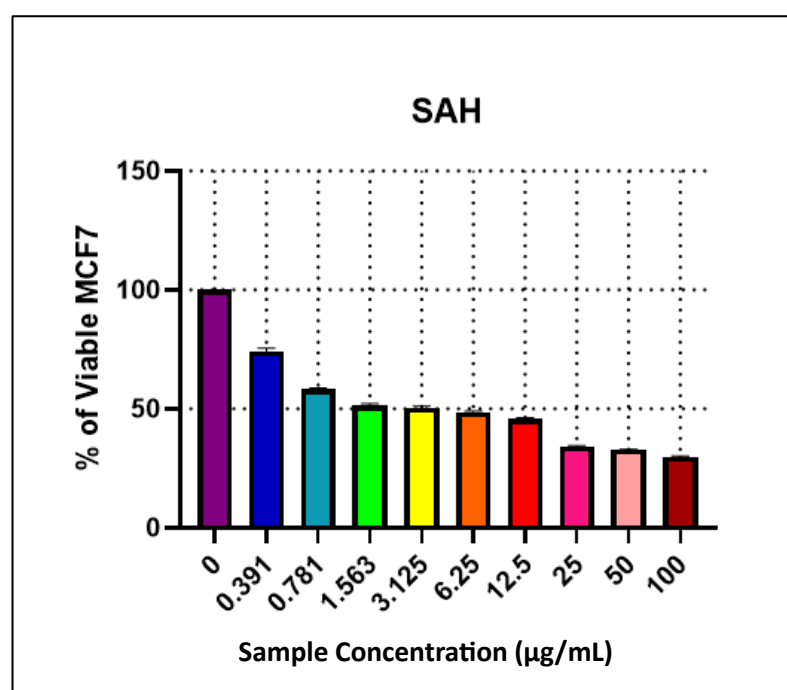


Figure 4.2.14 Percentage of viable MCF-7 for *S. alba* hexane extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

In contrast, for the L6 cell line, SAH concentrations from 0.39 µg/mL up to 50 µg/mL executed inhibitions below 35%. As can be seen in Table 4.2.8, only at 100 µg/ml, SAH inhibited more than half of the L6 population, up to 65.39%. As observed in Figure 4.2.15, SAH demonstrated weak toxicity against the normal cell line (L6) with the achieved IC₅₀ value of 74.13 µg/mL. SAH appeared to be low toxic to the normal cell line at low concentration, which further explained the trend in Figure 4.2.16, where the viability of L6 remained more than 60% throughout the 3-day treatment incubation period.

Table 4.2.8 Percentage of L6 inhibition for *S. alba* hexane extract after 72 h incubation period. The 0 µg/ml refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (µg/mL)	% of L6 Inhibition, n=3 (Mean)
0	0
0.391	1.934
0.781	6.826
1.563	10.82
3.125	13.78
6.25	16.30
12.5	18.13
25	26.56
50	35.13
100	65.39

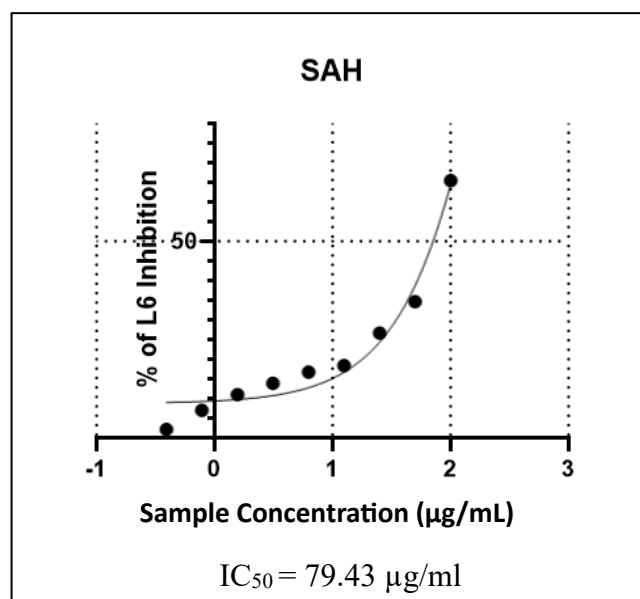


Figure 4.2.15 Log₁₀ sigmoidal-dose responses of *S. alba* hexane extract against L6 cell inhibition after 72h. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

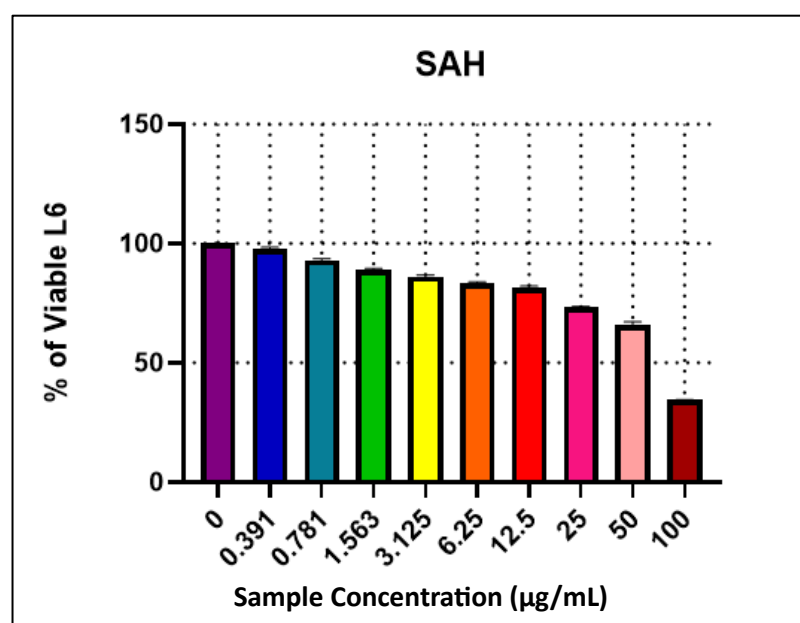


Figure 4.2.16 Percentage of viable L6 for *S. alba* hexane extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

4.2.5 *Sonneratia alba* Ethyl Acetate Extract (SAEA)

SAEA induced MCF-7 inhibitions in a dose-dependent manner. With the highest treatment dosage of 100 µg/mL, 82.24% of MCF-7 were found inhibited (Table 4.2.9). At the second highest dosage of 50 µg/mL, 69.56% of MCF-7 were found inhibited, which signifies more than 50% of the MCF-7 population was inhibited due to the treatment. However, concentrations of 25 µg/mL below were found unable to inhibit MCF-7 up to 50%. Referring to the viability graph in Figure 4.2.18, the percentage of MCF-7 viability decreased significantly with an increase in treatment concentration. Therefore, as presented in Table 4.2.17, SAEA prevailed with significant cytotoxicity against MCF7 with a weak IC₅₀ value of 27.8 µg/mL.

Table 4.2.9 Percentage of MCF-7 inhibition for *S. alba* ethyl acetate extract after 72 h incubation period. The 0 µg/ml refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (µg/mL)	% of MCF-7 Inhibition, n=3 (Mean)
0	0
0.391	4.97
0.781	8.91
1.563	23.41
3.125	30.99
6.25	38.09
12.5	41.89
25	44.91
50	69.56
100	82.24

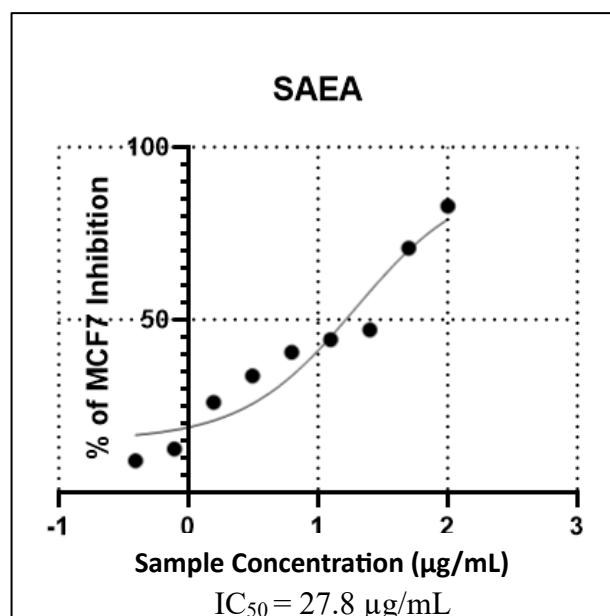


Figure 4.2.17 Log₁₀ sigmoidal-dose responses of *S. alba* ethyl acetate extract against MCF-7 cell inhibition after 72 h. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

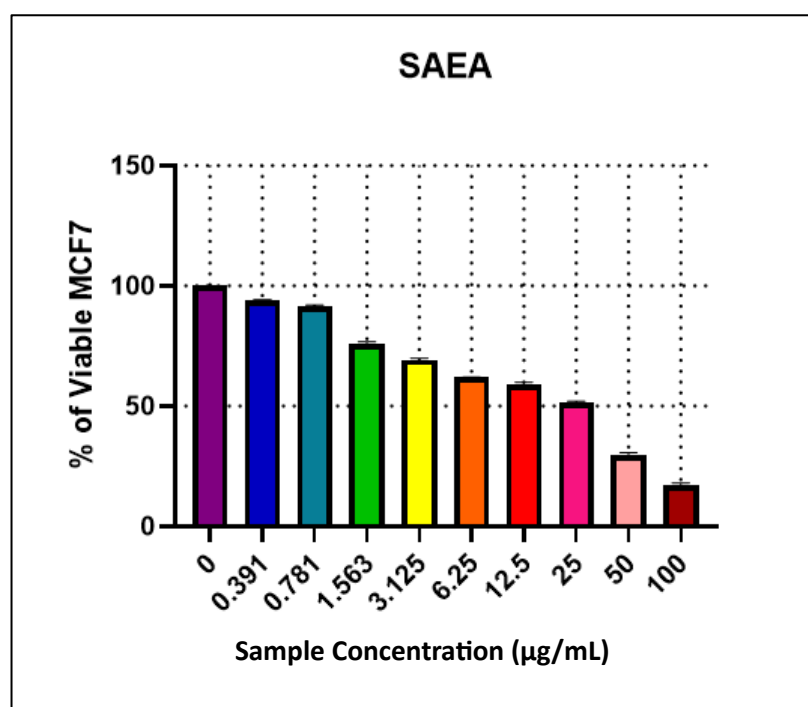


Figure 4.2.18 Percentage of viable MCF-7 for *S. alba* ethyl acetate extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

This cytotoxicity assay prevailed SAEA as low toxic against normal cells, as only 65.15% of L6 were inhibited at the highest concentration of 100 $\mu\text{g/mL}$ (Table 4.2.10). SAEA was found unable to reach 50% inhibition of L6 from the second highest concentration downward. This signified that 50 $\mu\text{g/mL}$ of SAEA were found to inhibit only 39.36% of L6. This observation pretty much elucidated that SAEA displayed weak toxicity against the normal cell line with the acquired IC_{50} value of 63.09 $\mu\text{g/mL}$. Therefore, SAEA was deemed to be weakly toxic against normal cells. Hence, more than 60% of L6 cells were found viable with 0.391 $\mu\text{g/mL}$ - 50 $\mu\text{g/mL}$ SAEA treatments after 72 h treatment period (Figure 4.2.20).

Table 4.2.10 Percentage of L6 inhibition for *S. alba* ethyl acetate extract after 72 h incubation period. The 0 $\mu\text{g/mL}$ refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration ($\mu\text{g/mL}$)	% of L6 Inhibition, n=3 (Mean)
0	0
0.391	10.77
0.781	15.40
1.563	17.64
3.125	22.90
6.25	26.38
12.5	27.71
25	28.50
50	39.36
100	65.15

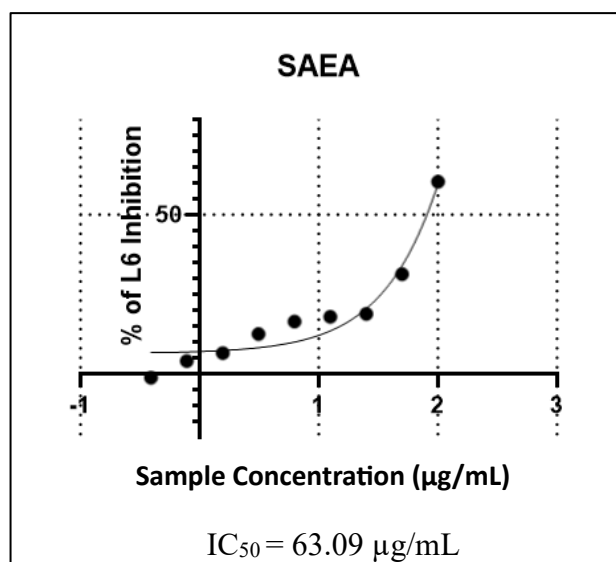


Figure 4.2.19 Log₁₀ sigmoidal-dose responses of *S. alba* ethyl acetate extract against L6 cell inhibition after 72 h. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

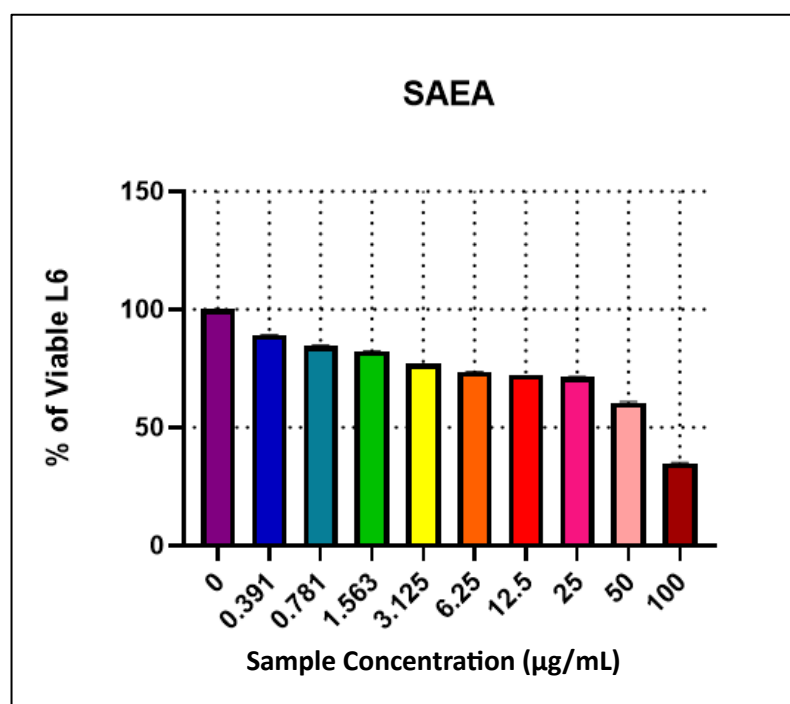


Figure 4.2.20 Percentage of viable L6 for *S. alba* ethyl acetate extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

4.2.6 *Sonneratia alba* Methanol Extract (SAM)

According to Table 4.2.11 below, generally SAM induced inhibition of MCF-7 in a dose-dependent manner. However, even after 72 h treatment period, at the highest concentration of 100 µg/mL, SAM was found to inhibit only 44.84% of breast cancer cells, MCF-7 (Table 4.2.11). As SAM was found unable to inhibit MCF-7 up to 50% in accordance with Figure 4.2.21, SAM failed to achieve an IC₅₀ value. Thus, this finding determined SAM inadequate to act as a potential anticancer agent against breast cancer. This explains the viability of MCF-7 remained more than 50% at all SAM concentrations after three days of treatment.

Table 4.2.11 Percentage of MCF-7 inhibition for *S. alba* methanol extract after 72 h incubation period. The 0 µg/ml refers to untreated cell (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (µg/mL)	% of MCF-7 Inhibition, n=3 (Mean)
0	0
0.391	5.48
0.781	8.52
1.563	10.48
3.125	16.78
6.25	17.36
12.5	20.51
25	33.35
50	43.36
100	44.84

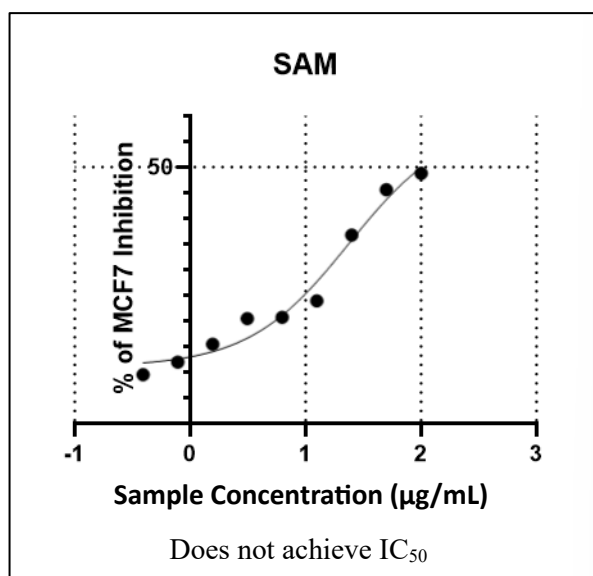


Figure 4.2.21 Log₁₀ sigmoidal-dose responses of *S. alba* methanol extract against MCF-7 cell inhibition after 72 h. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

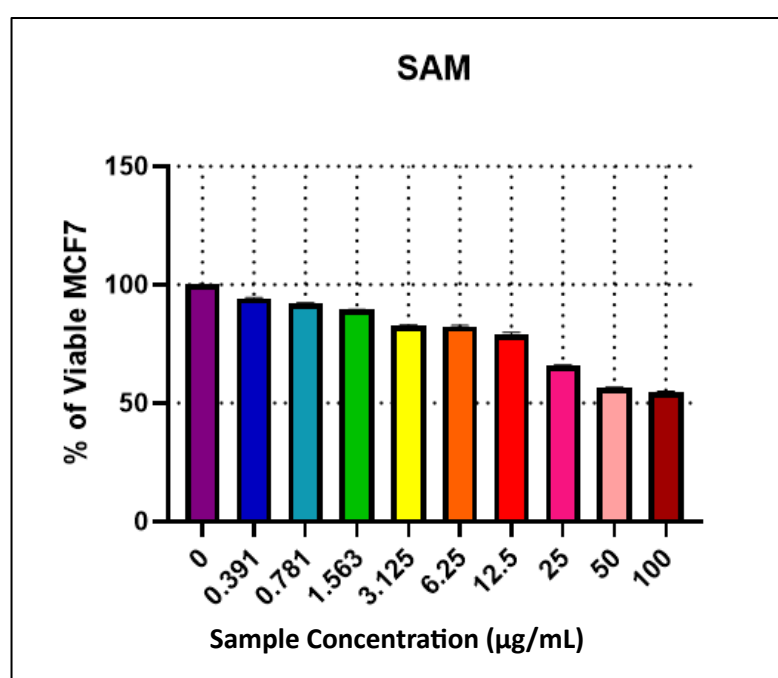


Figure 4.2.22 Percentage of viable MCF-7 for *S. alba* methanol extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

In the same manner, SAM was found inadequate to achieve half-maximal inhibition concentration against L6 as well (Figure 4.2.23). The highest concentrations of 100 µg/ml SAM just exhibited 47.77% inhibition against the normal cell line L6 (Table 4.2.12). This finding proves that SAM was non-toxic against normal cell proliferation. Therefore, L6 cell line viability remained higher than 50% throughout the 3-day treatment period as presented in Figure 4.2.24.

Table 4.2.12 Percentage of L6 inhibition for *S. alba* methanol extract after 72 h incubation period. The 0 µg/ml refers to untreated cell (negative control). Each value represented Mean (n=3) (three-independent experiments).

Sample Concentration (µg/mL)	% of L6 Inhibition, n=3 (Mean)
0	0
0.391	1.97
0.781	10.86
1.563	15.61
3.125	16.46
6.25	18.21
12.5	25.31
25	27.39
50	33.39
100	47.77

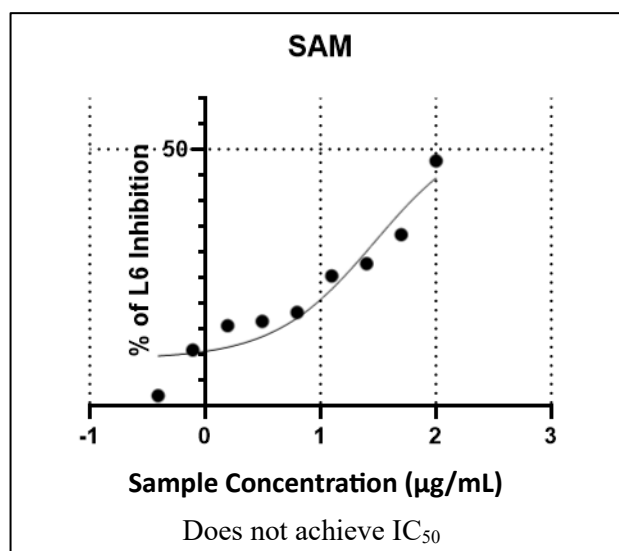


Figure 4.2.23 Log₁₀ sigmoidal-dose responses of *S. alba* methanol extract against L6 cell inhibition after 72 h. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

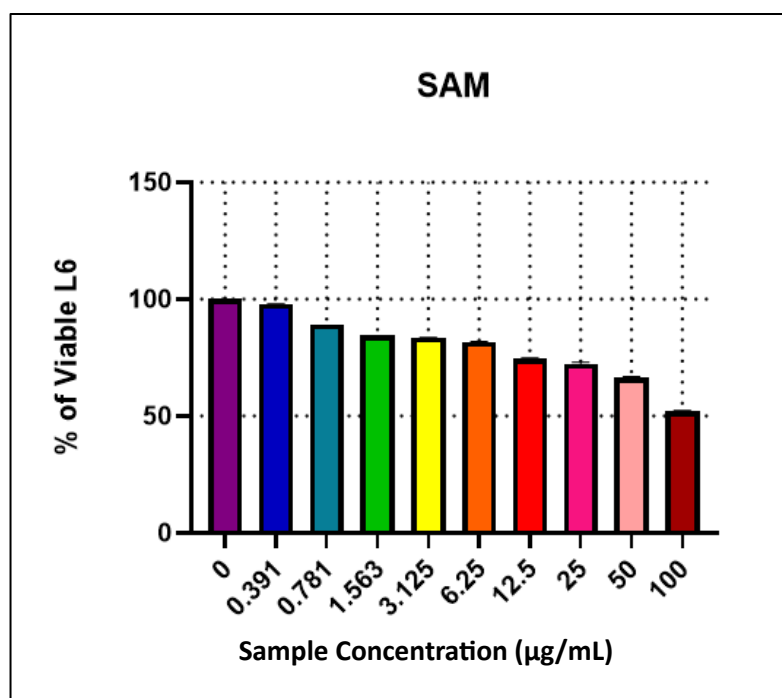


Figure 4.2.24 Percentage of viable L6 for *S. alba* methanol extract after 72 h incubation period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

4.2.7 Vincristine Sulphate (VS)

In this cytotoxicity assessment, vincristine sulphate (VS) served as a positive control for successful anticancer effects against breast cancer. VS is the second most consumed cytotoxic chemotherapy and is clinically used in the treatment of several cancer types, such as advanced breast cancer, acute lymphatic leukaemia, neuroblastoma, lymphoma, Wilms tumour, and lung cancer (Awuku-Sowah et al., 2022; Liu et al., 2024; Shukla et al., 2024, p. 202). Derived from plants, *Catharanthus roseus*, or commonly known as Madagascar periwinkle, vincristine is an antitumor drug that belongs to the vinca alkaloid group.

However, the natural yield of vincristine from *C. roseus* is naturally low, approximately 0.0005% of the plant's dry weight (Birat et al., 2022). Therefore, to accommodate the needs of the drugs, alternative ways are utilised, such as producing vincristine semi-synthetic and its analogue at an industrial scale (Zhang et al., 2022). Thereby, vincristine sulphate is chosen in this study to compare the effects of semi-synthetic and pure natural product anticancer drugs on both breast cancer and normal cell line proliferations. Figure 4.2.25 presented the MTS assay result conducted in this study of MCF-7 and L6 percentage inhibition after treatment with vincristine sulphate over 72 h treatment period.

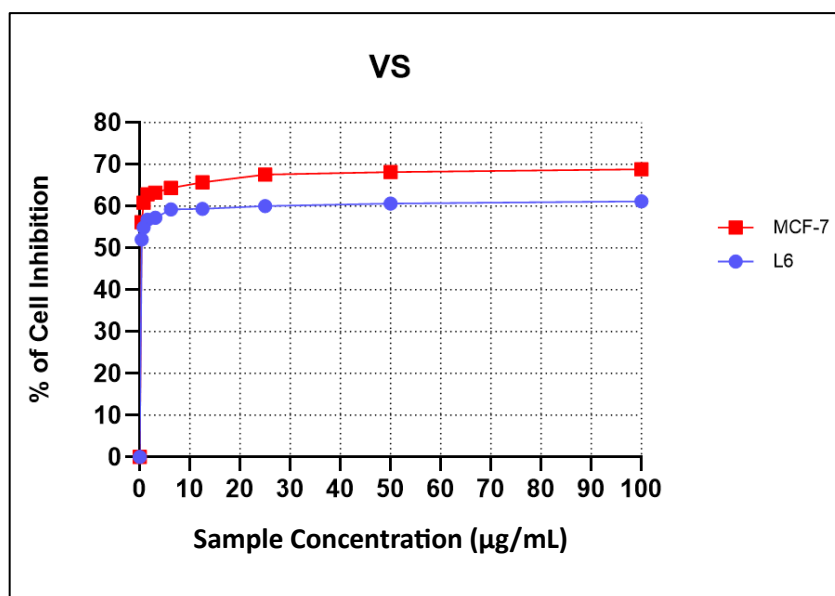


Figure 4.2.25 MCF-7 & L6 inhibition treated with vincristine sulphate after 72 h treatment period. Each value represented Mean $\pm$ S.D (n=3) (three-independent experiments).

Inhibition of VS against cell lines was plotted in a different graph as VS is a pure compound. Thus, starting concentrations for VS dilutions differ from crude extracts. Referring to Table 4.2.25, VS showed aggressive inhibitions towards both MCF-7 and L6 cell lines as more than 50% inhibition already occurred at the lowest concentration treatment of 0.391 $\mu\text{g/mL}$. Apparently, at the highest treatment concentration of 100 $\mu\text{g/mL}$, VS were found to inhibit 61.23% (L6) and 69.87% (MCF-7) in a dose-dependent manner. Against MCF-7, VS acquired a very strong IC_{50} value of 0.063 $\mu\text{g/mL}$. Therefore, VS appeared to be a highly potent anti-cancer agent. Additionally, in L6, VS displayed the same trend of a very strong IC_{50} value of 0.104 $\mu\text{g/mL}$.

Surprisingly, the trend of VS inhibition against MCF-7 was just about 9% higher compared to L6. This finding prevailed that VS was not just highly toxic against cancerous cells but appeared to be very toxic against normal cell lines as well. This study aligns with the claims that vincristine is definitely one of the best available options in battling breast cancer (Chen et al., 2023). However, a new insight on semi-synthetic chemotherapeutic drug side effects was added as this study provided similar outcomes with multiple clinical trials reporting vincristine is closely related with limited clinical use due to high toxicity incidences (Chen and Menezes, 2025).

Inclusive, all six crude twig extracts from *B. cylindrica* and *S. alba* inhibited both MCF-7 and L6 in a dose-dependent manner. Several crude extracts from these two selected species were found to exert anticancer effects against breast cancer cell lines. The IC₅₀ value obtained from all mangrove extracts utilised in the MTS assay in this study was summarised in Table 4.2.13 below along with the positive control, vincristine sulphate, for comparison purposes.

Table 4.2.13 IC₅₀ value of respective mangrove extracts against MCF-7 and L6. Each assay was conducted in triplicate, n=3. The IC₅₀ values are stated in Mean ± S.D

Sample	IC ₅₀ ± S.D (µg/mL)	
	MCF-7	L6
BCH	7.76 ± 0.18	75.86 ± 0.21
BCEA	-	-
BCM	94.5 ± 0.14	-
SAH	3.47 ± 0.20	74.13 ± 0.17
SAEA	27.8 ± 0.11	63.09 ± 0.17
SAM	-	-
VS	0.063 ± 0.12	0.104 ± 0.12

The dash symbol (-) represented sample failed in achieving IC₅₀. Vincristine sulphate IC₅₀ value serves as comparison between sample and positive control.

Among the three extractive solvents used in this study, hexane extract showed higher efficacy in delivering anticancer effects against the MCF-7 cell line. As anticancer compounds such as terpenoids, several alkaloids, and phenols appear to be nonpolar and lipid-soluble; this clarifies the high anticancer activities in hexane solvent. This finding is supported by a study conducted by Koh et al. (2017), which stated that nonpolar and hydrophobic solvents like hexane have the ability to extract anticancer compounds. Furthermore, in a study conducted by Albinhassan et al. (2021), they discovered that hexane extracts of *Avicennia marina* leaves are a potential candidate as an anticancer drug due to the inhibition of several cancer lines such as HCT-116, HepG-2, and MCF-7.

For mangrove anticancer activity against MCF-7, *Sonneratia alba* exert promising anticancer effect in two solvent which is hexane and ethyl acetate. *S. alba* in hexane extract showed very strong IC₅₀ values of 3.47 ± 0.20 µg/mL meanwhile *S.*

alba in ethyl acetate extracts prevailed weak IC₅₀ value of 27.8 ± 0.11 µg/mL. This finding aligned with Shafira et al. (2024) where fruit of *S. alba* were reported to moderately inhibit breast cancer cell (MCF-7). Similar to this study, Latief et al. (2020) proved that root extracts of *S. alba* and its β-sitosterol isolate are very toxic against brine shrimp lethality test and hold a promising value as oncology drug biomaterial.

Cytotoxicity screening also prevailed that *Bruguiera cylindrica* in hexane extracts showed a strong IC₅₀ value of 7.76 ± 0.18 µg/mL against MCF-7. This finding shows the same trend as the Chaudry et al. (2020) study, where they clarified that *B. gymnorrhiza* leaves prevailed with varied IC₅₀ values corresponding to the extractive solvent, where the butanol extract (3.39 µg/mL), diethyl ether extract (16.22 µg/mL), and methanol extract (37.15 µg/mL) in arresting MCF-7 proliferation. Kumari and Kasturi (2024) reported the same trend with 7-(dimethyl amino)-3-ethyl-8-oxo-5-thia-1-azabicyclo (4.2.0)-Oct-2-ene-2-carboxylic acid” (LS2) isolated from *B. cylindrica* extract inhibiting MCF-7 and A549 cell lines at IC₅₀ values of 58.81 µg and 70.8 µg, respectively.

Bruguiera cylindrica in methanol extract prevailed weak IC₅₀ value of 94.5 ± 0.14 µg/mL. Similarly, *B. gymnorrhiza* fruit and root extracts appear as weak and non-toxic against A549 cell line with IC₅₀ value of 130 µg/mL and 140 µg/mL respectively, as demonstrated in Bibi Sadeer et al. (2021) study. Interestingly there are still no report and scientific evidence of BCH, BCM, SAH and SAEA as significantly cytotoxic against breast cancer as per elucidated in this study. Therefore, to further compare the significance of differences between treatment group that achieved IC₅₀ with control group (Table 4.2.13), statistical analysis was conducted through one-way ANOVA and Wilcoxon Signed ranked test, where $p < 0.05$ is statistically significant.

Figure 4.2.26 showed the determination of significant anticancer agents of BCH, BCM, SAH, and SAEA cytotoxicity effects compared with the negative control (complete media) that implicated no relative toxicity against the MCF-7 cell line proliferation after 72 h incubation period. BCH, SAH, and SAEA were found to be statistically significant; meanwhile, BCM appeared to be insignificant. The significance of the mangrove sample was found to be proportional to the obtained IC₅₀

value. SAH acquired ($p<0.001$), showing very strong differences against complete media, meaning SAH showed very strong inhibition towards MCF-7. BCH was found to be strongly different from complete media ($p<0.01$), suggesting that BCH strongly inhibited MCF-7. With ($p<0.05$), SAEA showed moderate inhibition towards MCF-7 in comparison with complete media. Meanwhile, BCM was analysed to be insufficient as an anticancer agent ($p>0.05$), indicating its effects are not much different from complete media.

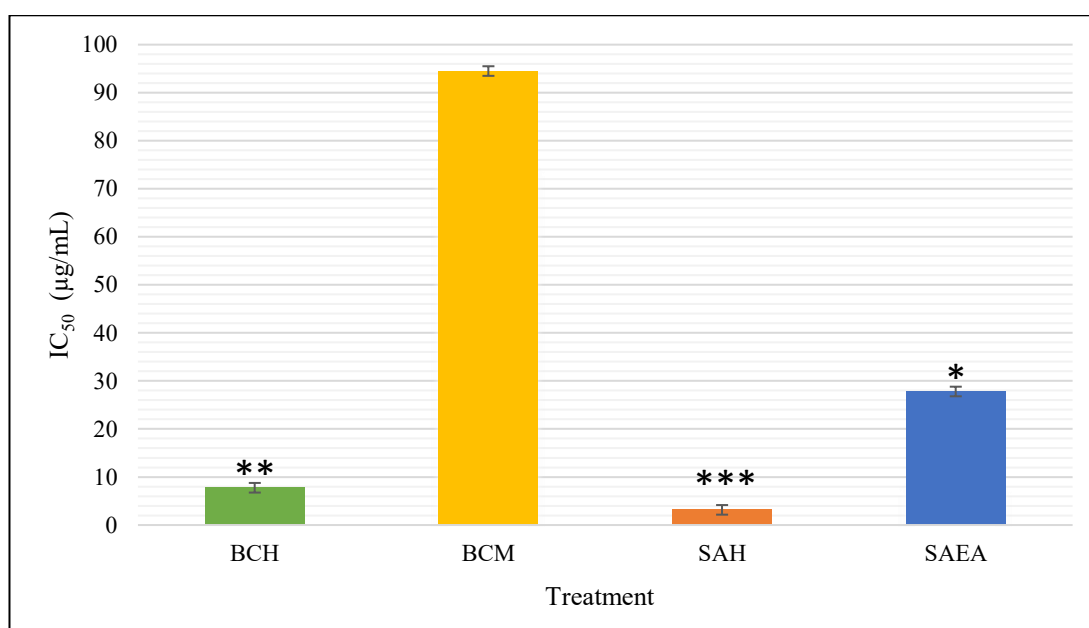


Figure 4.2.26 Active IC₅₀ value obtained from MTS assay of mangrove samples against MCF-7 cell lines. The value was expressed in µg/mL ($n=3$, mean $\pm$ S.D). Treatments were considered statistically significant (* $p<0.05$), (** $p<0.01$) and (***) $p<0.001$). The efficacy of mangrove extract as a potent anticancer agent against MCF-7 was determined based on comparison to complete media, with no inhibitory effects.

Interestingly, all six crude mangrove extracts utilised in this study exerted weak-to-non-toxic effects against normal cell proliferation (Table 4.2.13). Similarly extracts of *Rhizophora mucronata* did not inhibit progression of normal human fetal lung fibroblast cells (WI-38) with with IC₅₀ values of 932 ± 30 µg/mL (leaves) and 629 ± 3 µg/mL (stems). Therefore, in Figure 4.2.27, BCH, SAH, and SAEA with positive anticancer potential were relatively compared with the positive control (vincristine sulphate) to statistically determine the effects of natural products and semi-synthetic chemotherapy drugs against normal cell proliferation, where $p<0.05$ is

statistically significant. Figure 4.2.27 shows that all three extracts significantly differed from vincristine sulphate, with a p-value of less than 0.05. This finding indicated that SAH, BCH, and SAEA were statistically non-cytotoxic against normal cell line proliferation. Therefore, mangrove extracts were not likely to deliver the same toxic effects as vincristine sulphate, such as unpredictable and uncertain pharmacological nature, which leads to neurotoxicity, myelosuppression, alopecia, and gastrointestinal toxicity cases (Awosika et al., 2023).

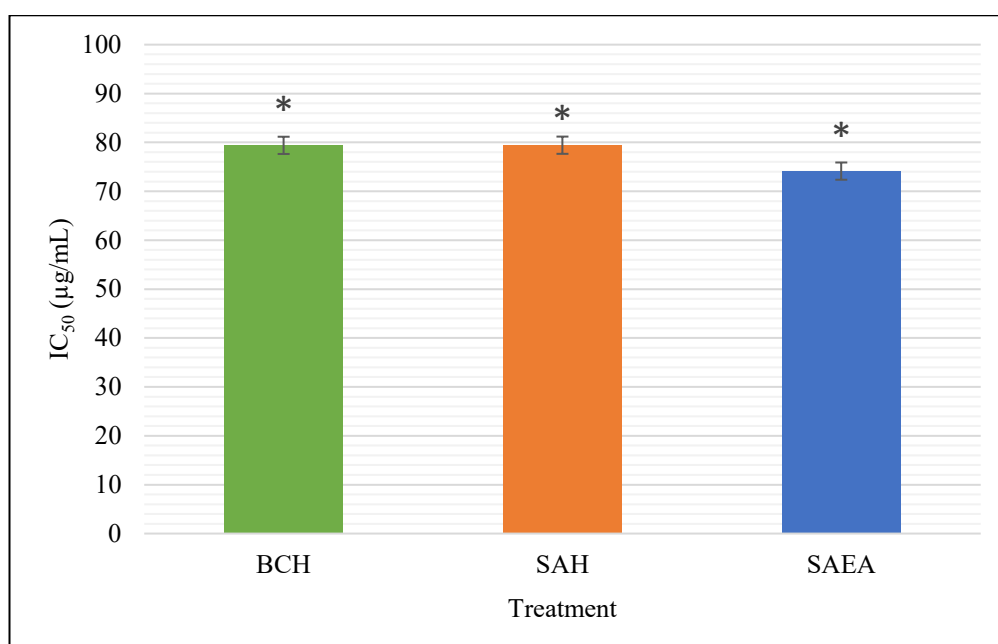


Figure 4.2.27 Active IC₅₀ value obtained from MTS assay of mangrove samples against L6 cell lines. The value was expressed in µg/mL (n=3, Mean ± S.D). Treatments were considered statistically significant (*p<0.05). The statistical analysis compared the non-toxicity of selected mangrove extracts to vincristine sulphate, which was highly toxic to normal cell proliferation.

Overall, this cytotoxicity screening prevailed that spectacular anticancer activities were observed with *Bruguiera cylindrica* in hexane extract (BCH), *Sonneratia alba* in hexane extracts (SAH) and *Sonneratia alba* in ethyl acetate extract (SAEA). In accordance to the guideline set by the American National Cancer Institute (NCI), BCH, SAH and SAEA carries the potential to be further developed into anticancer drug as the achieved IC₅₀ values were below than 30 µg/mL. Thus, these three selected extracts were further investigated in apoptosis assay to determine cell death mechanism of MCF-7.

4.3 Apoptosis Assay

In light of current oncological research, the major strategy for arresting cancer proliferation is to trigger the targeted apoptosis pathways. Apoptosis is deemed important in cancer control because it maintains the balance in cellular homeostasis while preventing the accumulation of mutagenic cellular components (Bock and Riley, 2023). Researchers have fully confirmed that apoptosis is the primary characteristic of a successful chemotherapeutic drug because it signals anti-inflammatory responses in the eradication of cancer cells (Chaudhry et al., 2022; Pfeffer and Singh, 2018). Indeed, apoptosis does not trigger injury in adjacent cells, as it effectively prevents the release of degradative enzymes and cytokines into the extracellular matrix (Vegting et al., 2021). Therefore, understanding apoptosis and its underlying mechanism could facilitate development of a more efficient and targeted cancer therapy.

Programmed cell death, or apoptosis, is a cysteine aspartyl-specific protease (caspase)-dependent signalling pathway that encompasses two primary routes: the intrinsic pathway and extrinsic pathway (Peng et al., 2022). The intrinsic pathway is mitochondrial dependent and is usually activated in cases of intracellular stresses such as DNA damage, hypoxia and oxidative stress conditions (Rodríguez-González and Gutiérrez-Kobeh, 2023). Meanwhile, extrinsic pathway activation is triggered by binding of extracellular signals on death receptors (Krasovec et al., 2024). While intrinsic and extrinsic pathways can be distinguished by the activation of initiator caspases-8/9, both pathways ultimately converge at the executioner signals of caspase-3 (Pfeffer and Singh, 2018). Caspase-3 is a pivotal in apoptosis as it cleaves key cellular protein leading to DNA fragmentation, cell shrinkage, and the formation of apoptotic bodies in compromised cells (Dou et al., 2024; Sun, 2024).

Interestingly, this study prevailed that treated MCF-7 at achieved IC_{50} concentration of BCH ($7.76 \pm 0.18 \mu\text{g/mL}$), SAH ($3.47 \pm 0.20 \mu\text{g/mL}$), and SAEA ($27.8 \pm 0.11 \mu\text{g/mL}$) were observed to undergo distinct morphological change prior death. As can be seen in Figure 4.3.1 below, MCF-7 were found losing adherence, becoming rounded, blebbing in the cell membrane, shrunken in size and losing volumes. These mentioned morphological changes positively aligned with apoptotic

cell characteristics. Thus, to further affirmed BCH, SAH and SAEA induced cytotoxicity in breast cancer cell line through apoptosis pathway, these three extracts were subjected into apoptosis assay.

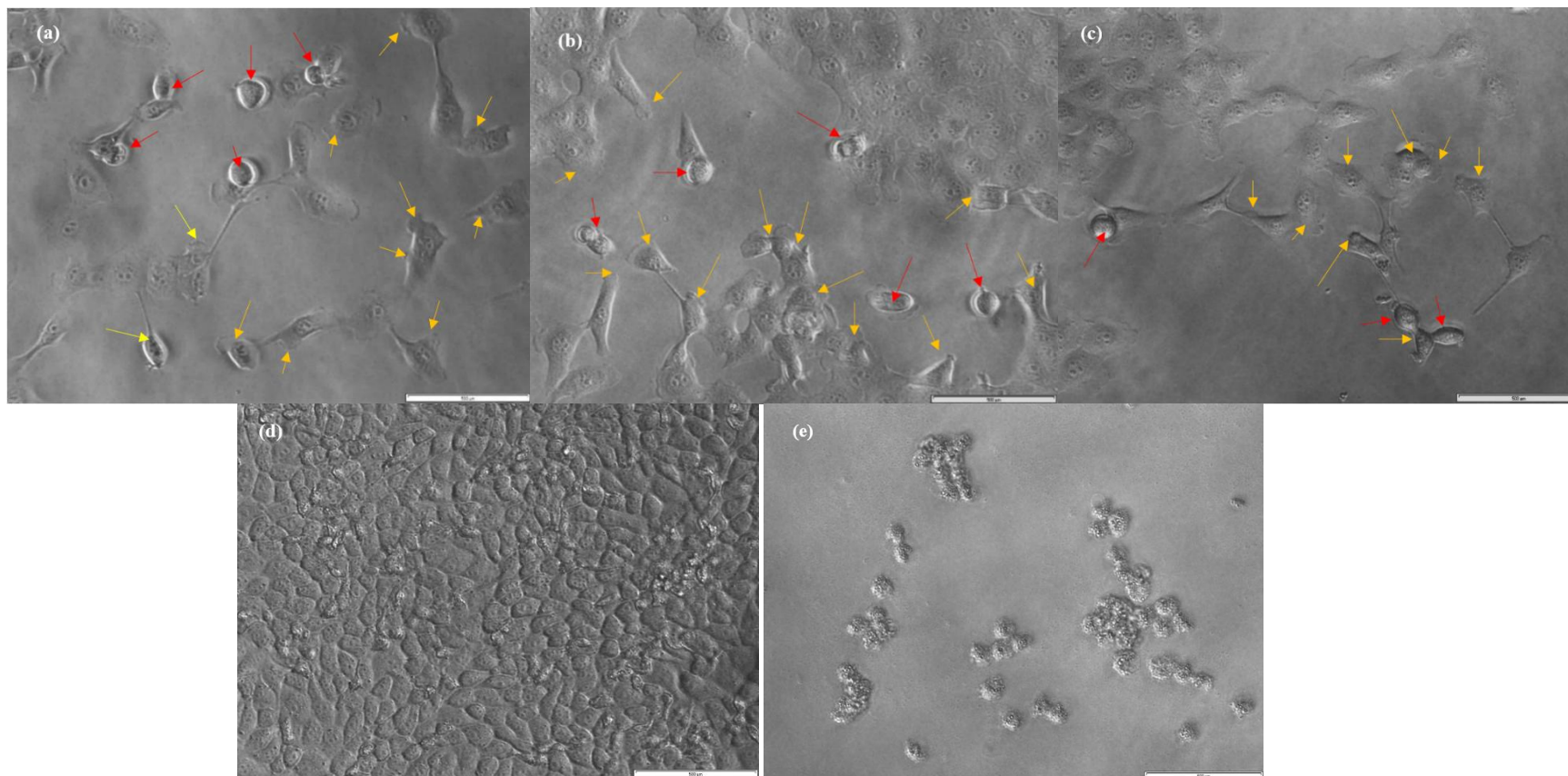


Figure 4.3.1 The presence of apoptosis sign in treated MCF-7 at IC_{50} value, observed under light microscope at 200X magnification. (a) MCF-7 treated with BCH, (b) MCF-7 treated with SAH (c) MCF-7 treated with SAEA (d) Negative control (e) Positive control. The arrow $\rightarrow$ indicates apoptotic body while $\rightarrow$ indicates membrane blebbing in MCF-

This study opted for the Annexin V FITC binding assay, as it is one of the most sensitive and widely used methods in identifying events of early and late apoptosis in drug discoveries (Costigan et al., 2023). The Annexin V Apoptosis Detection Kit FITC involved staining of treated MCF-7 with two different fluorescence dyes, namely Annexin V FITC (green-fluorescent protein stain) and propidium iodide (red-fluorescent DNA stain). Annexin V FITC is a member of the annexin family of intracellular proteins that bind to phosphatidylserine of apoptotic membrane plasma (Alshehade et al., 2024). Meanwhile, propidium iodide (PI) is a stain that can only bind with the DNA of dying cells but appears to be completely impermeable to the membranes of living cells (Crowley et al., 2016). Thus, positive staining of both Annexin V FITC and PI indicated apoptosis events.

The achieved qualitative result of apoptosis assay can be seen in Figure 4.3.2 below, compiling the apoptosis activities in MCF-7 induced by selected mangrove extract at IC_{50} concentration; BCH (7.76 $\mu\text{g/ml}$), SAH (3.47 $\mu\text{g/ml}$) and SAEA (27.8 $\mu\text{g/ml}$). In this experiment, vincristine sulphate was selected as a positive control, as it is a semi-synthetic plant-based chemotherapy which is proven to mediate cell cycle arrest in progressive cancer by triggering the apoptosis pathway (Awosika et al., 2023). Non-treated MCF-7 in complete media served as a negative control, indicating no apoptotic death occurrence. The observations were made under a fluorescence microscope, at 100X magnification, every 2 h interval in a 12 h treatment duration.

This study clearly demonstrated that BCH, SAH, and SAEA trigger MCF-7 cell death via the targeted apoptotic route, similar to vincristine sulphate. Relating to the negative control, there was no occurrence of apoptosis over the 2-hour treatment period. Apoptosis in treated MCF-7 cells was observable over the 4 to 12-hour treatment period. Fair staining of Annexin V FITC in MCF-7 phosphatidylserine can be observed in a 4 h – 6 h treatment period, this indicated early apoptotic event (Singh et al., 2009). At the 8 h treatment period, the stain of both Annexin V FITC and PI became moderately visible, indicating an event of mid-apoptosis (Singh et al., 2009). Late apoptotic events may be noticed after a 10 h and 12 h treatment period, as shown by the great visibility of overlapping staining in both the membrane and nucleus, and

the strongly stained red nucleus without the green stained membrane in MCF-7.
(Ibrahim et al., 2018; Singh et al., 2009)

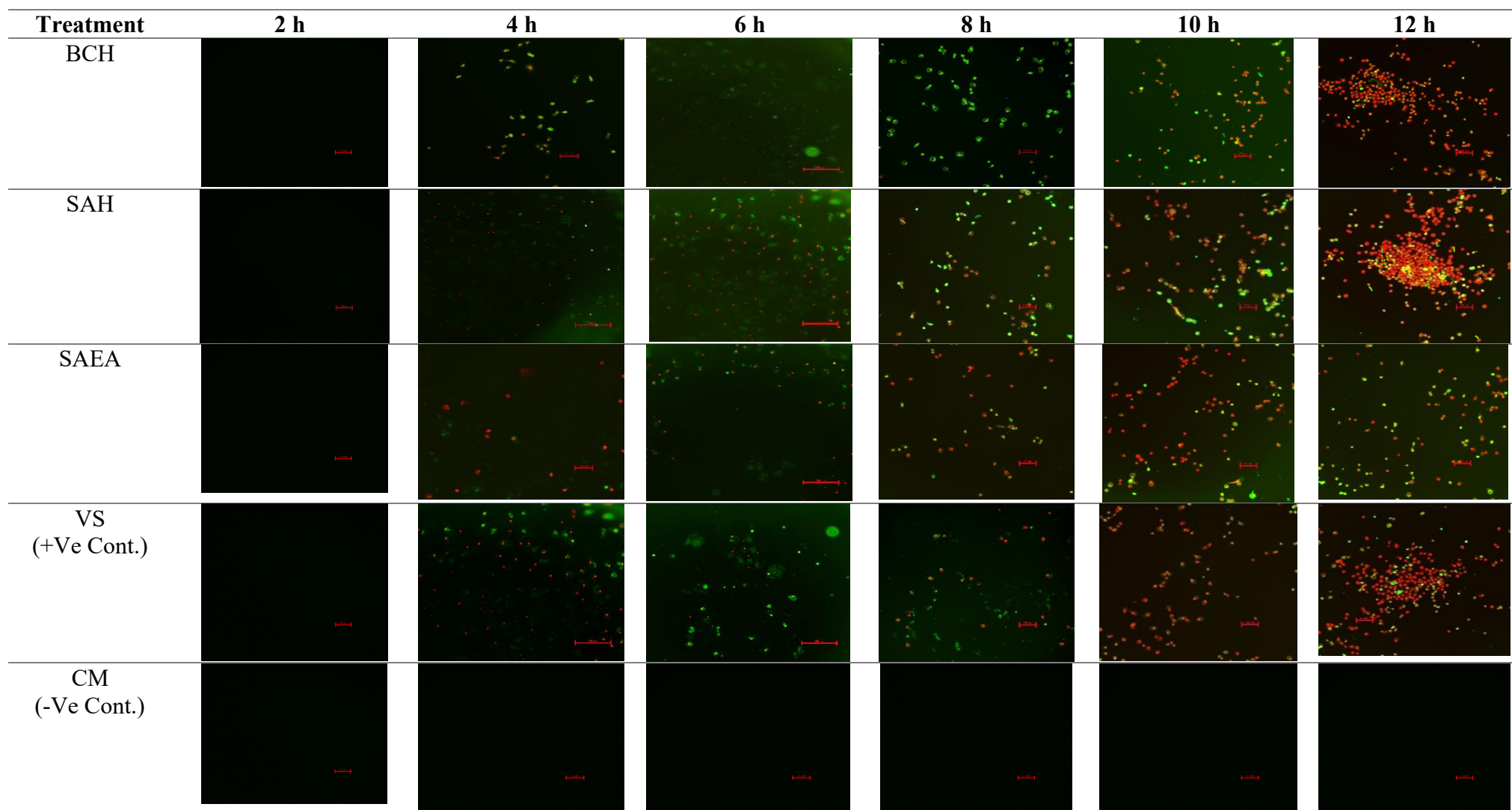


Figure 4.3.2 Detection of apoptosis in MCF-7 treated with BCH, SAH, and SAEA at achieved IC_{50} concentration achieved from MTS assay. The observations were made at every 2 h treatment period for 12 h under fluorescence microscope (Magnification 100X), relatively to positive apoptosis activity (vincristine sulphate), and non-treated cell in complete media (negative control).

Referring to the Figure 4.3.2, apoptosis is a multistep molecular pathway that unfolds in three stages of early, mid and late events. In early apoptosis, calcium-dependent scramblase mediated rearrangement of lipid in the plasma membrane, resulting in translocation of phosphatidylserine to the outer leaflet of the cells (Sivagnanam et al., 2017). The exposure of phosphatidylserine serves as a crucial signal in apoptosis, as it enables phagocytes to recognise and rapidly clear the dying cell (Furuta and Zhou, 2023; Shlomovitz et al., 2019). Afterward, as mitochondria lose their membrane potential and rupture, pro-apoptotic factors such as cytochrome C, Smac/DIABLO and AIF are released into cytosol (Peng et al., 2023). These pro-apoptotic factors activate initiator caspases, including caspase-8 for the extrinsic pathway and caspase-9 for the intrinsic pathway (Aral et al., 2019).

In mid apoptotic event, the initiator caspase-8/9 further signals downstream apoptosis pathway by activating executioner caspase-3, caspase-6 and caspase-7 (McIlwain et al., 2013; Wu et al., 2015). The executioner caspase-3 demonstrates the cleavage of various key structural proteins, including those involved in nuclear changes such as poly (ADP-ribose) polymerase (PARP) (Li et al., 2022). The cleavage of these key structural proteins contributed to irreversible morphological disassembly in dying cells, such as shrinking in size (Zubair and Bokhari, 2025). Afterward, nuclease such as caspase-activated deoxyribonuclease (CAD) or DNA fragmentation 40 (DFF40) activation occurs to prepare dying cells for DNA fragmentation (Larsen and Sørensen, 2017, p. 2).

In late apoptotic events, degradation of nuclear protein occurs due to cleavage of DNA at the internucleosomal linker site (Gheyas and Menko, 2023; Zhang and Xu, 2000). Disintegration of the nucleus leads to nuclear collapse of dying cells characterised by breakage of the nuclear envelope, chromatin condensation and fragmentation of DNA strands (Toné et al., 2007). Further disassembly of dying cells leads to membrane blebbing and protrusion of small membrane-bound vesicles known as apoptotic bodies (Aoki et al., 2020). These apoptotic bodies have intact plasma membranes that tightly enclose the fragments of damaged cellular contents, preventing them from being released into extracellular space (Wickman et al., 2013).

Last stage of apoptosis successfully occurs when the apoptotic bodies being rapidly engulfed by phagocytic cells such as macrophages (Kim et al., 2022).

Furthermore, this study prevailed that there was an observable trend between treatment dosage and incubation period with apoptosis activity. Therefore, analysis was made based on quantification of apoptotic cell number from the microscopy image in Figure 4.3.2 using ImageJ software. The number of apoptotic MCF-7 cells throughout the 12 h treatment incubation period of the respective extract can be referred to in Figure 4.3.3 below.

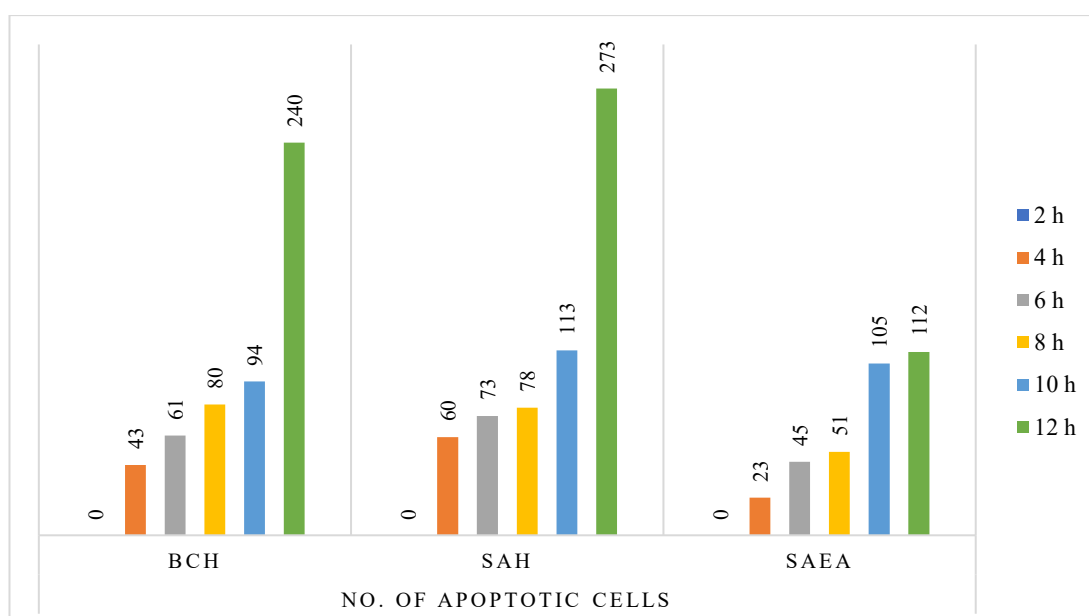


Figure 4.3.3 No. of MCF-7 undergone apoptosis in treatment of BCH, SAH and SAEA at IC_{50} value. No. of cells was quantified using ImageJ software from Figure 4.3.2 in assessment of treatment incubation period and apoptosis capabilities of extracts.

This finding emphasised that the treatment concentration influences the rate of apoptosis. SAH with the very strong IC_{50} ($3.47 \pm 0.20 \mu\text{g/mL}$) was found to trigger the highest amount of apoptotic death. Quantitatively, the number of apoptotic cells was found to be lower in strong IC_{50} BCH ($7.76 \pm 0.18 \mu\text{g/mL}$) and weak IC_{50} SAEA ($27.8 \pm 0.11 \mu\text{g/mL}$). This signifies apoptosis as dose-dependent. Other than that, the apoptosis rate was found to be time-dependent in this *in vitro* study, as the death of MCF-7 increased proportionally with the treatment incubation period. The *in vitro*

study conducted by Khazei et al. (2017) reported a similar trend of apoptosis against several cancerous cells, namely breast, cervical and liver cell lines.

This finding also aligned with research conducted by Reddy and Ratna (2018), where they stated that the methanolic extract of *Bruguiera gymnorhiza* exerted apoptosis in breast cancer and liver cancer cell lines. In addition, *Bruguiera* sp. was found to exert apoptosis in cancerous cell lines, including MCF-7, HepG-2 and HeLa (Chaudhry et al., 2022; Warsinah et al., 2012). A study by Ragasa et al. (2015) demonstrated a comparable finding, as triterpenes and sterols from *Sonneratia alba* root, fruit and leaves extract were found to induce apoptosis in H22 and BCG-803 cell lines. Interesting to note, there is limited availability on apoptosis mechanisms exerted by mangrove plant extracts, including on the two selected species, *S. alba* and *B. cylindrica*. Also, this finding aligned with a study conducted by Thirunavukkarasu et al. (2014) which reported that *B. cylindrica* leaf extract shows great potential in the inhibition of gastric cancer through the oxidative stress and apoptosis pathway.

The apoptosis assay conducted in this study confirmed that BCH, SAH, and SAEA induced favoured apoptosis in arresting MCF-7 cancer cell line proliferation. This finding strengthened the effectiveness of selected mangrove species as supplementary anticancer agents against breast cancer. To further demonstrate the efficacy of BCH, SAH, and SAEA as potent anticancer agents, these three extracts at the IC₅₀ concentration were tested in a cell viability assay to investigate their capabilities in exerting a reduction of the MCF-7 population, by day.

4.4 Trypan Blue Exclusion Assay

The cell viability assay plays a vital role in drug development as it evaluates the safety and effectiveness of potential compounds (Aslantürk, 2018). Performing a cell viability evaluation after cytotoxicity testing is essential, as it offers researchers a more comprehensive understanding of experimental data by examining cellular responses in relation to the drug's potentials (Cai et al., 2024; Riss et al., 2004). The frequency of cell viability measurement is crucial in cancer research, as it facilitated the determination of a more effective anticancer compound and further improved drug-administration strategies (Sánchez-Díez et al., 2025). Unlike the cytotoxicity test that determines the half-maximal inhibitory dosage of a potential drug, the viability assay provided insights into the effects of treatment on living cells. Therefore, further evaluation through a cell viability assay was conducted to determine the effects of BCH, SAH, and SAEA at IC₅₀ concentration on MCF-7 viability.

In this study, the viability of treated MCF-7 cells was evaluated using trypan blue exclusion assay. Trypan blue appears to be the gold standard in viability assays (Chan et al., 2020). This simple and quick assay is considered useful in assessing cell viability because it provides selective staining, visual differentiation, and is inexpensive. The trypan blue exclusion assay is a type of dye exclusion assay that can provide quantitative and qualitative results via cell imaging and cell counting. Essentially, trypan blue is a large, negatively charged staining molecule that can only enter ruptured or membrane-compromised cells (Sbrana et al., 2022). Therefore, trypan blue can distinguish between living and dead cells *in vitro* studies, with dead cells stained dark blue and living cells remains unstained (Madar et al., 2022).

In this study, MCF-7 treated with IC₅₀ concentration of BCH (7.76 ± 0.18 µg/mL), SAH (3.47 ± 0.20 µg/mL), and SAEA (27.8 ± 0.11 µg/mL), were stained with trypan blue dye at ratio of 1:1, every 24 h, 48 h and 72 h. Images of automated counted cell (Figure 4.4.1, 4.4.2, 4.4.3, 4.4.4) and MCF-7 viability in respond to treatment (Table 4.4.1), were retrieved from automated cell counter, DeNovix CellDrop™.

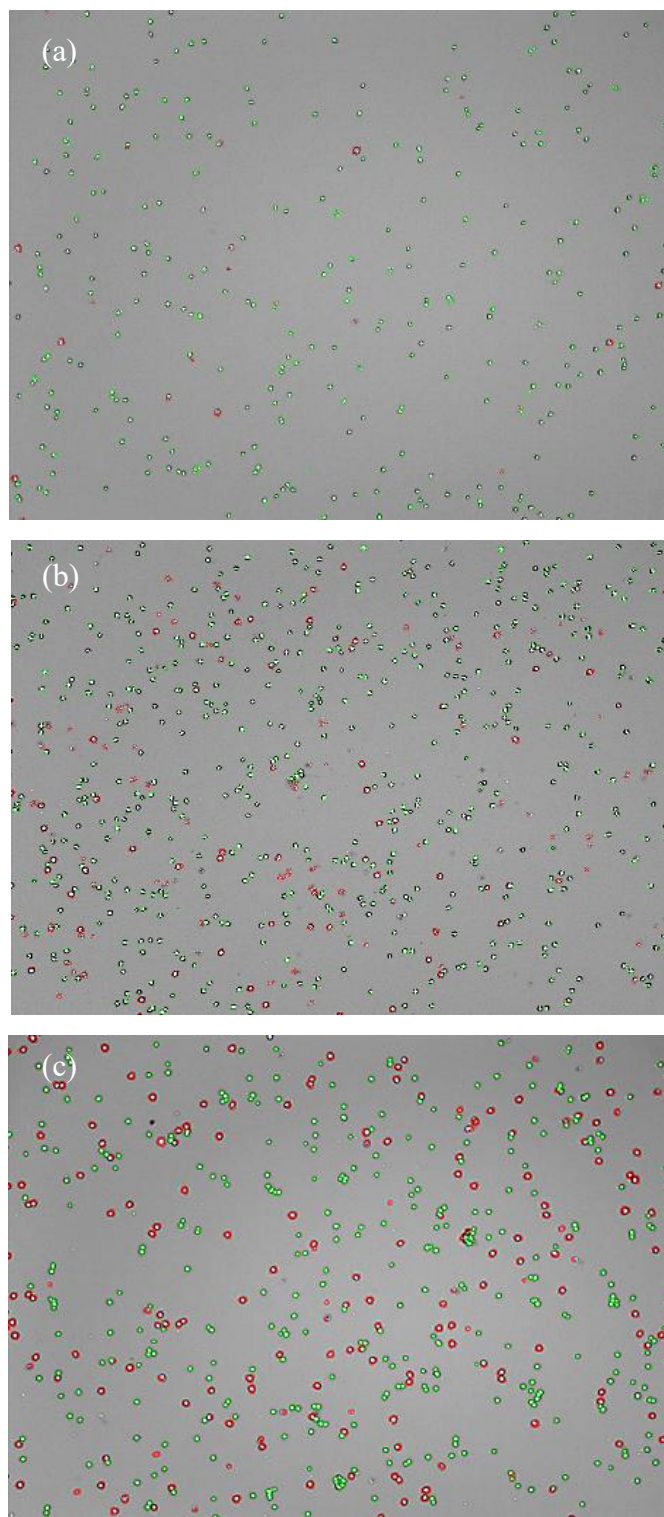


Figure 4.4.1 Counted MCF-7 with BCH treatment after 24 h (a), 48 h (b) and 72 h (c) incubation period. The symbol  marked living cell while  marked dead cell.

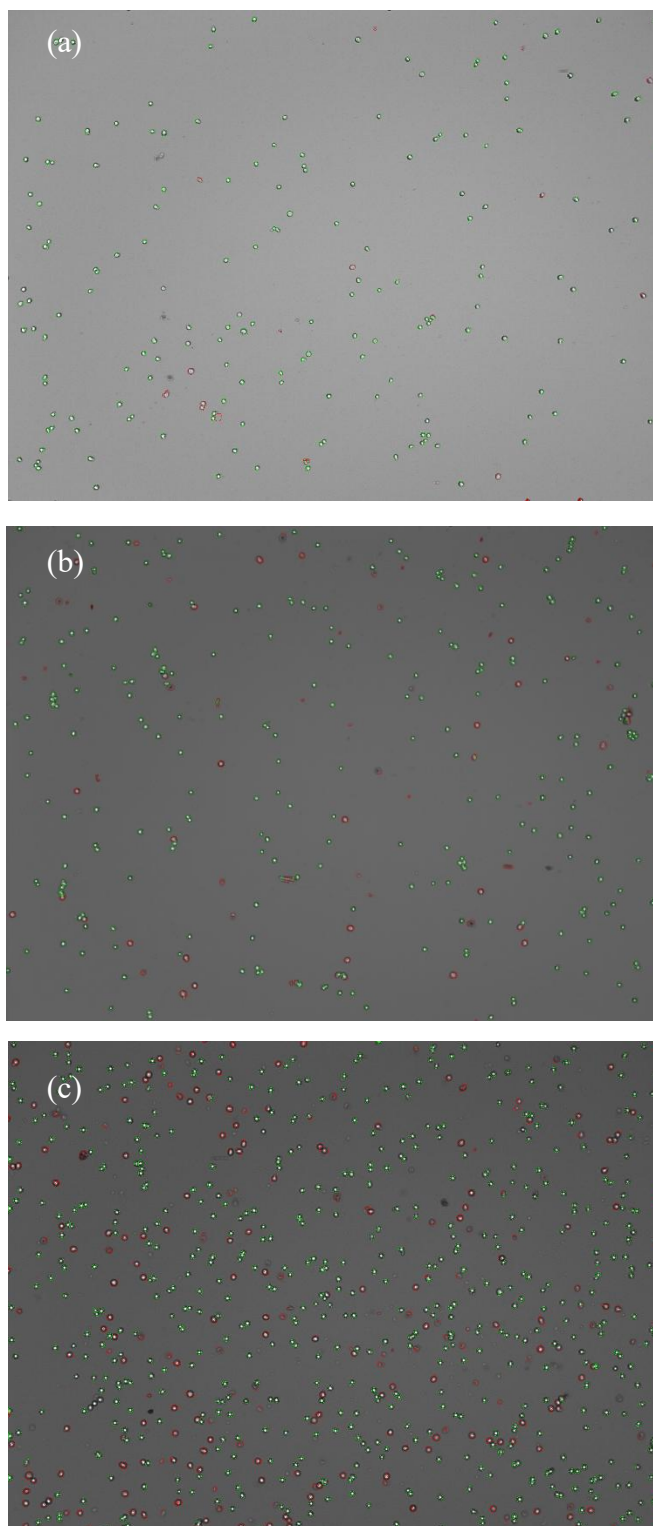


Figure 4.4.2 Counted MCF-7 with SAH treatment after 24 h (a), 48 h (b) and 72 h (c) incubation period. The symbol  marked living cell while  marked dead cell.

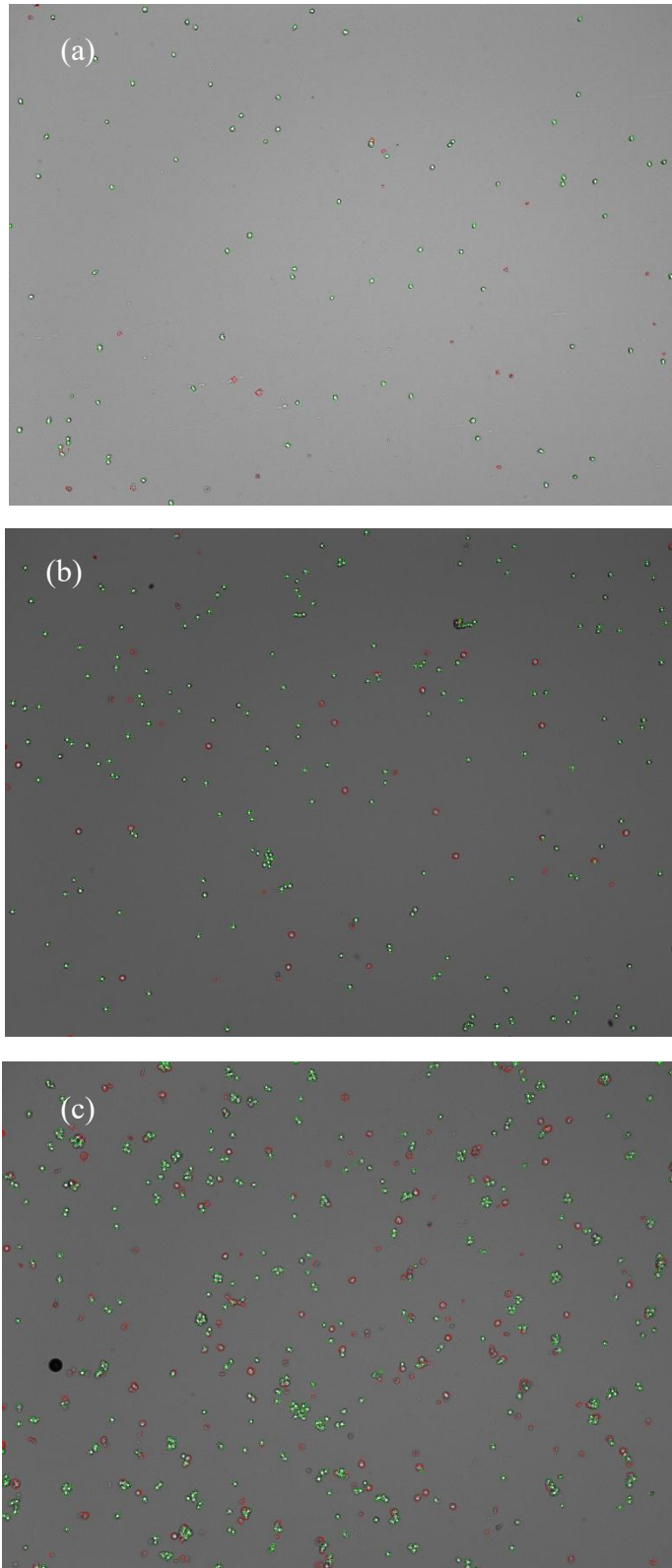


Figure 4.4.3 Counted MCF-7 with SAEA treatment after 24 h (a), 48 h (b) and 72 h (c) incubation period. The symbol  marked living cell while  marked dead cell.

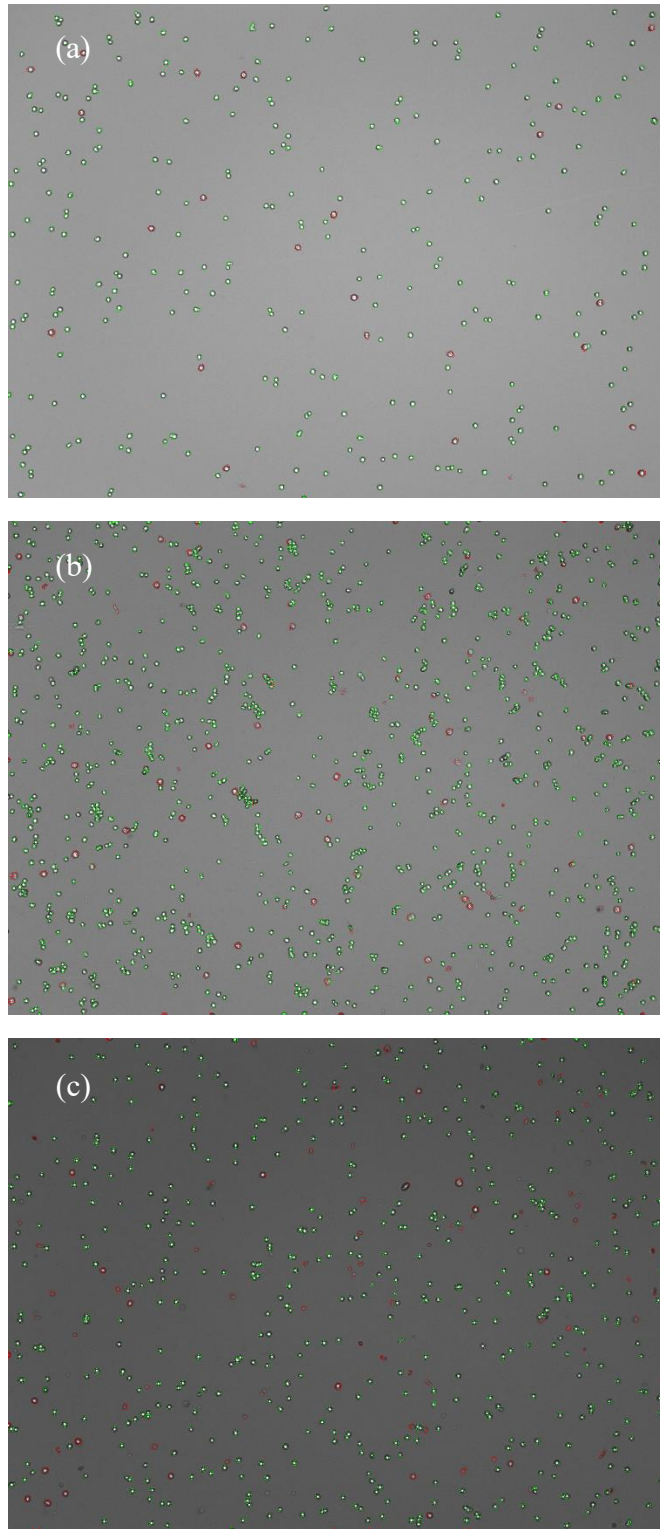


Figure 4.4.4 Counted untreated MCF-7 at 24 h (a), 48 h (b) and 72 h (c) incubation period. The symbol  marked living cell while  marked dead cell. Untreated MCF-7 served as negative control indicating MCF-7 proliferation under no treatment condition.

As can be observed in Figure 4.4.1, 4.4.2, 4.4.3 and 4.4.4 above, the number of living and dead MCF-7 were found to vary with the type of treatment and the treatment period. All three extracts were found to reduce MCF-7 viability in comparison to the negative control, where MCF-7 were found to be majorly viable. Within the three extracts, very strong IC₅₀ SAH was found to be the most efficient in reducing MCF-7 viability. MCF-7 was found to be moderately viable in strong IC₅₀ BCH and more viable in weak IC₅₀ SAEA. Additionally, this finding prevailed that MCF-7 viability decreases with an increase in the treatment period. Further quantitative result of trypan blue exclusion assay can be referred to in Table 4.4.1 below.

Table 4.4.1 Percentage of viable MCF-7 treated with BCH, SAH, and SAEA at achieved IC₅₀ concentration. The observation was made after 24 h, 48 h, and 72 h treatment period. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments). Treatments were considered statistically significant (*p<0.05), (**p<0.001) and (****p<0.0001). The control represented negative control (untreated MCF-7).

Treatment	% of MCF-7 Viability		
	24 h	48 h	72 h
BCH	92.15 $\pm$ 1.56*	63.96 $\pm$ 0.23**	48.76 $\pm$ 0.39****
SAH	90.70 $\pm$ 1.32*	64.05 $\pm$ 1.38**	47.66 $\pm$ 0.78****
SAEA	83.81 $\pm$ 1.29*	73.16 $\pm$ 2.13**	48.79 $\pm$ 0.37****
CM (-Ve Cont.)	97.81 $\pm$ 0.36*	96.44 $\pm$ 0.21**	96.15 $\pm$ 0.55****

Relative to complete media (negative control) that maintained MCF-7 viabilities within the range of 96% to 97% throughout 3 days of treatment; all three extracts were found to successfully reduce MCF-7 viability below 50% after 72 h. Conforming to the aforementioned discussion based on Figure 4.4.1, with BCH dosage, the viability of MCF-7 reduced from the initial 92.15% (24 h), 63.96% (48 h) and 48.76% (72 h). A similar trend can be observed with SAH treatment, where MCF-7 were found to be 90.70% viable in 24 h, decreased to 73.16% in 48 h, and were left only 47.66% viable after 72 h treatment. MCF7 in SAEA too was found to decrease from 83.81% to 73.16% and, lastly, to 48.79% throughout the 72-hour treatment period.

This finding aligned with Chaudry et al. (2020), where reduction in MCF-7 were observed to be time-dependant after being treated with *B. gymnorrhiza* leaves extracts. Huang et al. (2016) reported a comparable finding where mangrove ethyl acetate extract of *Avicennia marina* leaves reduces proliferation of breast cancer cell lines. This finding also aligned with Ramalingam and Rajaram (2018), where they reported that methanolic leaf extract of *Rhizophora apiculata* reduces lung cancer cell line (A549) proliferation. With the aforementioned supportive evidence, BCH, SAH and SAEA were confirmed to exert promising anticancer potential.

Therefore, to further compare the significance of differences between treatment group and control group throughout different treatment period, statistical analysis was conducted through paired t-test, where $p < 0.05$ is statistically significant. BCH, SAH, and SAEA were compared with the negative control (complete media) that implicated no viability inhibition towards MCF-7 cell line proliferation (Table 4.4.1). The selected extracts were found to be statistically significant throughout the 24 h, 48 h, and 72 h treatment periods in comparison with the negative control.

As referred in Table 4.4.1 the significance of the mangrove extracts was found to be proportional with the treatment period. The first 24 h treatment period achieved $p < 0.05$; this signifies there was a significant difference between the effects of the treatment group and the control group towards MCF-7 viability. 48 h of extract treatment was found to be strongly different from untreated cells ($p < 0.01$), suggesting that a longer treatment period implied higher MCF-7 reduction. P-value < 0.0001 for the 72 h treatment period, indicating there was a great difference between the viability of treated and untreated MCF-7. Thus, this finding confirms that a longer treatment period increases the efficacies of BCH, SAH, and SAEA as promising anticancer agents.

It is intriguing that this trypan blue exclusion assay results further supported and validated the previous results obtained from the MTS and apoptosis assays. This finding sufficiently elucidated that the twig extract of two native mangrove species, *B. cylindrica* and *S. alba*, confers promising anticancer value against breast cancer, as it was found to successfully reduce the viability of the breast cancer cell line *in vitro*. In

light of current cancer research approach of finding anticancer compound with additional effects that works together as potential antioxidant agent, BCH, SAH, and SAEA were further investigated in anti-oxidant assay since incidences of cancer is majorly contributed by oxidative stress.

4.5 DPPH Radical Scavenging Assay

Events of oxidative stress conditions are closely linked with carcinogenesis. High level of ROS is harmful and can indeed lead to cancerous cell death. Clinically approved chemotherapy such as from classes of anthracyclines, platinum derivatives and alkylating agents induced high oxidative stress conditions in mediating cell cycle arrest and apoptosis of cancer cells (Chiang et al., 2023). However, as cancer requires prolonged treatment periods, this condition further leads to the accumulation of free radicals in the patient's body. Therefore, in an approach to improve the current chemotherapy, it is essential to find an anticancer agent that can synergistically deliver both anticancer and antioxidant properties in arresting cancer progression at a balanced redox homeostasis condition.

Henceforth, this study selected in further assessing the anti-oxidant activity of BCH, SAH and SAEA through DPPH radical scavenging assay. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay is a type spectrophotometric method commonly used in analysis of anti-oxidant activities in natural product (Gulcin and Alwasel, 2023). This assay determine antioxidant activity by inhibition of stable free radicals (DPPH), signifies by observable change in colour from purple to light yellow (DPPH-H), after reduced by anti-oxidant (Baliyan et al., 2022; Sridhar and Charles, 2019). DPPH radical scavenging assay can be considered as efficient as it offers adequate information of compound anti-oxidant activities while being simple, rapid and cost-friendly (Kedare and Singh, 2011; Silva et al., 2024)

In this investigation, BCH, SAH, and SAEA, the three extracts that were positively confirmed to possess anticancer properties, were subsequently evaluated in an antioxidant assay to determine the dosage necessary to achieve half the minimal inhibitory effect of free radicals. In order to determine the antioxidant treatment dosage, expressed as IC₅₀, quantitative measurements were conducted at 517 nm using a multiplate reader to generate a dose-response curve. The anti-oxidant capabilities of BCH, SAH, and SAEA, in relative of positive control quercetin were further described in the following results and discussions.

***Bruguiera cylindrica* Hexane Extract (BCH)**

In accordance with Table 4.5.1, the lowest concentration of BCH (0.156 mg/ml) were found to only inhibited DPPH free radicals about 7.37%. Tested with concentration of 0.313 mg/ml, the radical inhibition percentage arose to 9.95%. Even with increasing concentration of samples from 0.625 mg/ml to the highest 10 mg/ml, BCH were only found to reduce up to 28.76% of free radicals. Therefore, as can be seen in dose responses curve in Figure 4.5, BCH failed to inhibit 50% of DPPH free radicals. This finding deems BCH as non- active anti-oxidant agent.

Table 4.5.1 Percentage of radical inhibition by *B. cylindrica* hexane extract. Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (mg/ml)	DPPH Radical Scavenging (%) n=3 (Mean)
0	0
0.156	7.37
0.313	9.95
0.625	10.81
1.25	10.39
2.5	17.13
5	22.35
10	28.76

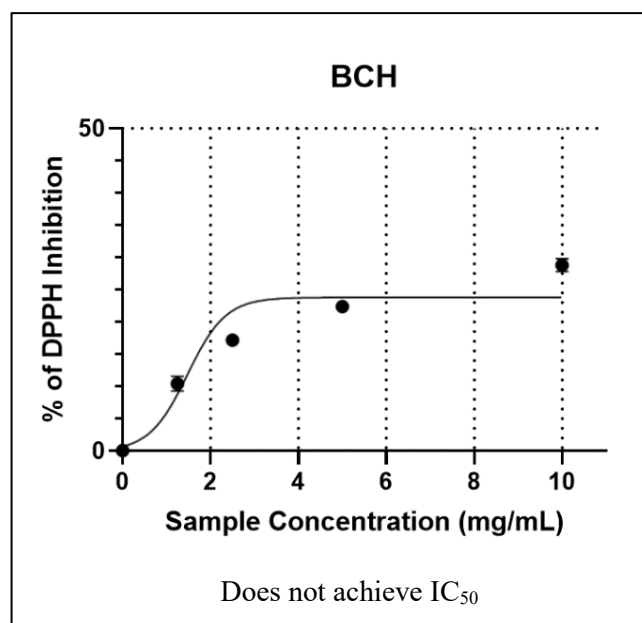


Figure 4.5.1 Dose-responses curve of *B. cylindrica* hexane extract against % of DPPH radical inhibition. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

***Sonneratia Alba* Hexane Extract (SAH)**

Referring to table 4.4.4, denoting the percentage of DPPH radical inhibition by SAH, the lowest inhibition (1.59%) was implicated with the usage of highest sample concentration of 10 mg/ml. Apparently, from the dosage of 0.156 mg/ml up to the highest 10 mg/ml, SAH unable to inhibit more than 50% of the free radicals. Dose-dependence curve Figure 4.5.2 indicated that SAH poorly inhibit free radical and no longer deem as an adequate anti-oxidant agent.

Table 4.5.2 Percentage of radical inhibition by *S. alba* hexane extract. Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (mg/ml)	DPPH Radical Scavenging (%) n=3 (Mean)
0	0
0.156	2.53
0.313	3.10
0.625	4.55
1.25	11.43
2.5	9.86
5	12.59
10	1.59

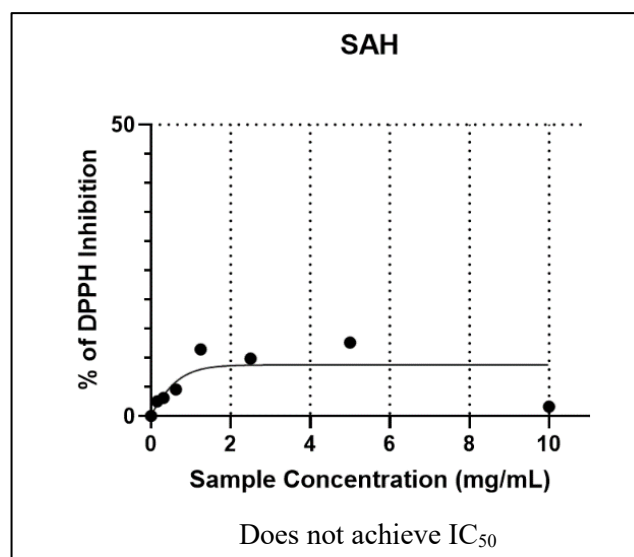


Figure 4.5.2 Dose-responses of *S. alba* hexane extract against % of DPPH radical inhibition. Each value represented mean $\pm$ S.D (n=3) (three-independent experiment).

***Sonneratia Alba* Ethyl Acetate Extract (SAEA)**

As per analysed in Figure 4.4.5, SAEA showed great potential as an anti-oxidant agent. DPPH radical scavenging assay revealed that SAEA implied inhibitions of free radical (56.55%) as early in 0.156 mg/ml sample. Specifically, with the usage of 0.313 mg/ml- 2.5 mg/ml, the inhibition rate remained >50% where the value of inhibition increases from 59%-69%. Sudden depletion of inhibition was observed with the usage of the two highest concentrations to 33.01% and 40.64% according to Table 4.5.3 prevailed SAEA as strong anti-oxidant agent.

Table 4.5.3 Percentage of radical inhibition by *S. alba* ethyl acetate extract. Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (mg/ml)	DPPH Radical Scavenging (%) n=3 (Mean)
0	0
0.156	56.55
0.313	59.16
0.625	62.35
1.25	64.25
2.5	69.17
5	33.01
10	40.64

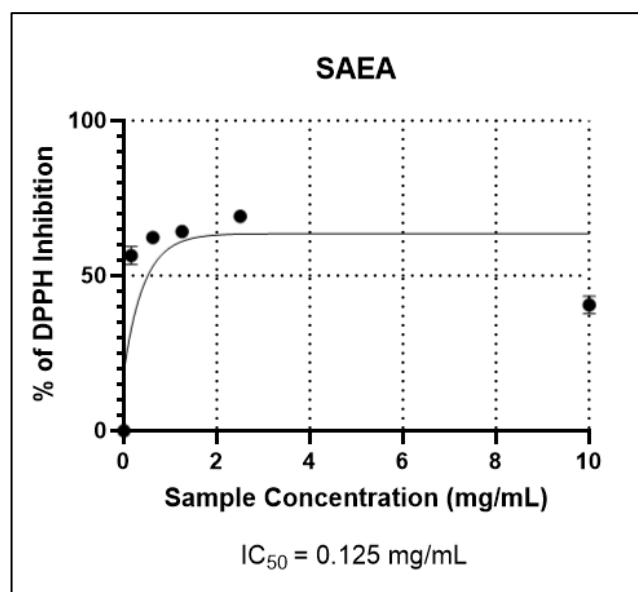


Figure 4.5.3 Dose responses of *S. alba* ethyl acetate extract against % of DPPH radical inhibition. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments)

Quercetin

Quercetin served as the positive control in the DPPH assay to quantify the anti-oxidant activities of the mangrove samples. Quercetin is chosen as the positive control because it is a strong anti-oxidant agent that are been studied extensively in cancer prevention as it was found capable of inhibiting several cancers such as breast, lung, colon, cervical and prostate (Asgharian et al., 2022). In this study, quercetin prevailed significant inhibition against free radical with the acquired IC_{50} value of 0.157 mg/ml as can be observed in Figure 4.5.4. The percentage of radical inhibition were found to proportionally increases with the increment of quercetin concentration as can be seen in Table 4.5.4 below with the highest inhibition of 68.89% at 10 mg/ml.

Table 4.5.4 Percentage of radical inhibition by standard quercetin (negative control). Each value represented mean (n=3) (three-independent experiments).

Sample Concentration (μg/ml)	DPPH Radical Scavenging (%) n=3 (Mean)
0	0
0.156	49.97
0.313	50.86
0.625	66.06
1.25	62.42
2.5	67.44
5	62.99
10	68.89

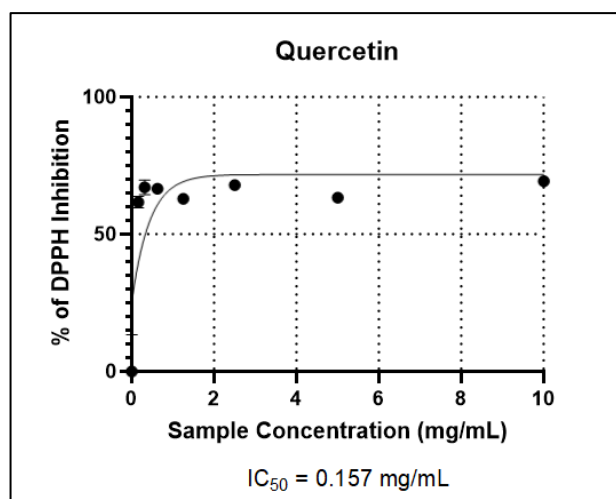


Figure 4.5.4 Dose responses of standard quercetin against % of DPPH radical inhibition. Each value represented mean $\pm$ S.D (n=3) (three-independent experiments).

This finding prevailed that not all three extracts with promising anticancer value could deliver antioxidant activity. Out of the extracts, only SAEA exerted potential antioxidant capability against DPPH free radicals, similarly to quercetin. It was observable that *S. alba* in mid-polar ethyl acetate possessed antioxidant activity, but not *S. alba* and *B. cylindrica* in hexane extracts. This finding indicated that mid-polarity solvent exerted better antioxidant effects compared to non-polar hexane solvent. Nurjanah et al. (2016) stated the same trend with *Bruguiera gymnorrhiza* sp., where the leaves methanolic extracts confer the highest antioxidant activity while n-hexane is the lowest. Fioroni et al. (2023) reported the same, where the antioxidant capacity is higher 2-18 times in polar solvents than in non-polar solvents. Indeed, higher polarity solvents were found effective in extracting strong antioxidants, which are mostly polar (Nawaz et al., 2020).

Table 4.5.5 IC_{50} value of DPPH Radical Scavenging Activity. (Each assay was conducted in triplicate, n=3. The IC_{50} values are stated in mean $\pm$ S.D. Treatments were considered statistically significant (****p<0.0001). SAEA and quercetin were compared with negative control, with no inhibition of DPPH free radicals.

Sample	$IC_{50} \pm S.D$ (mg/mL)
BCH	-
SAH	-
SAEA	0.125 ± 0.001 ****
Quercetin (+Ve Cont.)	0.157 ± 0.002 ****

The dash symbol (-) represented sample failed in achieving IC_{50} .

This study demonstrated that SAEA showed stronger antioxidant potential than quercetin, as the achieved IC₅₀ concentration of SAEA (0.125 ± 0.001 mg/ml) was lower compared to the positive control, quercetin (0.157 ± 0.002 mg/ml). Therefore, in the determination of SAEA as a significant antioxidant agent against negative control with no DPPH radical inhibition occurring, one-way ANOVA was conducted with $p < 0.05$ being statistically significant. Table 4.5.5 indicated that SAEA strongly differs from the negative control. This signifies SAEA showed strong inhibition towards DPPH free radicals, similarly to quercetin.

All-inclusive from previous assays, *Bruguiera cylindrica* in hexane extract, *Sonneratia alba* in ethyl acetate extract, and *Sonneratia alba* in hexane extract all exhibited promising features for the development of anticancer drugs. Additionally, *Sonneratia alba* in ethyl acetate extract was discovered to have synergistic anticancer and antioxidant effects. Consequently, liquid chromatography - mass spectrometry was performed on BCH, SAH, and SAEA to further identify the phytochemicals responsible for their anticancer and antioxidant effects.

4.6 Liquid Chromatography – Mass Spectrometry (LC-MS/MS)

Liquid-Chromatography-Mass Spectrometry (LC-MS/MS) is an analytical tool that is used in drug discovery. It is capable of physically separating target analytes and then selecting them based on their mass. LC-MS/MS is widely used in the discovery of bioactive compounds, as it enables the identification of unknown compounds from extracts by comparing them to known compounds in the database. This technique categorises chemical structures based on their fragmentation patterns. LC-MS/MS is highly advantageous in research due to its high sensitivity to analytes, which enables it to detect natural products at very low concentrations. Additionally, it is versatile and has a high throughput, as it can analyse a large number of samples quickly and provides a wide range of bioactive compounds with varying polarity and structure.

LC-MS/MS analysis was conducted in this study using BCH, SAH, and SAEA. Hundreds of bioactive compounds were identified in each extract, as mangroves are confirmed to be abundant in phytochemicals. In accordance with the current oncology approach of identifying anticancer compounds with synergistic effects, the raw data was employed to identify phytochemicals or bioactive compounds that exhibited both antioxidant and anticancer properties. These compounds were subsequently compiled into Tables 4.6.1, 4.6.2, and 4.6.3, which are discussed in more depth below.

***Bruguiera cylindrica* hexane extract**

Table 4.6.1 Phytochemicals responsible for both anticancer and antioxidant properties in BCH.

No.	Rt (Min)	m/z	Identified Compound	Class of Compound	Formula	Mode
1	21.581	318.30032	Phytosphingosine	1,3- aminoalcohols	C ₁₈ H ₃₉ NO ₃	+
2	17.344	338.34247	Erucamide	Fatty amides	C ₂₂ H ₄₃ NO	+
3	14.836	161.0596	4-Methylcoumarin	Coumarins and derivatives	C ₁₀ H ₈ O ₂	+
4	14.697	169.01318	Gallic acid	Gallic acids	C ₇ H ₆ O ₅	-
5	14.641	179.07013	4- Methoxycinnamic acid	Cinnamic acids	C ₁₀ H ₁₀ O ₃	+
6	14.394	487.34378	Asiatic acid	Triterpenoids	C ₃₀ H ₄₈ O ₅	-
7	11.41	301.21564	Grandifloric acid	Kaurane diterpenoids	C ₂₀ H ₂₈ O ₂	+
8	6.31	137.02356	4-Hydrobenzoate	Hydroxybenzoic acid derivatives	C ₇ H ₆ O ₃	-
9	6.177	135.11694	CAMPHOR (1R)	Bicyclic monoterpenoids	C ₁₀ H ₁₆ O	+
10	5.973	197.11717	Loliolide	Benzofurans	C ₁₁ H ₁₆ O ₃	+
11	5.459	173.08118	Suberic Acid	Medium-chain fatty acids	C ₈ H ₁₄ O ₄	-
12	5.196	153.05501	Vanillin	Methoxyphenols	C ₈ H ₈ O ₃	+

From BCH, 12 phytochemicals that can exert both anticancer and antioxidant effects were identified. Among these 12 compounds, BCH was found to possess major phytochemical classes such as coumarins, terpenoids and phenol. They were identified as 4-methylcoumarins (coumarins and derivatives), asiatic acid (triterpenoids), grandifloric acid (kaurene diterpenoids) and vanillin (methoxyphenols).

Wu et al. (2020) and Goel et al. (2007) reported that coumarins are very significant as anti-cancer agents, as they were found to target and inhibit several cell proliferation pathways such as PI3K, AKT, and mTOR, and activated apoptotic protein in lung cancer while regulating the ROS. Terpenoids and their derivatives have the ability to suppress tumours at early stages by cell cycle arrest and apoptosis, preventing cancer cells from further angiogenic and metastatic (Kamran et al., 2022). Maheswari and Sharma stated in 2023 that plant phenolics have the ability to alter cancer cell

genomic content, thus interrupting signal transduction leading to apoptosis. Phytosphingosine was found to induce cell cycle arrest at the G₂/M phase and mediate oxidative stress in cancer cells, leading to activation of the intrinsic apoptosis pathway in lung and breast cancer (Cai et al., 2024; Tripathy et al., 2023). Erucamide causes downregulation of UNC5B receptors, which signals for mitochondrial-dependent apoptosis (Hu et al., 2020).

***Sonneratia alba* hexane extract**

Table 4.6.2 Phytochemicals responsible for both anticancer and antioxidant properties in SAH.

No.	Rt (Min)	m/z	Identified Compound	Class of Compound	Formula	Mode
1	17.335	338.34232	Erucamide	Fatty amides	C ₂₂ H ₄₃ NO	+
2	16.644	149.02339	Phthalic anhydride	Phthalic anhydrides	C ₈ H ₄ O ₃	+
3	14.67	161.06	4-Methylcoumarin	Coumarins and derivatives	C ₁₀ H ₈ O ₂	+
4	14.67	179.07071	4-Methoxycinnamic acid	Cinnamic acids	C ₁₀ H ₁₀ O ₃	+
5	14.53	177.05444	Ferulate	Hydroxycinnamic acids	C ₁₀ H ₁₀ O ₄	+
6	12.756	297.24347	Ricinoleic acid	Lineolic acids and derivatives	C ₁₈ H ₃₄ O ₃	-
7	12.257	279.23218	Linolenic acid	Lineolic acids and derivatives	C ₁₈ H ₃₀ O ₂	+
8	11.019	170.09679	Diphenylamine	Aniline and substituted anilines	C ₁₂ H ₁₁ N	+
9	6.416	177.05496	Coniferyl aldehyde	Methoxyphenols	C ₁₀ H ₁₀ O ₃	-
10	6.379	179.07079	Coniferaldehyde	Methoxyphenols	C ₁₀ H ₁₀ O ₃	+
11	6.185	187.0966	Azelaic acid	Medium-chain fatty acids	C ₉ H ₁₆ O ₄	-
12	5.961	197.11781	Loliolide	Benzofurans	C ₁₁ H ₁₆ O ₃	+
13	5.317	173.08072	Suberic acid	Medium-chain fatty acids	C ₈ H ₁₄ O ₄	-
14	5.185	153.05498	Vanillin	Methoxyphenols	C ₈ H ₈ O ₃	+
15	3.227	122.09679	Phenylethylamine	Phenylethylamine	C ₈ H ₁₁ N	+

There were 15 compounds with both anticancer and antioxidant activities identified from SAH. The presence of 3 major phytochemicals was observed, such as coumarins, phenols and alkaloids. SAH was found to have 3 phenol derivatives (coniferyl aldehyde, coniferaldehyde, vanillin). One coumarin was identified as ferulate and 4-methylcoumarin. A type of alkaloid was identified from SAH, known as phenylethylamine. This finding prevailed that SAH consists of more major phytochemicals compared to BCH.

Coniferyl aldehyde exhibits anticancer activity through inhibition of the crucial cell-nucleus pathway, Janus Kinase –2 (JAK 2), while neutralising free radicals in cancer cells (Ismail et al., 2023; Mengie Ayele et al., 2022). Vanillin is conferred with the ability to inhibit cancer cell migration through suppression of the PI3K pathway, thus promoting apoptosis in cancer cells (Kafali et al., 2024; Lirdprapamongkol et al., 2009). Ferulate delivers anticancer properties through cell cycle arrest in the G₀/G₁ phase, further inhibiting cancer from proliferating and becoming invasive (Bao et al., 2023). Gupta et al. (2021) stated that ferulate promotes apoptosis by upregulation of the pro-apoptotic genes p53 and p51 in cancer cells. 4-methylcoumarin has the ability to inhibit cancer by suppressing hyaluronic acid synthesis and tumourigenesis, thus activating apoptosis (Kishk et al., 2022). Hamdan et al. (2013) proved 4-methoxycinnamic acid was found to cause DNA damage in several cancer cell lines, such as MCF-7, T47D and MDA-MB-231. Blaszczyk (2020) reported that ricinoleic acid causes DNA damage and apoptosis in HT29 cell lines.

***Sonneratia alba* ethyl acetate extracts**

Table 4.6.3 Phytochemicals responsible for both anticancer and antioxidant properties in SAEA.

No.	Rt (min)	m/z	Identified Compound	Class of Compound	Formula	Mode
1	21.564	318.30017	Phytosphingosine	1,3-aminoalcohols	C ₁₈ H ₃₉ NO ₃	+
2	10.601	269.04565	Aloe-emodin	Anthraquinones	C ₁₅ H ₁₀ O ₅	-
3	9.322	371.03943	Norstictic acid	Diaryl ethers	C ₁₈ H ₁₂ O ₉	+
4	7.536	271.06049	Naringenin	Flavanones	C ₁₅ H ₁₂ O ₅	-
5	7.099	285.03958	3',4',5,7-tetrahydroxyflavone	Flavones	C ₁₅ H ₁₀ O ₆	-
6	6.425	301.03467	Quercetin	Flavonols	C ₁₅ H ₁₀ O ₇	-
7	6.191	275.0914	Phloretin	2'-Hydroxy-dihydrochalcones	C ₁₅ H ₁₄ O ₅	+
8	6.148	177.05476	Coniferyl aldehyde	Methoxyphenols	C ₁₀ H ₁₀ O ₃	-
9	6.062	137.02354	4-Hydroxybenzoate	Hydroxybenzoic acid derivatives	C ₇ H ₆ O ₃	-
10	5.947	197.11711	Loliolide	Benzofurans	C ₁₁ H ₁₆ O ₃	+
11	5.883	193.04951	Ferulic acid	Hydroxycinnamic acids	C ₁₀ H ₁₀ O ₄	-
12	4.601	167.03429	Vanillic acid	M-methoxybenzoic acids and derivatives	C ₈ H ₈ O ₄	-
13	4.485	179.03415	Esculetin	6,7-dihydroxycoumarins	C ₉ H ₆ O ₄	+
14	4.467	177.01871	6,7-Dihydroxycoumarin	6,7-dihydroxycoumarins	C ₉ H ₆ O ₄	-
15	4.217	135.04417	4-Hydroxyacetophenone	Alkyl-phenylketones	C ₈ H ₈ O ₂	-
16	4.217	165.05452	3-hydroxy-1-(4-hydroxyphenyl)propan-1-one	Alkyl-phenylketones	C ₉ H ₁₀ O ₃	-
17	4.191	183.02901	Methyl gallate	Galloyl esters	C ₈ H ₈ O ₅	-
18	3.903	137.02312	Salicylic Acid	Salicylic acids	C ₇ H ₆ O ₃	-
19	3.19	122.09677	Phenylethylamine	Phenethylamines	C ₈ H ₁₁ N	+

About 19 bioactive compounds with synergistic effects of anticancer and antioxidant effects were identified from LC-MS/MS analysis. It is interesting to note that SAEA is capable of inducing very strong antioxidant activity in previous DPPH assays, as it was found to contain 3 strong antioxidant compounds, including aloe emodin, quercetin and phloretin.

Jiang et al. (2021) stipulated that aloe emodin induces intrinsic apoptosis in cancer cells by HCT-116 by downregulating the anti-apoptotic protein Bcl and upregulating the pro-apoptotic protein Bax, leading to the signalling of caspase 9 and 3 in colon cancer. Reported in a study by Ebrahim et al. (2016), norstictic acid was found to inhibit the MDA-MB-231 cancer cell line while showing low cytotoxicity towards the normal mammary cell line (MCF-10). Zhao et al. (2019) demonstrated that naringenin, a type of flavanone, causes cell cycle arrest at G₀/G₁ in MDA-MB-231, causing the cancer cell to die in the apoptosis pathway.

Interestingly, the LC-MS/MS analysis performed in this study revealed that *Bruguiera cylindrica* and *Sonneratia alba* contain an extensive variety of bioactive compounds capable of exerting multiple therapeutic effects. BCH, SAH, and SAEA have enormous potential for development as supplemental chemotherapeutic agents in cancer treatment, as evidenced by the results of all previous experiments in this study. This discovery also suggests that other mangroves in Malaysia contain bioactive compounds that could be developed into novel anticancer raw materials.

CHAPTER 5

CONCLUSION

In conclusion, *B. cylindrica* and *S. alba* were found to exhibit potential anticancer value towards breast cancer cell line, MCF-7 but appear to be non-toxic against normal cell line proliferation, L6. As these mangrove species possess major phytochemical classes such as coumarins, phenols, alkaloids, flavonoids, and terpenoids that able to induce both anticancer and antioxidant properties, they potentially might be a promising breakthrough as a supplementary chemotherapy against breast cancer. highlighted that mangroves are not only crucial for ecological well-being, but they appear to demonstrate significant therapeutic value that might mitigate cancer mortality burden globally. As this study prevailed that native mangrove species have the ability to deliver multiple therapeutic values with the usage of different extractive solvents, this research suggests that mangroves carry vast potential to be developed as a novel therapy. This research acknowledges the major limitation in conducting this study is vague information on mangrove anticancer mechanisms, as research of mangroves in medical studies, especially, is lacking. Therefore, this finding added an additional value of mangrove as a pharmacologically relevant natural product with proper scientific documentation. Furthermore, this research significantly provided understanding on native mangrove species and provided vast insight on sustainable drug development approaches. Thus, future study is recommended to optimise the extraction methods to increase phytochemical yield and conduct screening of local mangrove species both *in vivo* and *in vitro* to further confirm their underlying mechanism in battling cancer.

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Publications

- 1 Nadzrin, N., Mazlan, N. W., Kasim, M. N. I., Lakshminarayanan, R., and Sevakumaran, V. Phytochemicals from Mangrove Species: Prospective Saviour in Battle against Cancer? Natural Product Research (Accepted)
- 2 Nadzrin, N., Mazlan, N. W., Kasim, M. N. I., Tan, J. K., and Sevakumaran, V. Phytochemical Characterization and Mechanistic Analysis of Solvent-Derived Extracts from Malaysian Mangroves *Sonneratia Alba* and *Bruguiera Cylindrica* for Potential Anticancer Properties. Malaysian Journal of Analytical Sciences (In Review)

List of award

- 1 Best Presenter in International Conference on Environmental Biotechnology and Sustainability (ICEBS-25)