CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Optimization of Extraction of Active Compounds from Australian Native Plant Lemon Myrtle (*Backhousia citriodora*) and Analysis of In Vitro Anticancer Properties of this Towards Pancreatic Cancer

by

Zahra Khan

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I dedicate this thesis to my loving and supportive family and friends, whose unwavering support has been crucial in helping me achieve my life goals.

CERTIFICATE OF APPROVAL

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Abstract

Pancreatic cancer remains one of the most aggressive and deadly forms of cancer, with limited treatment options and a low survival rate. Natural compounds, especially those derived from plants, have garnered attention as potential therapeutic agents due to their bioactive properties. In this study, the Australian native plant *Backhousia citriodora* (Lemon Myrtle) containing antioxidant and antimicrobial compounds is examined for anticancer effects on pancreatic cancer. While there is considerable interest in the bioactivity of Lemon Myrtle, specifically its potential to reduce pancreatic cancer cell proliferation, the full capacity of the plant remains unknown and efficient approaches to extracting its bioactive components remain significantly unexplored. To that end, the study enhanced the yield of bioactive compounds from Lemon Myrtle leaves using a liquid–liquid factionation method. Phenolic content and antioxidant activities of the extracted compounds were determined by Total Phenolic Content (TPC), Ferric Reducing Antioxidant Power (FRAP), DPPH Radical Scavenging, and ABTS Radical Scavenging. Subsequently, these extracts were subjected to cytotoxicity against pancreatic cancer cells: MIA PaCa-2, BxPC-3, and non-cancerous pancreatic duct epithelial (HPDE) to assess their in vitro antitumor activity. The results revealed that the crude and butanol extract of Lemon Myrtle had the highest phenolic content (**46.41 mg GAE/g**) and exhibited superior antioxidant activity across multiple assays, with the FRAP assay showing the greatest reducing power (451.28 μmol Fe^{2+2} /g). The aqueous extracts had lower phenolic content and antioxidant activity, though the aqueous extract demonstrated strong radical scavenging in the DPPH assay (**5.14**%). These findings contribute to the growing body of research on natural products as potential therapeutic agents and highlight the importance of optimizing extraction methods to maximize bioactivity.

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Abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	Analysis of Variance
BxPC-3	Pancreatic Adenocarcinoma Cell Line
CUST	Capital University of Science and Technology
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalents
HCl	Hydrochloric Acid
HPDE	Human Pancreatic Duct Epithelial
HPLC	High-Performance Liquid Chromatography
KSFM	Keratinocyte Serum-Free Medium
LMEO	Lemon Myrtle Essential Oil
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
PBS	Phosphate-Buffered Saline
RPMI-1640	Roswell Park Memorial Institute Medium 1640
SD	Standard Deviation
TPC	Total Phenolic Content
TPT	Tripyridyl-s-triazine
UV–VIS	Ultraviolet-Visible Spectroscopy

Chapter 1

Introduction

Native to coastal Queensland, Australia, and lemon myrtle is an indigenous Australian shrub. One of the most well-known of the bush tucker plants derived from Australian outback. Australian Aboriginal people have utilised lemon myrtle leaves for ages. They appreciated it for its taste and for its therapeutic properties as well. Applied topically, it was used as a cough and common cold cure. Sometimes they kept the leaves submerged in water, burned the materials, then swallowed the vapours [1].

Scientifically, lemon myrtle plant is *Backhousia citriodora*. Although Ferdinand von Mueller gave the genus his name, the species was called James's Backhouse in respect of the Quaker missionary botanist. Arriving to Australia in 1847, Mueller was a German physician and accomplished botanist. Citral was the chemical causing the taste and scent of lemon myrtle discovered in 1889 by a German business under the name Schimmel & Co [1].

FIGURE 1.1: Lemon Myrtle Plant

Lemon myrtle has a taste that is close to that of lemongrass and citrus fruits type of fruits. It has a lemon and lime flavor, but not their sourness, so that it may be blended with dairy products without coagulation. It has been referred to as even having a lemon sharper than that of the lemon fruit as some will put it into the words. Lemon myrtle also can have a very strong taste and smell profile longer than almost any other source of its wonderful taste [1]. A well-known plant of the Australasian region, Lemon Myrtle is used by the Australian Aborigines both as a flavoring agent besides being medicinally useful. They are usually applied as dried flake or a protected flavor carrier – flavor concentrate which increases stability and is used for addition to vegetable oils, pasta, shortbread and etc. That however is largely used in teas, most especially as combinations. Furthermore, 'Lemon Myrtle' stands as a leavening for milk based on foods such as cheesecake or ice-cream because normal lemon fruits cause the milk to curdle [2]. Researches additionally clarified that the presence of essential oils in lemon myrtle has cytotoxic effects; this has also skin toxicity; this demonstrate antibacterial and antifungal activity and also influences bone formation [2]. With minimal cytotoxicity [3] the essential oil (steam distillate) from the Australian lemon myrtle has been shown to have antibacterial effects in vitro [4, 5]. More than three hundred essential oils of ethnic medicine in vitro, and the essential oil of lemon myrtle was discovered to exhibit strong cytotoxicity on many cancer cell lines. Native to tropical rainforests in the Queensland coastline area of Australians, B. citriodora, lemon myrtle, is extensively grown in the eastern portion of Australia and Yunnan province of China [6]. Food, folk medicine, aromatherapy, and aromatherapy

all benefit from the plant; it is also well-known for increasing blood circulation to release blood stasis, so reducing pain, so calming the soul, anti-bacterial, and anti-cancer [6, 7]. Originally used in Australia for anti-cancer treatment, anti-cancer, anti-inflammatory, headache, cough cold skin illness, and inducement of sleep [8] LMEO have been used in the antiseptic treatment of pimples and acne, noted the Complementary Medicines Evaluation Committee (CMEC) of the Australian Therapeutic Goods Administration (TGA). LMEIO has also been shown to have amniotic biologic characteristics including anti-oxidant, anti-bacterial and anti-inflammatory [9]. The writers of the latest investigations have also noted that LMEO has some therapeutic use for human conditions like diabetes, obesity, and heart ailments. From a study made by [10], it was revealed that LMEO possess an anti-obesity property by suppressing the differentiation and lipid accumulation in adipocyte. Infectious molluscum contagiosum in children has been found to be treated with the LMEO identified in clinical investigations [11]. The process of extracting active compounds from lemon myrtle can be considered optimal in terms of its efficiency because yield of bioactive elements plays crucial role for researchers and manufacturers of natural products with potential therapeutic properties. A number of extraction methodologies including solvent extraction, super critical fluid extraction, microwave assisted extraction, ultrasound assisted extraction have been used to extract bio active compounds from the leaves of lemon Myrtle or the essential oil. Optimization means establishing the most appropriate conditions, solvents, time, temperature and pressure that enhance the extraction process while considering the stability and effectiveness of the extract's active ingredients [12].

In addition, once optimum conditions of extraction is achieved, the extracts thus obtained should then be evaluated for its anticancer activity and more so on pancreatic cancer. Pancreatic cancer is a highly deadly form of cancer, which has several hallmarks including high aggressiveness, early metastasis and short survival time. Pancreatic cancer is a type of cancer that occurs in the cells of the pancreas and develops as the organ is located behind the stomach and is vital for digestion and glucose control. The two main types of pancreatic cancer are exocrine tumors, which account for the majority of cases, and less common endocrine

tumors (islet cell tumors).

The pancreas exocrine cancer is frequently located in the ducts of the pancreas and consists predominantly of adenocarcinomas. Some of the modifiable risk factors involve: Age, smoking, history of pancreatitis, history of familial pancreatic malignancies, and some specific cancer syndromes as hereditary pancreatitis and HNPCC [8].

Symptoms of pancreatic cancer are non-specific complaints which often do not alert the patient and/or the physician, and the disease is often diagnosed at an advanced stage, at which curative treatments are not feasible. The initial signs may be consistent with features of acute abdomen, clinical jaundice, weight loss, abdominal pain, vomiting and alterations in bowel habit. Imaging is done through CT, MRI, EUS and for histological confirmation biopsy may be done.

Management of pancreatic cancer is mainly based on the stage and it may be by surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy. However, a significant low overall survival rate for pancreatic cancer exists – to date, five-year survival stands at less than 10% due to the cancer's invasiveness and restricted therapeutic options available for the later stages [8].

The research plan aims at assessing the feasibility of utilizing Lemon Myrtle as a source of phytochemicals with anti-cancer characteristics against pancreatic cancer. This work target at getting optimum yield of active compounds from native Australian lemon myrtle and investigate the in vitro anti-cancer properties of these compounds on pancreatic cancer cells. After that, the study aims at determining the impact of Lemon Myrtle extracts on the pancreatic cancer cells in a petri dish to determine if they contain anticancer properties. In summary, this study meets the future demand for new targeted treatment approaches against PC, and taps the diverse treasury of Lemon Myrtle and its related plant traditions. From its results, the researchers claimed that it has broached the possibility of synthesizing new anticancer compounds using natural compounds as reference, which outreach the doomed cancer patients.

1.1 Problem Statement

The optimization of extraction techniques for active compounds from Lemon Myrtle (*Backhousia citriodora*) and subsequent assessment of their in vitro anticancer properties specifically targeting pancreatic cancers present a critical challenge. Lemon Myrtle contains potentially bioactive compounds that could serve as promising candidates for pancreatic cancer therapy. However, the extraction is optimized, but it isn't known what the active compounds are and so further analysis of these to determine is required.

1.2 Hypothesis

Lemon Myrtle extracts contain bioactive compounds with potential anticancer properties against pancreatic cancer cells. I hypothesize that the optimized extraction of active compounds from Lemon Myrtle leaves will yield extracts rich in phytochemicals, which, when tested in vitro, will demonstrate cytotoxic effects on pancreatic cancer cells further anticipate that causing death will mediate these effects, suggesting potential use of lemon myrtle extracts as creative pancreatic cancer therapeutic agents.

1.3 Research Gap

Despite the growing interest in natural products as potential sources of anticancer agents, there exists a notable research gap in the optimization of extraction methods and the subsequent analysis of the in vitro anticancer properties of Lemon Myrtle extracts specifically against pancreatic cancer. While some studies have explored the bioactivity of Lemon Myrtle extracts against various cancer types, including breast cancer, neither have focused specifically on pancreatic cancer, which presents unique challenges due to its aggressive nature and limited treatment options. Additionally, there is often suboptimal extraction performance that

is not optimised to its full extent for parameters like the choice of solvent, extraction time, or temperature, which brings an issue of non-comparison of the results in regards to different studies. Closing these research gaps is imperative to expand the understanding of Lemon Myrtle for pancreatic cancer therapy, and to establish feasible interventions founded on its bioactive compounds.

1.4 Scope

The objective of this work involves expanding on the best practices for extraction non-leachable bioactive compounds from Lemon Myrtle leaves, and then appraising there in vitro anticancer efficacy on pancreatic cancer cells. It indicates that the approach taken with the study will be to investigate the variety of extraction techniques which include liquid-liquid fraction and discover which techniques will provide the ideal yield strength and bioactivity for the product. Next, the mentioned bioactive compounds will be characterized by analytical tools such as HPLC to determine its chemical nature. These compounds will then be assessed for their efficacy in down regulating growth and viability of pancreatic cancer lines through cell based activity assays that examine such factors as proliferation, down regulation or facilitation of apoptosis, and cell cycle. Of greatest interest is the investigation of the anticancer properties of Lemon Mytle extracts. Collectively, this study seeks to establish anticipative awareness on the potential therapeutic effects of Lemon Myrtle extracts toward pancreatic cancer and on the natural product-based anticancer research which could be beneficial toward the eventual formulation of potential treatment options for this difficult type of cancer.

1.5 Aim and Objectives

The objectives of this study will be the optimization of the extraction process of active compounds in the native plant called lemon myrtle by assessing their in vitro anticancer properties on pancreatic cancer.

1.5.1 Objectives

- To determine the most efficient process of Liquid-Liquid fraction for extracting active compounds of Lemon myrtle leaves.
- To identify the structure of the extracted compounds by employing analytical method High-performance liquid chromatography.
- To evaluate the cytotoxic effects of the extracted compounds on pancreatic cancer cell lines (e.g. MIA PaCa-2, BxPC-3, HPDE (H6c7) using MTT assay or other appropriate cytotoxicity assays.

Chapter 2

Literature Review

2.1 Significance of Lemon Myrtle

Lemon myrtle (Backhousia citriodora) is an Australian native herb well known for its strong lemony flavor and pharmacological potential of phenolic acids, flavonoids and proanthocyanidins. They only add to its flavor but are important sources of antioxidants, a property that is sought after in food processing, pharmaceuticals and cosmetics. Lemon myrtle is usually associated with cooking; however, scientific research is now focusing on the possible health effects and, in particular, antioxidant action of lemon myrtle as applied to combating of oxidative stress and allied illnesses [13].

Essential oils find great application in food, drinks, cosmetics, fragrance, medicine, pharmacy, and aromatherapy. The growing global use of essential oils begs various issues regarding negative health consequences that need attention [13].

Essential oils produced by Australian native plants include tea tree oil (oil of Melaleuca alternifolia), which finds usage in consumer health goods including topical antiseptics, mouthwashes, and acne treatments [13]. Well reported are the broad-spectrum antibacterial action, chemistry, and in vitro cytotoxicity of tea tree oil [14].

Native to eastern Australia, the plant genus Backhousia falls under the family Myrtaceae [15]. First reported by the German company Schimmel and Co. in 1888 from a specimen discovered in the rainforests close to Imbill, southern Queensland, B. citriodora Highly aromatic plant containing mostly citral (3,7-dimethyl-2-7-octadienal) with two primary isomeric aldehydes neral and geranial is the most often occurring chemotype B. citriodora F. Mueller (oil of lemon myrtle) [15].

Essential oil, leaf, and lemon myrtle fruits have been somewhat popular in the last ten years as components for culinary flavourings, herbal teas, and fragrances [16]. Recently, this essential oil has been standardised with the publication [17] of the Australian Standard 4941-2001 for the oil of B. citriodora, citral type (lemon myrtle oil). This standard calls for an oil minimum concentration of 85% citral. There is no mention of antibacterial activity or toxicological/safety features of the oil within this standard. Lemon myrtle oil has moderate antibacterial action [18] and great germicidal activity [19]. Still, additional research on the oil's antibacterial power is still under progress. The main component in the oil, citral, was demonstrated to have broad-spectrum antibacterial action [20].

2.2 Impact of Drying Methods on Phytochemical Retention & Antioxidant Properties of Lemon Myrtle

Several drying techniques have been researched in a bid to try and preserve as many of the bioactive compounds in the lemon myrtle leaves as possible. Among them are the hot air dry, vacuum dry, microwave dry, freeze dry, sun dry and shade dry. Both methods differ in their influence on the content of phytochemicals and antioxidant activity, drying time and energy consumption, as well as the structure of the dried material.

2.3 Phytochemicals in Lemon Myrtle and Their Health Benefits

Lemon myrtle contains many phenolic compounds such as gallic acid and hesperetin which are numbered among the most effective bioactive fractions. These compounds show high antioxidant effect, which is responsible for protection of the cells from free radicals damage. The antioxidant capacity of plant based foods such as the lemon myrtle has been identified to possess a number of health positive attributes which include anti-inflammatory, anti-microbial and anti-cancer [21]. Numerous analytical ex vivo essays have shown that both alcoholic and aqueous extracts of LM possess high antioxidant activity that can be associated with the presence of phenolics, flavonoids, and proanthocyanidin [21].

2.4 Impact of Drying Methods on Bioactive Compound Retention

Temperature, duration, and the particular method of drying have strictly affect the content of bioactive compounds in plant materials. The drying method decides the level of moisture as well as heat inactivation of heat sensitive components such as phenolics and flavonoids. Below is an overview of how different drying techniques affect these compounds in lemon myrtle:

2.4.1 Freeze Drying

Method that has been especially effective in maintaining the phytochemical content is freeze drying, which involves freezing the plant material before evaporating the water by sublimation. This method conserve the maximum total phenolic content TPC, total flavonoid content TFC and antioxidant potential using a number of assays including FRAP, CUPRAC, ABTS and DPPR [22]. Freeze drying is not

very destructive to phenolic compounds because the process occurs at low temperature and therefore is suitable for research use or high value product purposes [22]. But its disadvantage is that freeze drying is also the most energy consuming and longest process making its applicability in large scale industrial uses slightly questionable.

2.4.2 Hot Air Drying

This method requires blowing heated air over the plant material in order to dry it. It was found that drying lemon myrtle at 90°C for 75 minutes exposed the product to relatively higher retention of phenolics and flavonoids compared to lower temperature values [23]. Greater drying temperatures cause shorter drying times for reducing the time exposure of heat-sensitive compounds and also help in the maintenance of antioxidant characteristics. Hot air drying can be defined as a process in energy saving and time consuming thus being fabricated for industrial uses but with tendency to cause loss of heat sensitive constituents [23].

2.4.3 Vacuum Drying

Vacuum drying works at low pressure, and hence water evaporates at a low temperature, thus beneficial to heat sensitive compounds. Lemon myrtle leaves contain high phenolic compounds and antioxidant activity even when prepared at 90°C in vacuum without the need to freeze dry the sample. This method is considerate of the bioactive compounds and is also friendly in the energy demand making it suitable for industrial production [23].

2.4.4 Microwave Drying

Microwave drying is a process in which the use of electromagnetic energy is employed to heat water molecules within the plant material in order to evaporate the water. It is the fastest method of drying and preserves a reasonable level of

phenolic content and antioxidant activity at 960 W for 7 minutes [24]. The short drying time reduces exposure to heat, thereby limiting the degradation of sensitive compounds like phenolics. Microwave drying also consumes less energy compared to hot air and vacuum drying, making it a viable option for industrial applications [24].

2.4.5 Sun and Shade Drying

These are traditional drying methods that rely on natural conditions, with sun drying exposing the material to direct sunlight and shade drying occurring in a shaded, ambient environment. These methods require significantly longer drying times (2 days for sun drying and 12 days for shade drying) and result in lower retention of bioactive compounds compared to mechanical drying methods. However, they are energy-efficient (requiring no electricity) and can be used for large-scale, low-cost production. Interestingly, shade drying is noted for retaining higher levels of hesperetin, a key flavonoid in lemon myrtle [25].

2.5 Antioxidant Assays and Correlation with Phytochemicals

The antioxidant capacity of dried lemon myrtle leaves is commonly evaluated using assays such as the Ferric Reducing Antioxidant Power (FRAP), Cupric Ion-Reducing Antioxidant Capacity (CUPRAC), ABTS radical scavenging capacity, and DPPH assays. Studies have shown a strong positive correlation between the total phenolic content and antioxidant capacity, indicating that phenolic compounds are major contributors to the antioxidant potential of lemon myrtle [26]. Flavonoids and proanthocyanidins also play a significant role, with CUPRAC showing the strongest correlation with these compounds [26].

2.6 Cancer Prevalence

Cancer remains a significant health concern worldwide, with Japan facing a particularly high burden, especially regarding breast cancer incidence among women. While various treatment modalities exist, including surgery, chemotherapy, and targeted therapies, they often come with debilitating side effects, impacting the quality of life for patients. Consequently, there is a growing interest in exploring alternative and adjunctive therapies with fewer adverse effects. Essential oils, derived from plants and rich in bioactive compounds, have garnered attention for their potential therapeutic properties, including antitumor effects [27].

Volatile molecules taken from plants, essential oils are well-known for their variety of uses including stress-relieving, antibacterial, and antiviral ones. Among their components, terpenes are highly important; monoterpenes, sesquiterpenes, diterpenes, and higher terpenoids all play roles. Notably, monoterpenoid aldehydes, such as citral (comprising neral and geranial isomers), exhibit a spectrum of biological activities, including antitumor properties. The rising prevalence of cancer globally has underscored the importance of exploring novel treatment avenues. While synthetic anticancer compounds dominate the market, a significant proportion of drugs are derived from or inspired by natural products. Terpenes and terpenoids found in essential oils have emerged as promising candidates for cancer therapy due to their diverse pharmacological activities, including antitumor effects. The low molecular weight and volatility of monoterpenes and sesquiterpenes make them particularly intriguing for drug discovery efforts in cancer treatment [27].

2.7 Lemon Myrtle in Breast Cancer

The antitumor effects of different essential oils were evaluated in a study by [28], and they reported significant results for their efforts against breast cancer cells. Of the 20 essential oils investigated, Lemongrass, Lemon Myrtle, Litsea and Melissa were notable because they were found to be particularly potent as inhibitors of

tumor growth. But remarkably, these essential oils could even do this when significantly diluted. Lemon myrtle was not only the most potent antitumor substance, but surprisingly, the transpiration component was the most active, and showed very little cytotoxicity with normal peripheral blood mononuclear cells (PBMCs). This differential cytotoxicity underscores the specificity of these essential oils towards cancer cells, highlighting their potential as targeted therapeutic agents. Further analysis revealed that each of the identified essential oils contained substantial quantities of citral, a monoterpenoid aldehyde known for its diverse biological properties. When citral was volatilized from these essential oils, it exhibited potent antiproliferative and invasion-inhibiting effects against breast cancer cells. The inhibitory concentration 50 (IC50) values for citral against breast cancer cells were notably low, indicating a high degree of efficacy. Specifically, the IC50 values for geranial and neral, the two isomers of citral, were determined to be 1.67 μL/mL and 1.31 μL/mL [28].

2.8 Lemon myrtle Induced S-phase Cell Cycle Arrest and Apoptosis in HepG2 Cells

In a study conducted by [29], Lemon myrtle essential oil (LMEO) exhibited significant cytotoxicity against various cancer cell lines, with considerable variation in IC50 values across different cell types. Specifically, LMEO demonstrated IC50 values of 40.90 $\pm$ 2.23 μg/mL for the liver cancer HepG2 cell line, 58.60 $\pm$ 6.76 μg/mL for the human neuroblastoma SH-SY5Y cell line, 68.91 $\pm$ 4.62 μg/mL for the human colon cancer HT-29 cell line, and 57.57 $\pm$ 7.61 μg/mL for the human non-small cell lung cancer A549 cell line. These results therefore reveal that LMEO displays broad spectrum cytotoxicity against many different cancer types. Further analysis revealed that citrals, 74.9% of HLMEO content, were the major cytotoxic constituents. Network pharmacological analysis highlighted several potential cytotoxic targets of LMEO, including apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1), androgen receptor (AR), cyclin-dependent kinase 1 (CDK1), nuclear factor erythroid 2-related factor 2 (Nrf-2), fatty acid synthase (FASN), epithelial

growth factor receptor (EGFR), estrogen receptor 1 (ERα), and cyclin-dependent kinase 4 (CDK4). These targets are involved in fundamental cellular functions including cell movement, cell division and cell death [24]. The protein p53 showed the highest confidence in co-associating with the target genes identified in this study.

The result of the current study indicated that LMEO suppressed HepG2 cell migration in a time- and dose-dependent manner suggesting a possible antimetastatic role. Moreover, LMEO caused S phase cell cycle arrest and enhanced apoptosis in HepG2 cells. Furthermore, p53 protein level, Cyclin A2 and Bax proteins increased and Cyclin E1 and Bcl-2 proteins level decreased in western blot analysis which just explain the fascinating cytotoxicity profile of LMEO. These observations provide significant insights on how LMEO might be used for anti-cancer therapy and particularly on facets of pathways which would fuel tumor development and spread [29].

2.9 Anti-Inflammatory and Anti-Oxidative Activities Of Lemon Myrtle

Oyaizu [30] wildly researched the anti inflammatory and anti oxidative effects of alcoholic lemon myrtle extract (LME). Analysis of LME (total polyphenol, 118.77 mg/g extract; flavonoid, 14.53 mg/g extract) demonstrated that LME contained a high antioxidant profile. Apart from that, LME also showed highly notable anti oxidative activity, as it showed the reactions against 2, 2 diphenyl 1 picrylhydrazylhydrate (DPPH) and 2,2' azinobis / (3 ethylbenzthiazolyne 6 sulfonic acid) (ABTS) radicals [30]. Furthermore, anti inflammatory activity of LME was determined in the RAW 264.7 mouse macrophage cells under lipopolysaccharide stimulated condition. For instance, pretreatment with LME at non cytotoxic levels (10–100 g/mL) significantly reduced inflammatory mediator production, particularly nitric oxide (NO). Later enzyme linked immunosorbent assay (ELISA) and reverse transcriptase polymerase chain reaction (RT PCR) analyses

subsequently showed a concentration dependent suppression of pro inflammatory cytokines including IL 6 and tumour necrosis factor (TNF α) [30].

Chapter 3

Materials and Methods

3.1 Preparation of Lemon Myrtle Extract

3.1.1 Harvesting of Lemon Myrtle Leaves

Approximately 300 grams of fresh lemon myrtle leaves were harvested from healthy, mature trees. To ensure the quality of the leaves, careful attention was given to select leaves free from visible signs of damage or disease. Sharp pruning shears or scissors were used to delicately cut the leaves from the branches, minimizing bruising or tearing.

3.1.2 Preparation for Drying

Lemon myrtle leaves were harvested and placed straight into clean containers with tight closings or airtight bags to minimize compromise of quality. In order to preserve the interleafing, the leaves were taken to the laboratory as soon as possible. The equipment for using liquid nitrogen also that for evaporation of the solvent and drying chamber that should follow the extraction process were arranged

3.1.3 Treatment with Liquid Nitrogen and Drying

A drying chamber was pre-cooled to a temperature of -46.6^{o}C in a conservation chamber that was opened in a ventilated room, with good temperature controlled atmosphere and humidity. The leaves from the lemon myrtle plant were initial-lypurified using liquid nitrogen as a means of flash freezing them. The leaves are laid on trays and put in the drying chamber after which they are frozen, after which the thawed leaves are spread uniformly on trays to allow proper air circulation. They were freeze-dried or lyophilization for 24-48 hours in case of the leaves. To facilitate adequate removal of moisture in the drying chamber, temperature and vacuum levels were well regulated. Maintenance inspections were performed to evaluate the drying conditions with corresponding modifications made on the parameters involved in the drying process in order to promote uniformity in the drying process.

3.1.4 Post-Drying Handling

After the completion of drying period, the trays with dried lemon myrtle leaves were taken out from the drying chamber. The leaves were examined for proper dryness in which the leaves' texture are crispy and free from moisture content. The leaves were then put into clean tight plastic bags or airtight containers in order to minimize their exposure to moisture and dust. These containers were appropriately coded to indicate the specific data of drying and batch no for tracking purposes. The dried lemon myrtle leaves were put into an environment with low temperature free from direct sunlight until further processing.

3.1.5 Grinding of Lemon Myrtle Leaves

The fresh lemon myrtle leaves where dried thereafter the dried sample was grounded using a grinder which was in accordance to the manufacturers' instructions which creates a powder from the leaves. To ensure consistency the leaves were grounded in small portions, not to fill the grinder to allow for equal distribution of the

pressure. To reduce particle size of the drug to a constant size the achieve larger the powders were put through a fine mesh sieve. The sieved powder was visually examined in order to check its homogeneity as well as absence of large fractions. This powdered lemon myrtle was then sealed and stored in an airtight container to minimized contact with moisture, light and air which negatively impacts on the bioactive contents of the product.

3.1.6 Preparation of Extraction Solvent

The working solution of 50% acetone was made by combining acetone and distilled water in 1: 1 proportions. One hundred milligrams of finely ground lemon myrtle leaf powder was weighed out a transfer to a 500 mL conical flask was made and the volume made up to 500 mL with 5% acetone. It was carried out in a round bottom flask to avoid the loss of solvent vapor during the extraction of the mixture through the use of reflux condenser. The flask was placed in a 60^{o}C water bath and heated for fifty minutes. They were blended during this time to make sure efficient extraction of the above mentioned bioactive compounds. The reflux condenser to which the solution is led allowed for condensation of the solvent and return to the flask thus maintaining its volume constant. The solution was then left to cool to room temperature after the heating period of extraction has elapsed.

3.1.7 Centrifugation of Extract

To the cooled extract mixture was transferred into the centrifuge tubes then balanced and the tubes were then centrifuged for 20minutes at 4000 rpm. This particular step was relevant and necessary to ensure that the solids and the liquids where well separated after the plant particles were ground. The supernatant was then poured into a different container while being very cautious not to mix the solid pellet at the base of the tubes.

3.1.8 Filtration of the Extract

Vacuum filtration was done in a Buchner funnel using filter paper as the adsorbent layer. The liquid constituent that was obtained from the process of centrifugation was decanted through the filter paper and the vacuum was used to suck the rest of the solid particle. The filtrate was gathered to a flak in which there was no grains and residues in the liquid extract.

3.1.9 Rotary Evaporation

The filtered extract was transferred into a rotary evaporator flask the solvents in the filtered extract were evaporated at forty degrees Celsius in the rotary evaporator. Heating the sample was done at a low temperature of 40 degree Celsius in a water bath. The flask was then placed onto rotary evaporator that came with reduced pressure. The flask was swirled around in order to provide additional surface area on which acetone could evaporate easily. Following this the flask contained the concentrated lemon myrtle extract after acetone has evaporated.

3.1.10 Treatment with Liquid Nitrogen and Air Drying

The concentrated lemon myrtle extract was frozen by immersing it in liquid nitrogen; this technique of freezing facilitated faster and uniform freezing which helped in retaining the content and potency of the extracts bioactive content. The frozen extract was then removed from the instrument and placed into an air dryer with a temperature maintained at around 25^oC. The extract was left under these conditions until the liquid nitrogen had evaporated and the extract thus became a crude powder.

3.1.11 Post-Drying Handling

After drying the crude powder was gently taken out of the air dryer used as shown after the process. To ensure that dilution with the powder was perfect, the

powder was visually checked to make sure it was completely dry, and no moisture was present. The crude powder was then kept in air tight containers in order to avoid unwanted external interferences and dampness. A relevant information was given on these containers, including drying date and batch number for tracing. The crude lemon myrtle powder was kept in a cool, dry and dark place to avoid degrading of essential oils in the sample.

3.2 Liquid-Liquid Fractionation Methodology

3.2.1 Sample Preparation

The lemon myrtle leaves were ground finely, and thirty grammes of the powder were precisely weighed. The powdered material was combined in a suitable container with 600 mL of 50% acetone solution. To enable the extraction of bioactive molecules, the container was then submerged in a water bath and heated for fifty minutes at 60^{o}C. The mixture was heated then centrifuged at 3000 rpm for 20 minutes to remove the solid wastes. After filtration to produce a clear extract, the supernatant was sent to a rotary evaporator. Under low pressure, acetone evaporated at below 40^{o}C, leaving an aqueous solution behind.

3.2.2 Liquid-Liquid Fractionation

The fractionation process was carried out using two solvents: Butanol, and water. The procedure for both fractions is detailed below:

3.2.2.1 Butanol Fractionation

A separatory funnel was then filled with another 200 mL of the aqueous solution and 200 mL of butanol. The same shaking and settling process was used as with the hexane fractionation. Separated and arranged in marked containers were the aqueous and butanol layers.

3.2.2.2 Aqueous Fractionation

Together with another 200 mL of water, the remaining 200 mL of the aqueous solution was piped into a separatory funnel. The aqueous layer was gathered in a marked container and the mixture shook and settled using the same technique.

3.2.3 Concentration of Fractions

Both Butanol, and aqueous fractions were concentrated separately using a rotary evaporator since the solvent was removed under reduced pressure at temperature not exceeding 40^{o}C. The concentrated residues were then liquated into individual tubes and immediately frozen at the rate of liquid nitrogen. The frozen residues were then placed in a freeze dryer for 2 days, yielding powdered extracts. The powdered extracts were stored at -30^{o}C for future use or analysis.

3.3 Characterization of Extracted Compounds

3.3.1 Total Phenolic Content (TPC) Assay

3.3.1.1 Preparation of Solutions

- **Folin-Ciocalteu Reagent Solution (10% v/v):**

  Diluting the Folin-Ciocalteu reagent to a 10% (v/v) concentration produced Measuring 10 mL of Folin-Ciocalteu reagent, one put it to a volumetric flask. One then added deionised water till the whole amount came to 100 mL. Thorough mixing guaranteed homogeneity in the solution.

  Sodium Carbonate Solution (7.5% w/v):

- Using an analytical balance, 7.5 grammes of sodium carbonate were precisely weighed to ready the sodium carbonate solution. The sodium carbonate was

then put in a 100 mL volumetric flask. Gradually adding deionised water under constant stirring, the total amount came to 100 mL. To guarantee perfect dissolution of the sodium carbonate, the solution was carefully stirred.

Gallic Acid Standard Solution (0.5 mg/mL):

- An analytical balance let 0.05 grammes of gallic acid be precisely weighed. The gallic acid then was moved to a 100 mL volumetric flask. Gradually adding deionised water to the flask under constant stirring, the total amount eventually came to 100 mL. To guarantee total gallic acid breakdown, the solution was carefully stirred. Triplicate preparation of this standard solution guaranteed precision and repeatability.

3.3.1.2 Serial Dilutions of Gallic Acid

- **100 μg/mL Solution:**

  Using a pipette, 5 mL of the 0.5 mg/mL gallic acid standard solution was measured and then pipled to a 25 mL volumetric flask. Up till the entire amount in the flask came to 25 mL, deionised water was added.

- **75 μg/mL Solution:**

  Measuring 3 mL of the 100 μg/mL solution using a pipette, it was then pipled into a 4 mL volumetric flask. Up till the whole amount came to 4 mL, deionised water was added. The solution was completely mixed.

- **50 μg/mL Solution:**

  Measuring 2 mL of the 100 μg/mL solution using a pipette, it was then pipelled to a 4 mL volumetric flask. Added till the entire amount came to 4 mL was deionised water. The solution was fully combined.

- **25 μg/mL Solution:**

  Measuring 2 mL of the 50 μg/mL solution using a pipette, it was then pipled into a 4 mL volumetric flask. Up till the whole amount came to 4 mL, deionised water was added. The solution was properly blended.

- **12.5 μg/mL Solution:**

  Measuring 2 mL of the 25 μg/mL solution using a pipette, it was then pipled to a 4 mL volumetric flask. Up till the whole amount came to 4 mL, deionised water was added. The solution was well blended.

- **6.25 μg/mL Solution:**

  2 mL of the 12.5 μg/mL solution was measured using a pipette and transferred to a 4 mL volumetric flask. Deionized water was added until the total volume reached 4 mL. The solution was mixed thoroughly.

3.3.2 Preparation of Lemon Myrtle Extract Samples

- **Crude Powder Extract:**

  0.1 g of crude powder was weighed and dissolved in 100 mL of 50% methanol.

- **Butanol Extract:**

  0.1 g of butanol extract powder was weighed and dissolved in 100 mL of 50% methanol.

- **Aqueous Extract:**

  0.1 g of aqueous extract powder was weighed and dissolved in 100 mL of deionized water.

3.3.2.1 TPC Assay Procedure

Supplies of each lemon myrtle extract sample and each gallic acid standard solution were put in triplicates, 1mL of each was transferred into separate test tubes, 5 mL of the 10% Folin-Ciocalteu reagent was added to each test tube, and the reagent was mixed thoroughly. Each test tube was allowed to stand at room temperature for 8 minutes before 4 mL of the 7.5% sodium carbonate solution was added to each test tube. Incubation for 60 minutes at room temperature was given to the mixtures. Each sample had its absorbance measured at 765 nm with a spectrophotometer.

3.4 Ferric Reducing Antioxidant Power (FRAP) Assay

3.4.1 Preparation of Trolox Standard Solution

To prepare a 1 mM (1000 μM) Trolox standard solution, 0.01265 g of Trolox was weighed and dissolved in 100 mL of methanol.

3.4.2 Preparation of Serial Dilutions of Trolox

- 500 μM Solution: A 1000 μM Trolox solution was diluted to 5 mL with 7.5 mL methanol.
- 250 μM Solution: 7.5 mL methanol was added to 5 mL 500 μM Trolox solution.
- 125 μM Solution: 250 μM Trolox solution (5 mL) was diluted by methanol (7.5 mL).
- 62.5 μM Solution: The 125 μM Trolox solution was then diluted with 7.5 mL of methanol and added 5 mL of Trolox.
- 25 μM Solution: 7.5 mL of methanol was added to 5 mL of the 62.5 μM Trolox solution.

3.4.3 Preparation of 300 mM Acetate Buffer (pH 3.6)

A 300 mM acetate buffer was synthesized by dissolving 3.1 grams of sodium acetate trihydrate ($NaC_2H\ O_2\ 3H_2O$) in 16 ml of glacial acetic acid. Lastly the total solution was stirred well, brought up to 1 litre with deionized water and then stirred.

3.4.4 Preparation of TPT Reagent

First, a 40 mM HCl solution was prepared by adding 3.4 mL of concentrated HCl (36%) to 1 liter of deionized water. Then, 0.3123 g of 2,4,6-tripyridyl-s-triazine (TPT) was dissolved in 100 mL of the prepared 40 mM HCl solution, ensuring the TPT was fully dissolved.

3.4.5 Preparation of 20 mM Ferric Chloride Solution

To prepare the 20 mM ferric chloride solution, 5.406 g of ferric chloride (FeCl) was dissolved in deionized water and adjusted to a total volume of 1 liter.

3.4.6 Assay Procedure

The FRAP working solution was prepared by mixing 100 mL of the 300 mM acetate buffer, 10 mL of the prepared TPT reagent, and 10 mL of the 20 mM ferric chloride solution. The solution was warmed to 37^{o}C before use.

- **Sample Incubation:**

  150 μL of each Trolox standard dilution (500 μM to 25 μM) and lemon myrtle extract (crude powder, butanol, aqueous, and fresh extracts) were pipetted into separate tubes in triplicate. Following this, 2850 μL of the warmed FRAP working solution was added to each tube.

- **Reaction Time:**

  The tubes were incubated at room temperature for 30 minutes.

- **Absorbance Measurement:**

  After incubation, the absorbance was measured at 593 nm against a blank solution.

3.5 DPPH Radical Scavenging Capacity Assay

3.5.1 Preparation of DPPH Stock Solution

To prepare the DPPH stock solution, 0.024 g of DPPH (molecular weight: 394.32 g/mol) was dissolved in 100 mL of methanol. The solution was stored in the dark to prevent degradation.

3.5.2 Preparation of DPPH Working Solution

On the day of the assay, 20 mL of the DPPH stock solution was diluted with 90 mL of methanol. The absorbance of the working solution was adjusted to 1.1 ± 0.02 at 515 nm.

3.5.3 Preparation of Trolox Standard Solution

As per the FRAP assay, 0.01265 g of Trolox was dissolved in 100 mL of methanol to prepare a 1 mM (1000 μM) Trolox standard solution.

3.5.4 Preparation of Serial Dilutions of Trolox

- **750 μM Solution:** 7.5 mL of the 1000 μM Trolox solution was diluted with 2.5 mL of methanol.
- **500 μM Solution:** 7.5 mL of the 1000 μM Trolox solution was diluted with 7.5 mL of methanol.
- **250 μM Solution:** 7.5 mL of the 500 μM Trolox solution was diluted with 7.5 mL of methanol.
- **125 μM Solution:** 7.5 mL of the 250 μM Trolox solution was diluted with 7.5 mL of methanol.

- **62.5 μM Solution:** 7.5 mL of the 125 μM Trolox solution was diluted with 7.5 mL of methanol.

- **25 μM Solution:** 7.5 mL of the 62.5 μM Trolox solution was diluted with 7.5 mL of methanol.

3.5.5 Assay Procedure

- **Sample Incubation:**

  150 μL of each Trolox standard dilution (750 μM to 25 μM) and lemon myrtle extracts were pipetted into separate tubes in triplicate. 2850 μL of the DPPH working solution was added to each tube.

- **Reaction Time:**

  The samples were incubated at room temperature for 30 minutes in the dark.

- **Absorbance Measurement:**

  The absorbance of the samples was measured at 515 nm, and the percentage inhibition of DPPH radical scavenging was calculated.

3.6 ABTS Radical Scavenging Activity Assay

A stock solution was prepared by mixing 8 mL of 7.4 mM ABTS solution with 8 mL of 2.6 mM potassium persulfate solution. The mixture was incubated in the dark for 16 hours to allow the formation of ABTS radical cations (ABTS+).

3.6.1 Preparation of ABTS Working Solution

The ABTS stock solution was diluted with methanol at a ratio of 1:60 until an absorbance of 1.1 $\pm$ 0.02 was reached at 734 nm.

3.6.2 Preparation of Trolox Standard Solution

As per the DPPH and FRAP assays, 0.01265 g of Trolox was dissolved in 100 mL of methanol to prepare a 1 mM (1000 μM) Trolox standard solution.

3.6.3 Preparation of Serial Dilutions of Trolox

- **750 μM Solution:** 7.5 mL of the 1000 μM Trolox solution was diluted with 2.5 mL of methanol.
- **500 μM Solution:** 7.5 mL of the 1000 μM Trolox solution was diluted with 7.5 mL of methanol.
- **250 μM Solution:** 7.5 mL of the 500 μM Trolox solution was diluted with 7.5 mL of methanol.
- **125 μM Solution:** 7.5 mL of the 250 μM Trolox solution was diluted with 7.5 mL of methanol.
- **62.5 μM Solution:** 7.5 mL of the 125 μM Trolox solution was diluted with 7.5 mL of methanol.
- **25 μM Solution:** 7.5 mL of the 62.5 μM Trolox solution was diluted with 7.5 mL of methanol.

3.6.4 Assay Procedure

- **Sample Incubation:**

  150 μL of each Trolox standard dilution (750 μM to 25 μM) and lemon myrtle extracts were pipetted into separate tubes in triplicate. 2850 μL of the ABTS working solution was added to each tube.

- **Reaction Time:**

  The tubes were incubated at room temperature for 6 minutes in the dark.

- **Absorbance Measurement:**

  The absorbance of the samples was measured at 734 nm, and the percentage inhibition of ABTS radical scavenging was calculated.

3.7 High-Performance Liquid Chromatography (HPLC) Analysis of Crude, Butanol, & Aqueous Extracts of Lemon Myrtle

The high-performance liquid chromatography (HPLC) analysis for the lemon myrtle extract, as well as crude butanol and aqueous extracts, was conducted as follows:

According to the method described by Saifullah et al. [36] the HPLC analysis was carried out. In brief, solvents A and B used were 0.2% formic acid in deionized Milli-Q water and 100% acetonitrile, respectively. The separation was using a C18 (2) reversed phase column (Luna 100Å 5 μm, 250 mm $\times$ 4.6 mm; Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia) in an HPLC system (CBM-20A, Shimadzu Australia, Rydalmere, NSW, Australia).

The gradient solvent flow was set to 1 mL/min with the following gradient program: From 0 to 5 minutes 100% A, 5 to 15 minutes 30% B, 15 to 20 minutes 70% B and 20 to 25 minutes 100% B. 20 μL were used for injection. Levels of the compounds in the lemon myrtle extract and in the crude butanol and aqueous extracts were detected by means of a UV–VIS detector.

LabSolutions software (Shimadzu Corporation, Kyoto, Japan) was used for recording and analyzing the chromatographic data for chromatographic profiling.

3.8 Cell Culture

3.8.1 HPDE (H6c7) Cell Line

For culturing HPDE (H6c7) cells, Keratinocyte Serum-Free Medium (KSFM) plus epidermal growth factor (EGF) and bovine pituitary extract was prepared. The cells were thawed rapidly in 9 mL of pre-warmed KSFM in a bead bath at 37^{o}C and transferred to a 15 mL conical tube. The cell suspension was centrifuged to 300 x g for 5 minutes, and the supernatant discarded. The cell pellet was resuspended in 13 ml freshKSFM and seeded in a T25 flask. The flask was then allowed to stand at 37 degree centigrade under 5% CO2. We changed medium every 2 to 3 days and at 70 - 80

For passaging, serum media was removed and replaced with PBS with 2% FBS and aspirated, 2 mL of Trypsin-EDTA was added to the flask to detach the cells, and placed in 37^{o}C incubator until detached. The trypsin action on the cells was stopped by addition of fresh KSFM medium; cells were then either split into a new culture flask or prepared for freezing. For the purpose of storage, the cells were trypsinized and resuspended in a freezing media comprising of 45mls of the KSFM and 5 mls of DMSO. Using a 1:1 ratio of freezing medium to fresh KSFM, 1.5 mL aliquots were cryovially housed at -80^{o}C.

3.8.2 BxPC-3 Cell Line

Ten percent FBS and one percent penicillin-streptomycin supplemented RPMI-1640 medium housed BxPC-3 cells. Thawed cells were centrifuged at 300 x g for five minutes in a 15 mL conical tube filled with nine millilitres of full RPMI-1640 medium. The supernatant was thrown away, and before being seeded into a T25 flask the cell pellet was resuspended in 13 mL of new RPMI-1640 medium. Cells were incubated at 37^{o}C with 5% CO2; medium were replaced every two to three days until the cells attained 70–80

Cells were washed with 4 mL PBS containing 2% FBS then added 2 mL of Trypsin-EDTA to separate them for passaging. After full RPMI-1640 media neutralising detached cells, they were transferred to fresh flasks for further cryopreservation or growth. The cells were resuspended in freezing medium including 45 mL RPMI-1640 and 5 mL DMSO for long-term preservation. Cryovials containing aliquots of 1.5 mL of freezing and fresh media were kept at -80^{o}C.

3.8.3 MIA PaCa-2 Cell Line

MIA PaCa-2 cells were cultured in DMEM medium supplemented with 10% FBS, 2.5% horse serum, and 1% penicillin – streptomycin, quickly thawed in a 37^{o}C bead bath in a 15 mL conical tube with 9 mL of total DMEM. Supernatant was thrown away after centrifuging the cell suspension at 300 x g for five minutes. The cell pellet was resuspended in 13 mL fresh DMEM medium and sown into a T25 flask. Incubated under 37^{o}C (with 5% CO2), the flask was changed every two to three days until enough cells were reached to be 70–80% confluent.

Cells were passaged using 4 mL PBS with 2% FBS, washed. The cells were detached by trypsin-EDTA (2 mL), then incubated at 37^{o}C. Trypsin was neutralised with whole DMEM medium upon detachment, and the cells were moved to fresh flasks for either cryopreservation or further growth. The cells were resuspended in freezing medium including 45 mL DMEM and 5 mL DMSO for long-term preservation. Placed in cryovials, cells were frozen using 1.5 mL aliquots of fresh medium and 1.5 mL of freezing media at -80^{o}C.

3.9 Treatment of Cancer Cell Lines with Extracts

Three different extracts crude powder, butanol extract, and aqueous extract were prepared by dissolving them in RNase-free water to achieve a stock concentration of 100 μg/mL. Serial 10-fold dilutions were then made to obtain a range of final concentrations from 100 μg/mL to 0.00001 μg/mL for each extract.

3.9.1 Cell Seeding

Cell viability was confirmed following trypsinization, followed by cell detachment, with trypan blue exclusion and hemocytometer counting. Cell suspensions were prepared to achieve the desired cell density for each well: For MIA PaCa-2: cells per well = 3,000; for BxPC-3: cells per well = 7,000; for HPDE: cells per well = 10,000. Media containing 100 μL was added to each well in a 96 well plate, and plates were incubated at 37^{o}C with 5% CO for 18-24 hours to allow cell attachment.

3.9.2 Extract Treatment

After the attachment period, 100 μL of the prepared extract dilutions were added to each well containing adherent cells. Each concentration of extract was tested in triplicate to ensure accuracy. Control wells were included in each plate setup, consisting of:

- Positive control (e.g., gemcitabine)
- Untreated control (cells without extract)
- Negative controls for each diluent used (e.g., RNase-free water, PBS, DMSO)

3.9.3 Resazurin Assay

To assess cell viability 68 hours post-treatment, a resazurin assay was performed using the following steps:

- **Reagent Preparation:** The required volume of resazurin reagent was added to a reagent reservoir.
- **Reagent Addition:** 20 μL of the resazurin solution was pipetted into each well.

- **Incubation:** The plates were incubated for 4 hours at 37^{o}C.
- **Measurement:** Fluorescence was measured in microplate reader at an excitation/emission wavelength of 544/590 nm after incubation. We set the temperature of the plate reader to 25^{o}C. Also to account for possible autofluorescence interference, absorbance readings were taken at 600 nm and 570 nm. From the absorbance at 570 nm minus absorbance at 600 nm, a corrected measure of resorufin production was obtained as a direct indicator of cell viability.

3.9.4 Data Processing and Analysis for Cell Viability Assay

For data analysis, raw absorbance readings at both 570 nm and 600 nm were first recorded. To ensure accuracy, the following data normalization steps were performed:

- **Blank Averaging:** To correct for background absorbance, an average value of blank wells was calculated.
- **Negative Control Adjustment:** These blank adjusted values were then normalized to negative controls to provide blank corrected values for each cell line.
- **Absorbance Difference Calculation:** Cogdaone smith1999 obtained blank corrected values for 570 nm and 600 nm readings and further subtracted 600 nm from 570 nm for the focused resorufin signal with minimal interference.
- **Negative Control Averaging:** Based on viability assessment, we calculated an average of the negative control wells as a baseline.
- **Percent Growth Analysis:** Percent increase obtained after the relative calculation to average negative control Using GraphPad Prism software, this value helped us determine the IC50 values and assess the dose dependent effects of each extract.

This processing approach allowed for the reduction of background noise and enhancement of signal reliability, enabling accurate comparisons across experimental conditions.

3.9.5 Validation of Measurements

To ensure that plate reader measurements aligned with actual cell conditions, visual inspection using a microscope was conducted to assess cell morphology and density in treated versus control wells. Regular checks for contamination were also performed prior to concluding the experiment.

Chapter 4

Results and Discussion

4.1 Total Phenolic Content (TPC) Assay

Table 4.1: Absorbance values of gallic acid at different concentrations measured at 765 nm (TPC Assay)

Concentration (μg/mL)	**Replicate 1 (Abs)**	**Replicate 2 (Abs)**	**Replicate 3 (Abs)**	**Wavelength (nm)**
Control (0 μg/mL)	0.0752	-	-	765
100	0.2953	0.1587	0.0667	765
75	0.3711	0.2567	0.0676	765
50	0.2155	0.2135	0.1567	765
25	0.5148	0.5470	0.5210	765
12.5	1.1655	1.4370	1.3302	765
6.25	1.5150	1.5470	1.6070	765

Table 4.2: Absorbance values of different extract solutions diluted in 25 mL deionized water measured at 765 nm (TPC Assay)

Extract Type	**Replicate 1 (Abs)**	**Replicate 2 (Abs)**	**Replicate 3 (Abs)**	**Wavelength (nm)**
Crude Powder	0.3537	0.4106	0.4189	765
Butanol Extract	0.4196	0.4197	0.4196	765
Aqueous Extract	0.1733	0.1743	0.1734	765

4.2 Ferric Reducing Antioxidant Power (FRAP) Assay

TABLE 4.3: Absorbance values of Trolox dilutions (500 μM to 62.5 μM) with FRAP working solution measured at 593 nm

Concentration (μM)	**Replicate 1 (Abs)**	**Replicate 2 (Abs)**	**Replicate 3 (Abs)**	**Wavelength nm**
Control (0 μM)	0.0794	-	-	593
500	0.9030	0.9020	0.9027	593
250	0.5032	0.5033	0.5034	593
125	0.2892	0.2888	0.2888	593
62.5	0.1851	0.1853	0.1853	593

TABLE 4.4: Absorbance values of 150 μL lemon myrtle extracts measured at 593 nm (Frap Assay)

Sample Type	**Replicate 1 (Abs)**	**Replicate 2 (Abs)**	**Replicate 3 (Abs)**	**Wavelength nm**
Control	0.0769	-	-	593
Crude Powder	0.7959	0.7954	0.7954	593
Butanol Extract	0.7866	0.7867	0.7668	593
Aqueous Extract	0.5832	0.5833	0.5832	593

4.3 DPPH Scavenging Capacity Assay

TABLE 4.5: Absorbance values of Trolox dilutions (750 μM to 31.5 μM) with DPPH working solution measured at 515 nm

Sample Type	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Wavelength (nm)**
Zero (Methanol)	0.0746	-	-	515.0
Reagent Blank	0.8408	-	-	515.0
750 μM Trolox	0.3259	0.3256	0.3257	515.0
500 μM Trolox	0.4963	0.4964	0.4965	515.0
250 μM Trolox	0.5251	0.5252	0.5252	515.0
125 μM Trolox	0.6028	0.6029	0.6029	515.0
62.5 μM Trolox	0.7153	0.7154	0.7155	515.0

Table 4.5 continued from previous page

Sample Type	Replicate 1	Replicate 2	Replicate 3	Wavelength (nm)
31.5 μM Trolox	0.8118	0.8118	0.8118	515.0

TABLE 4.6: Absorbance values of 150 μL lemon myrtle extracts with DPPH working solution measured at 515 nm

Sample Type	Replicate 1	Replicate 2	Replicate 3	Wavelength (nm)
Crude Powder	0.6022	0.6021	0.6017	515.0
Butanol Extract	0.5362	0.5361	0.5361	515.0
Aqueous Extract	0.4315	0.4315	0.4315	515.0

4.4 ABTS Radical Scavenging Activity Assay

TABLE 4.7: Absorbance values of Trolox dilutions (750 μM to 31.5 μM) with ABTS working solution measured at 734 nm

Sample Type	Replicate 1	Replicate 2	Replicate 3	Wavelength (nm)
Zero (Methanol)	0.0568	-	-	734.0
Reagent Blank	0.9586	-	-	734.0
750 μM	0.2937	0.2936	0.2936	734.0
500 μM	0.3810	0.3810	0.3810	734.0
250 μM	0.4082	0.4036	0.4041	734.0
125 μM	0.5434	0.5431	0.5432	734.0
62.5 μM	0.6810	0.6821	0.6814	734.0
31.5 μM	0.7937	0.7929	0.7926	734.0

TABLE 4.8: Absorbance values of 150 μL lemon myrtle extracts with ABTS working solution measured at 734 nm

Sample Type	Replicate 1	Replicate 2	Replicate 3	Wavelength (nm)
Crude Powder	0.6186	0.6190	0.6194	734.0
Butanol	0.4583	0.4588	0.4589	734.0
Aqueous	0.5977	0.5978	0.5979	734.0

4.5 Data Analysis

All results were analyzed using descriptive statistics and presented as the mean of all repeats ± standard deviation (SD), which were determined using Microsoft Excel 365 ©. To compare the mean values, one-way ANOVA (analysis of variance) followed by Tukey's Honest Significant Difference (HSD) test was performed using JMP PRO 16® statistical software (SAS Institute Inc.), with an alpha level set to $p < 0.05$ to determine statistical significance. The tables for each assay, including the mean, standard deviation, standard curves, and charts for the extracts, and JMP PRO results are presented below.

4.5.1 Total Phenolic Content (TPC) Assay

TABLE 4.9: Mean Absorbance Values of Standard Concentrations for TPC Assay

Concentration (μg/mL)	**Mean TPC (Absorbance)**
6.25	0.2516
12.5	0.3641
25	0.4145
50	0.5136
75	0.6751
100	0.7969

4.5.1.1 Standard Curve

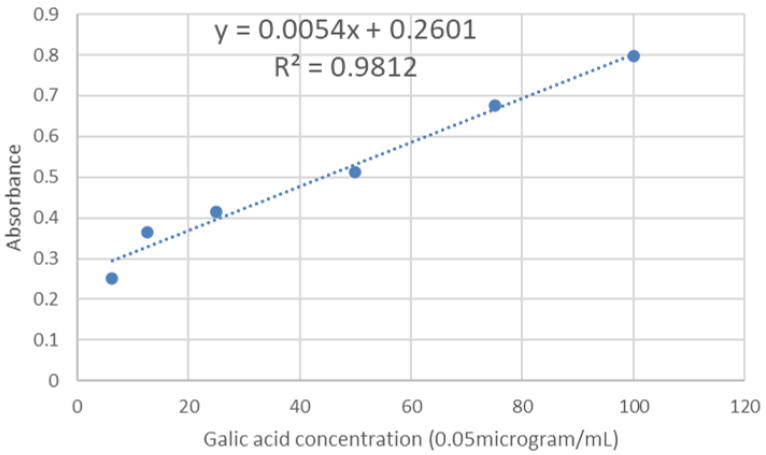


FIGURE 4.1: Standard curve of galic acid (TPC Assay)

The standard curve for the TPC assay, constructed using gallic acid, showed a strong linear relationship between concentration and absorbance values (y = 0.0054x + 0.2601), indicative of the assay's accuracy and sensitivity in quantifying phenolic compounds. This linearity is consistent with other studies that employed similar UV-vis spectrophotometric methods in estimating phenolic content, Cheok et al. [6] previously explained the effect of solvent type and procedure on phenolic quantification. In addition, Xu and Chang [4] emphasized that due to its sensitivity, a different extraction solvent and even conditions may produce different phenolic profile, and micro variation in extraction conditions may affect the result of such assays.

The high R^2 value again substantiates the assay accuracy in the present study as evident from the similar studies related to different plant extracts for phenolic content, as executed by Kim et al [21]. Similar to the findings of Nguyen et al. [31] and Sawant [32] who did a study on Australian native botanicals especially the lemon myrtle, the current study established that UV-vis spectrophotometry was effective in phenolic analysis and it can even capture a slight change in phenolic content due to extraction and drying conditions.

TABLE 4.10: Mean and Standard Deviation of Total Phenolic Content (TPC) for Crude, Butanol, and Aqueous Extracts

Extract Type	**Mean TPC (mg GAE/g)**	**Standard Deviation (SD)**
Crude Extract	46.41	0.0283
Butanol Fraction	29.54	0.0107
Aqueous Fraction	21.03	0.1020

4.5.1.2 Total Phenolic Content

Following butanol extract (21.03 ± 0.102 mg/g GAE), the TPC test findings showed that the crude extract had the highest total phenolic content (46.41 ± 0.028 mg/g GAE). These variations imply that, maybe because of their efficacy in extracting phenolic chemicals, phenolic compounds were higher concentrated in the crude and butanol extracts. Xu and Chang [4] have found similar results:

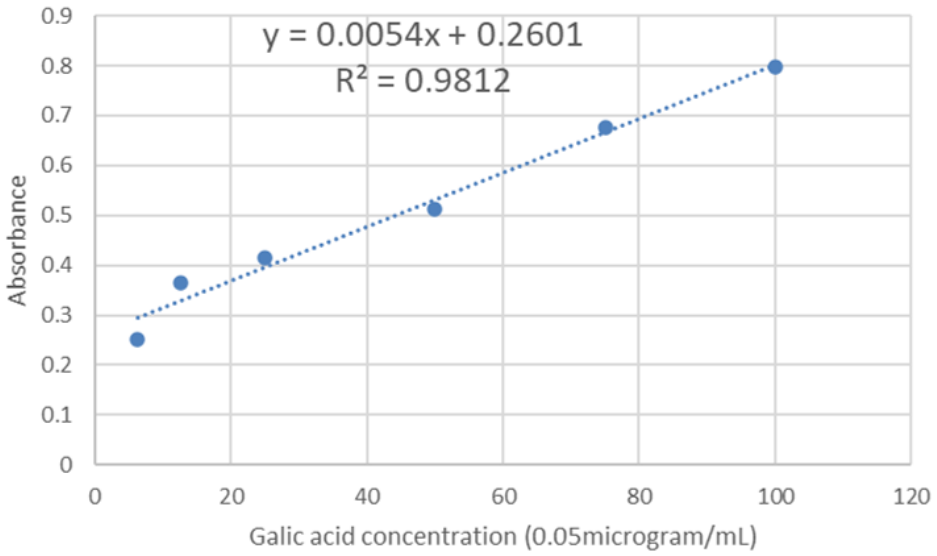


FIGURE 4.2: Total phenolic content (TPC) of the three extracts. Data are of means ± Standard Deviations. Data haring similar letters are not significantly different at $p<0.05$.

solvent choice greatly affects phenolic yield; organic solvues usually produce more phenolic extractions than aqueous ones.

Strong antioxidant qualities of phenolic compounds are well known [6], which might help to explain the higher antioxidant activity seen in the crude and butanol extracts. Cheok et al. [6] underlined that solvents such as butanol often remove more phenolic content, which corresponds with the data shown here.

Moreover, Kang et al. [7] showed that the phenolic-rich lemon myrtle (Backhousia citriodora) extracts showed substantial antioxidant and anti-inflammatory action, thereby strengthening the link between phenolic content and bioactivity.

Further corroborating the high phenolic content seen in the crude and butanol extracts in this study, studies on Australian native herbs and spices as investigated by Konczak et al. [22] showed that phenolic content directly corresponds with antioxidant capacity.

4.5.1.3 One-Way ANOVA and Tukey-Kramer HSD Analysis

The results associated with one-way ANOVA demonstrated that the variety of TPC differed significantly ($p< 0.05$) between the crude extract, butanol fraction,

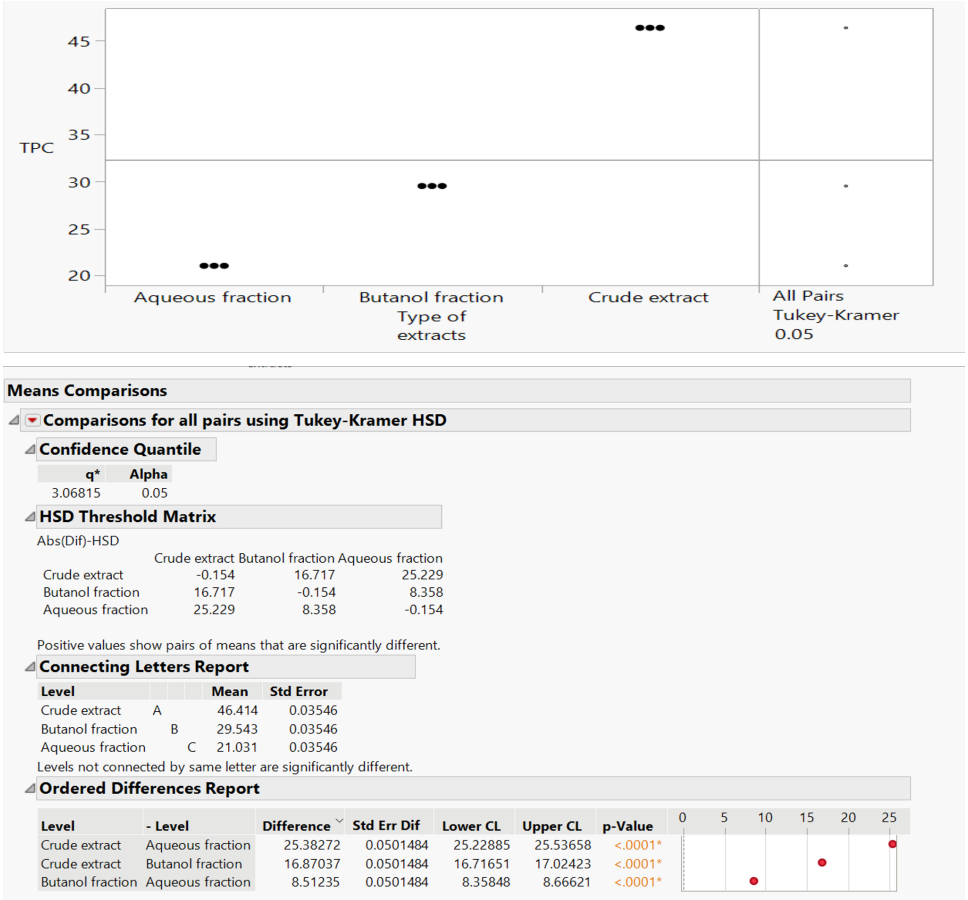


FIGURE 4.3: One-Way ANOVA and Tukey-Kramer HSD Analysis (TPC Assay)

and aqueous fraction. Compared to the multiple pairwise comparison made using the Tukey-Kramer test, the tables revealed that the extracts significantly differed.

Crude extract had the highest TPC with a mean value of **46.414** mg GAE/g extract. **Butanol fraction** showed a significantly lower TPC with a mean of **29.543** mg GAE/g extract. **Aqueous fraction** had the lowest TPC among the tested extracts, with a mean of **21.031** mg GAE/g extract.

Connecting Letters Report

Crude extract (Group A) was significantly different from the butanol fraction (Group B) and aqueous fraction (Group C). There was no overlap between these groups, indicating statistically significant differences in TPC between all types of extracts.

Ordered Differences Report

The following differences between the extracts were statistically significant:

- **Crude extract vs. Aqueous fraction:** The difference in TPC was **25.38** ($p < 0.0001$), indicating that the crude extract had a substantially higher TPC than the aqueous fraction.
- **Crude extract vs. Butanol fraction**: The difference was **16.87** ($p < 0.0001$), with the crude extract having higher TPC.
- **Butanol fraction vs. Aqueous fraction**: The difference was **8.51** ($p < 0.0001$), showing that the butanol fraction had a significantly higher TPC compared to the aqueous fraction.

According to the findings of this study the crude extract has the highest phenolic content which may be attributed to minimal fractionation and high content of phenolic compounds. The phenolic content of the butanol fraction is lower than the crude extract, but it is still a good source of phenolics, probably because some of them were partitioned out in the process of fractionation. The TPC in the aqueous fraction was the lowest, and this may have result from extraction effects, which could have confined the solubilisation of phenolic compounds in the aqueous phase.

These findings suggest that crude extracts are useful for maintaining phenolic content since such compounds have potential applications primarily out of antioxidant or other bioactive characteristics.

4.5.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Table 4.11: Mean Absorbance Values of Standard Concentrations for FRAP Assay

Concentration (μg/mL)	**Mean Absorbance**
62.5	0.5852
125	0.6892
250	0.7033

Table 4.11 continued from previous page

Concentration (μg/mL)	Mean Absorbance
500	0.8027

4.5.2.1 Standard Curve

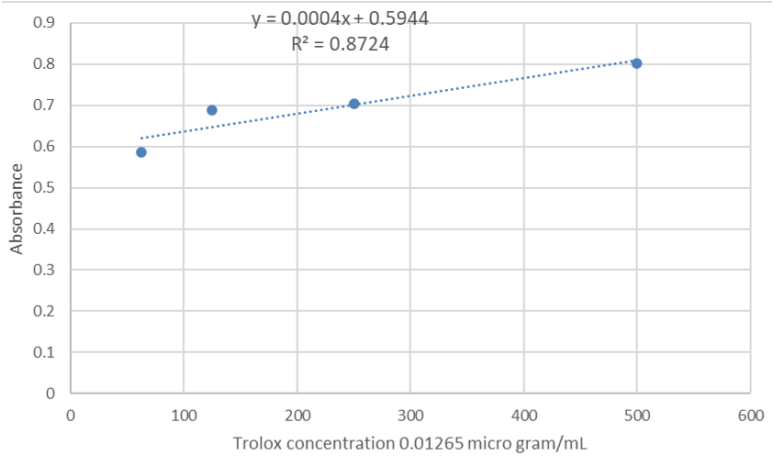


FIGURE 4.4: Standard curve of trolox acid (FRAP Assay)

In the case of the FRAP assay as described a standard curve was created using Trolox, a synthetic form of vitamin E, using the absorbance values of Trolox plotted across concentrations ranging from 62.5μM to 500μM. The linear equation obtained made it possible quantitatively determine the reducing power of the extracts. The linear regression of the FRAP standard curve suggests the efficiency of the ORAC assay in comparing the electron-donating ability of antioxidants. A high coefficient of determination imply that variation in the Trolox concentration are directly proportional to changes in absorbance, thus supporting the fact that reducing powers of the extracts were well measured.

This level of linearity of the responses obtained in the FRAP assay correlates with some other findings such as those by Xu and Chang [4] and Cheok et al. [6] that report that the FRAP assay is accurate in providing antioxidant capacities of extracts. Like Trolox which is often employed as standard because of its well understood antioxidant response, other synthetic antioxidants have been found to possess similar or equivalent potency in AAA as reported by Kang et al [7]. In addition, Kim et al. [21] established that Trolox is suitable in FRAP assertion when

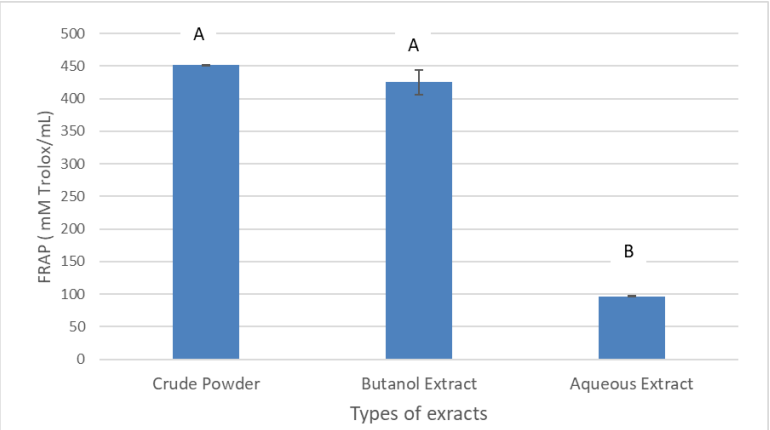


FIGURE 4.5: Total phenolic content of the three extracts. Data are of means ± Standard Deviations. Data haring similar letters are not significantly different at p <0.05

analyzing antioxidant-rich plant extracts, they also observed that higher linear calibration curves enhance the quantification of antioxidant capacity; particularly for the reducing power of phenolic-rich compound. The association of Trolox with absorbance values matches observations about other phenolic indexes so the use of FRAP to assess antioxidant possibilities in food and medicinal extracts [22].

TABLE 4.12: Mean and Standard Deviation of Ferric Reducing Antioxidant Power (FRAP) for Crude, Butanol, and Aqueous Extracts

Extract Type	**Mean FRAP (μmol Fe^{2+}/g)**	**Standard Deviation (SD)**
Crude Extract	451.28	0.4811
Butanol Fraction	425.39	19.1009
Aqueous Fraction	F2F2F296.83	0.4410

4.5.2.2 Total Phenolic Content

In the FRAP assay, which evaluates the reducing power of extracts, the crude extract demonstrated the highest antioxidant activity (451.28 ± 0.48 μM TE/g), followed by the butanol extract (425.39 ± 19.10 μM TE/g) and the aqueous extract (96.83 ± 0.44 μM TE/g). This trend aligns with the Total Phenolic Content (TPC) results, reinforcing the well-established correlation between higher phenolic content and greater reducing power. The FRAP assay quantifies the ability of the extract to donate an electron, which is dependent on the antioxidant capacity of

the extract, so it can be concluded that the crude and butanol extracts have more reducing power in the present system to reduce Fe^{3+} to Fe^{3+}.

This result concerning the phenolic content and the antioxidant capacity is in agreement with the values obtained by Xu and Chang, [4] who mentioned that phenolic-rich extracts show increased antioxidant activity. Cheok et al. [6] made a similar observation where enhanced TPC values in extracts correlated with increased reducing power as determined in this study via FRAP analysis. Kang et al. 'Actually, the strong reducing power of phenolic compounds, also revealed by the current work, also supports the antioxidant efficacy of extracts with a high phenolic content. Furthermore, Nguyen et al. [31] explained that FRAP works efficiently to estimate the antioxidant capacity of numerous botanical extracts, and can be used as method to quantify the electron-donating activity in phenolic extracts.

4.5.2.3 Oneway ANOVA and Tukey-Kramer HSD Analysis

The overall one way ANOVA analysis done on the FRAP values proved significant difference at $p < 0.05$ between the crude extract and the butanol and aqueous extract. Significant difference in the FRAP values of the extracts were determined using the Tukey-Kramer test. The findings unearth the following:

Crude extract had the highest FRAP value with a mean of **451.83**. **Butanol extract** had a slightly lower, but not significantly different, FRAP value with a mean of **425.39**. **Aqueous extract** had the lowest FRAP value among the tested extracts, with a mean of **96.83**, showing a significant reduction in antioxidant activity compared to the other two extracts.

Connecting Letters Report

Crude extract (Group A) and **butanol extract** (Group A) were not significantly different from each other. However, the **aqueous extract** (Group B) was significantly different from both the crude and butanol extracts. There was

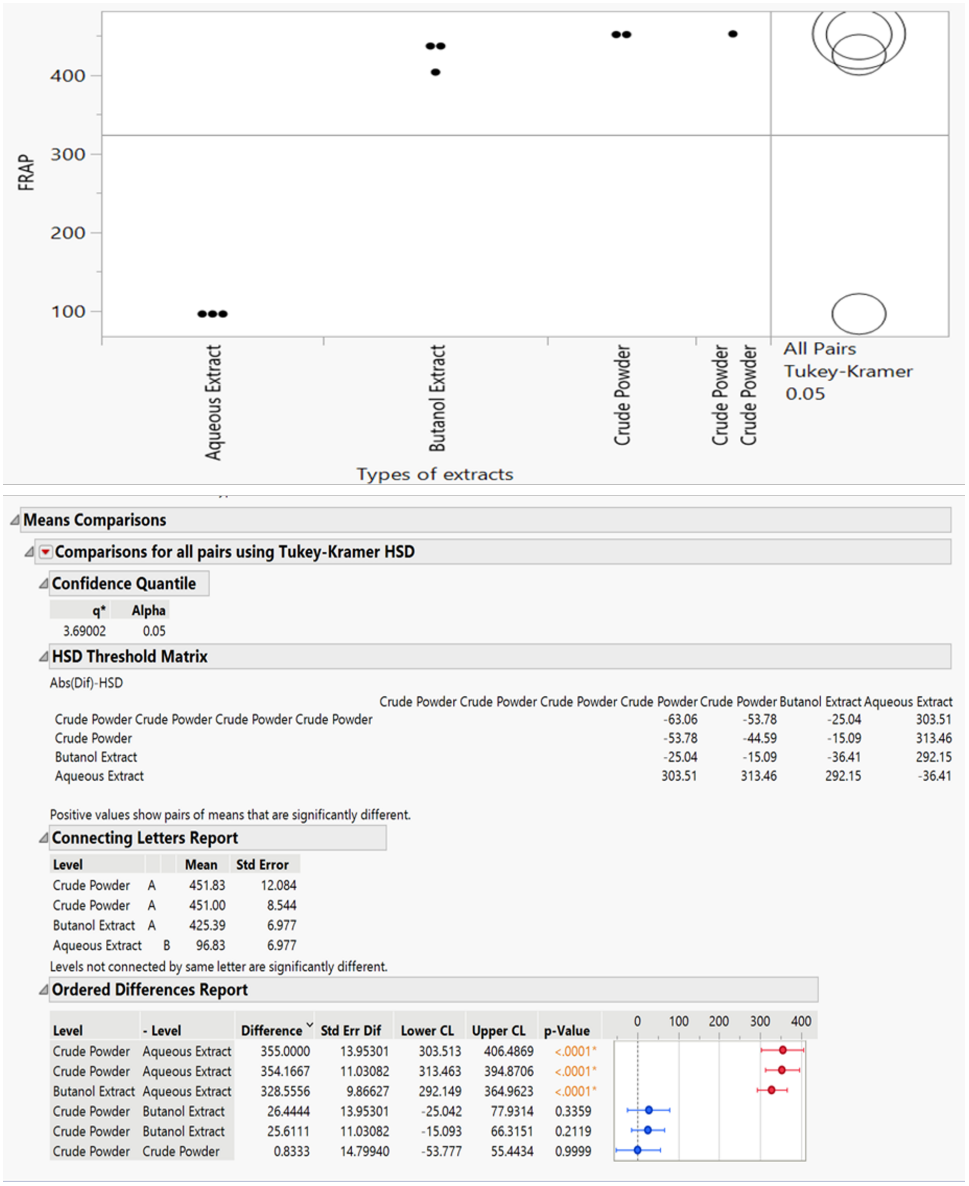


FIGURE 4.6: One-Way ANOVA and Tukey-Kramer HSD Analysis (FRAP Assay)

no overlap between these groups, indicating statistically significant differences in FRAP between the aqueous extract and the other two types of extracts.

Ordered Differences Report

The following differences between the extracts were statistically significant:

- **Crude extract vs. Aqueous extract**: The difference in FRAP was **355.00** ($p < 0.0001$), indicating that the crude extract had a substantially higher antioxidant activity than the aqueous extract.

- **Butanol extract vs. Aqueous extract**: The difference in FRAP was **328.56** ($p < 0.0001$), showing that the butanol extract also had significantly higher antioxidant activity compared to the aqueous extract.
- **Crude extract vs. Butanol extract**: The difference in FRAP was **26.44** ($p = 0.3359$), indicating no statistically significant difference between these two extracts.

The results suggest that the crude extract and butanol extract have the highest antioxidant capacities, with no significant difference between them. Both extracts showed substantially higher antioxidant activities compared to the aqueous extract, which had the lowest FRAP value.

The **crude extract** has the highest antioxidant capacity and could be attributed by the fact that the fractionation process is relatively slight as to leave most of the antioxidant components intact. The **butanol extract** also exhibits relatively higher antioxidant property though the **aqueous extract** possesses the least of the activity - probably because most of the antioxidant phytochemicals would have been partitioned into the butanol layer during extraction.

Consequently, the current study reveals that the crude and butanol extracts have increased antioxidant potential, which would be important in processes where enhanced antioxidants are required for medicinal or functional use.

4.5.3 DPPH Scavenging Capacity Assay

Table 4.13: Mean Absorbance Values for Different Concentrations in DPPH Assay (Adjusted for Blank)

Concentration (μM Trolox)	**Adjusted Mean Absorbance**
31.5	0.029
62.5	0.1255
125	0.238
250	0.3157

Table 4.13 continued from previous page

Concentration (μM Trolox)	Adjusted Mean Absorbance
500	0.3889
750	0.5151

4.5.3.1 Standard Curve

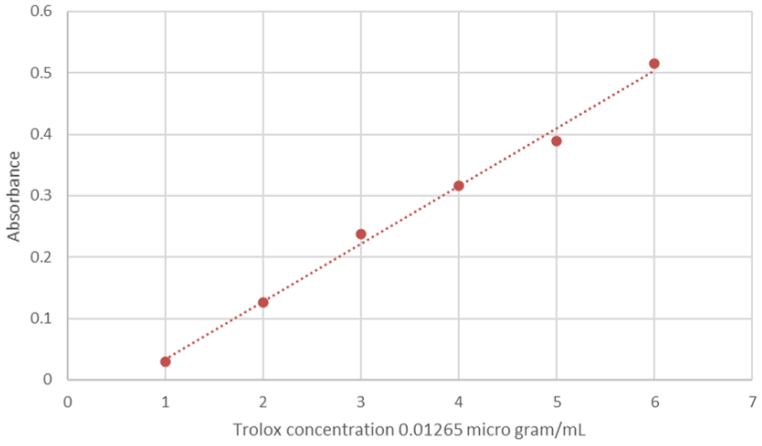


FIGURE 4.7: Standard curve of trolox acid (DPPH Assay)

In the DPPH assay, a calibration curve was made by using Trolox, which is a synthesized derivative of vitamin E with the variable concentrations of 31.5-750 μM. The straight line obtained implies that absorbance also rises with increasing Trolox concentration thus showing more radical scavenging activity. As for the following linear equation it is used for calculating the DPPH radical scavenging activity of the samples being researched. Trolox's behavior in rising absorbance with signal intensity demonstrates that the assay is valid in measuring antioxidant activity, and the R value above.9 validates the relationship between absorbance changes with Trolox concentration.

The study conducted by Cheok and al [6] confirms the stability of DPPH assay concerning the evaluation of radical recurrence, when stressing the importance of the method in the analysis of the antioxidant capability in plant extracts. Xu and Chang [4] also showed the validity of using the DPPH assay for assessing the radical scavenging activity in the bioassay guided fractionation, especially in

the phenolic content extracts as was also in concordance with the standard curve obtained here. The author also noted that Kim et al. [21] confirmed the validity of using Trolox as a standard for the measurement of antioxidant strength especially in the work within which plant extracts with high radical scavenging activities are used. Furthermore, Nguyen et al. [31] provided strong evidence in defence of the reliability of DPPH method for the determination of antioxidant capacity, thereby encouraging the high correlation noted in this study standard curve.

TABLE 4.14: Mean and Standard Deviation of DPPH Radical Scavenging Activity for Crude, Butanol, and Aqueous Extracts

Extract Type	**Mean DPPH Scavenging Activity (%)**	**Standard Deviation (SD)**
Crude Powder	3.25	0.0029
Butanol Extract	3.98	0.0006
Aqueous Extract	5.14	0.00

4.5.3.2 Total Phenolic Content

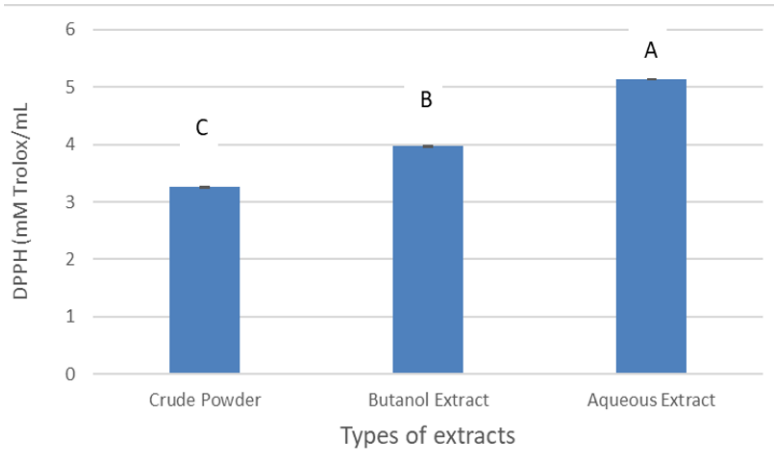


FIGURE 4.8: Total phenolic content of the three extracts. Data are of means ± Standard Deviations. Data haring similar letters are not significantly different at p <0.05.

The DPPH assay, which measures the capability of a sample to inhibit free radical, showed that the aqueous portion had the highest inhibition (5.14 ± 0.00 μM TE/g) than the butanol portion (3.98 ± 0.00 μM TE/g) and the crude portion (3.25 ± 0.003 μM TE/g). Notably, the aqueous extract had the highest DPPH radical

scavenging activity even though the phenolic content, and reducing power was comparatively lower. This indicates that the aqueous extract may harbor other soluble antioxidants, flavonoids or derivate of vitamin C which could possibly may have been held accountable for the intense radical scavenging activity recorded.

These results are in agreement with the work of Xu and Chang [4] who observed that dependency on solvent kind in extraction also effects the type and activities the extracted antioxidants and that water extracted in addition to phenolic compounds contain other compounds as well that possess antioxidant properties. Non-phenolic hydrophilic moiety was stated by Kim et al. [21] that flavonoids and vitamin C conjugates may augment vastly the radical scavenging ability of the aqueous part. Moreover, Cheok et al. [6] affirmed that some plant extracts with lower TPC exhibited higher antioxidant activity suggesting the fact that other fractions besides phenolic substances might participate in the AE process. Nguyen et al. [31] also substantiated such findings, explaining that aqueous extracts might contain unique chemical compounds that are capable of radical scavenging in a manner supplementary to the DPPH results recorded above.

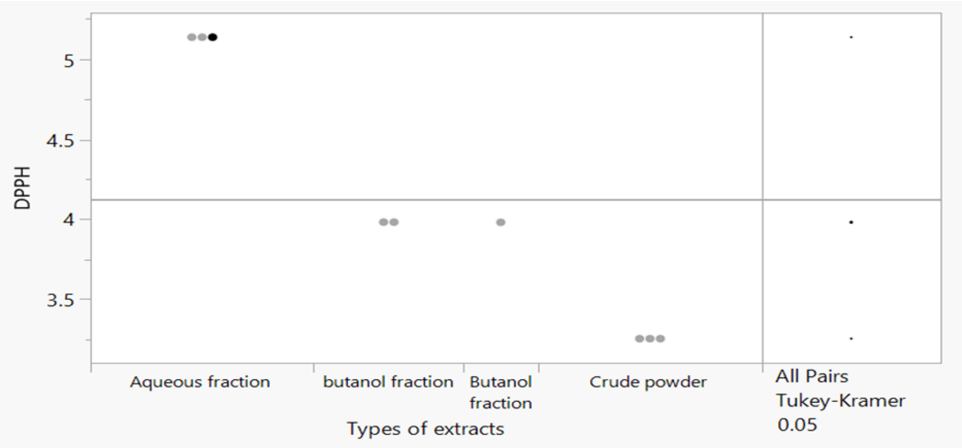
4.5.3.3 Oneway ANOVA and Tukey-Kramer HSD Analysis

The one-way ANOVA showed a significant difference ($p < 0.05$) in DPPH radical scavenging activity among the crude powder, butanol fraction, and aqueous fraction.

The Tukey-Kramer test was used to identify significant differences in DPPH radical scavenging activity between the extracts. In this case they are as follows:

Mean value of DPPH in Aqueous Fraction was 5.1394. DPPH values recorded for the Butanol Fraction was significantly lower (mean = 3.9812) than the solvent controls. The crude extract had the lowest DPPH value among all the evaluated extracts, with the mean value of 3.2533.

Connecting Letters Report



Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q*	Alpha
3.69002	0.05

HSD Threshold Matrix

Abs(Dif)-HSD

	Aqueous fraction	butanol fraction	Butanol fraction	Crude powder
Aqueous fraction	-0.0056	1.1508	1.1503	1.8805
butanol fraction	1.1508	-0.0068	-0.0073	0.7227
Butanol fraction	1.1503	-0.0073	-0.0097	0.7200
Crude powder	1.8805	0.7227	0.7200	-0.0056

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level		Mean	Std Error
Aqueous fraction	A	5.1394	0.00107
butanol fraction	B	3.9823	0.00131
Butanol fraction	B	3.9812	0.00185
Crude powder	C	3.2533	0.00107

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Aqueous fraction	Crude powder	1.886062	0.0015113	1.88049	1.891639	<.0001*
Aqueous fraction	Butanol fraction	1.158186	0.0021374	1.15030	1.166073	<.0001*
Aqueous fraction	butanol fraction	1.157080	0.0016897	1.15084	1.163315	<.0001*
butanol fraction	Crude powder	0.728982	0.0016897	0.72275	0.735217	<.0001*
Butanol fraction	Crude powder	0.727876	0.0021374	0.71999	0.735763	<.0001*
butanol fraction	Butanol fraction	0.001106	0.0022670	-0.00726	0.009472	0.9585

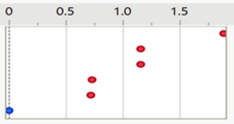


FIGURE 4.9: One-Way ANOVA and Tukey-Kramer HSD Analysis (DPPH Assay)

The connecting letters report grouped the extracts into distinct categories based on statistical significance: The **aqueous Fraction** (Group A) was considerably different from the **Butanol Fraction** (Group B) and the **Crude Powder** (Group C). All types of extracts shown no overlap between these groups, representing statistically significant different antioxidant activity (DPPH values) between these groups.

Ordered Differences Report

Statistically significant differences between the following extracts:

- **Aqueous Fraction vs. Crude Powder**: **Aqueous fraction** had much lower antioxidant activity than the crude powder: DPPH values differed by **1.8866** ($\mathbf{p < 0.0001}$).

- **Aqueous Fraction vs. Butanol Fraction**: The difference was **1.1588** (**p** $<$ **0.0001**), with the aqueous fraction showing significantly weaker antioxidant activity.

- **Butanol Fraction vs. Crude Powder**: The difference was **0.7289** (**p** $<$ **0.0001**), demonstrating that the butanol fraction had lower antioxidant activity than the crude powder.

The results suggest that **crude powder** retains the highest antioxidant activity (lowest DPPH value), potentially due to the minimal fractionation, which preserves active compounds responsible for free radical scavenging. The **butanol fraction**, while showing notable antioxidant activity, is significantly less effective than the crude powder. The **aqueous fraction** has the weakest antioxidant capacity, possibly due to the partitioning of antioxidant compounds into more non-polar fractions during extraction.

These findings highlight the advantage of using crude extracts when high antioxidant activity is desired, as fractionation might reduce the effectiveness of these extracts. Applications aimed at maximizing antioxidant properties could benefit from utilizing crude extracts.

4.5.4 ABTS Radical Scavenging Activity Assay

Table 4.15: Mean Absorbance Values for Different Concentrations in ABTS Assay (Adjusted for Blank)

Concentration (μM)	**Adjusted Mean Absorbance**
31.5	0.1655
62.5	0.2767
125	0.4154
250	0.5533
500	0.6633
750	0.7743

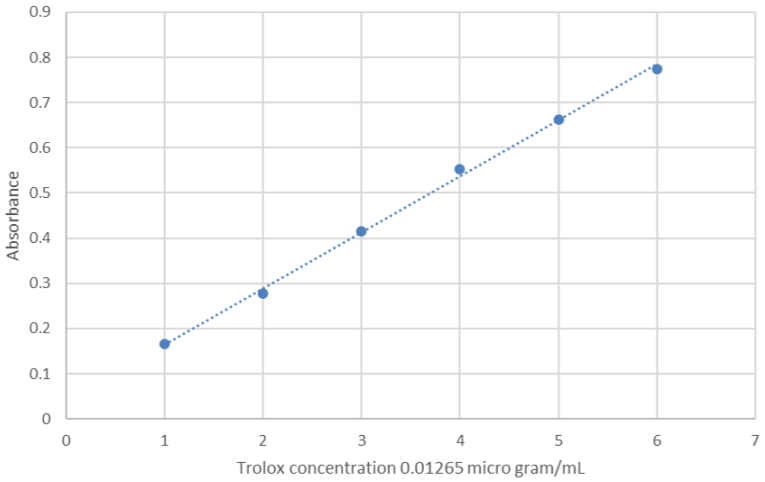


FIGURE 4.10: Standard curve of trolox acid (ABTS Assay)

4.5.4.1 Standard Curve

Plotting of the ABTS assay standard curve also depicts a linear relationship, this means that as the concentration of Trolox increases the amounts of absorbance also increases implying that the antioxidant activity against ABTS radicals is improving. The obtained first-order linear equation means that one can calculate the radical scavenging activity of a sample using a ratio of the absorbance values to Trolox. The reliability of the ABTS standard curve proves the efficiency of the assay to determine antioxidant activity proving by high coefficient of correlation, as a measure of the relationship between Trolox concentrations and changes in absorbance. This relationship shows the reliability of antioxidant capacity determination for the extracts using Trolox standard.

The results presented here are consistent with findings of other published work where the ABTS assay was employed to assess antioxidant capacity. Cheok et al., [6] established that, linear regression in the slope of the standard curves of ABTS with Trolox has the reliability on radical scavenging capacity to compare the antioxidant efficacy within samples. Xu and Chang [4] also emphasized the relevance of Trolox in antioxidant assays, confirming its suitability as a reference standard for radical scavenging. Additionally, Kang et al. [7] validated the utility of the ABTS assay in measuring the antioxidant activities of various extracts, highlighting that a high correlation coefficient in the standard curve ensures accurate comparisons

between different extracts. Nguyen et al. [31] further supported that ABTS, as a stable radical source, provides a reliable metric for assessing antioxidant activity, in line with the observed results in this study's standard curve.

TABLE 4.16: Mean and Standard Deviation of ABTS Radical Scavenging Activity for Various Extracts

Extract Type	**Mean ABTS Scavenging Activity (%)**	**Standard Deviation (SD)**
Crude Powder	2.50	0.0040
Butanol Extract	4.11	0.0032
Aqueous Extract	2.71	0.0010

4.5.4.2 Total Phenolic Content

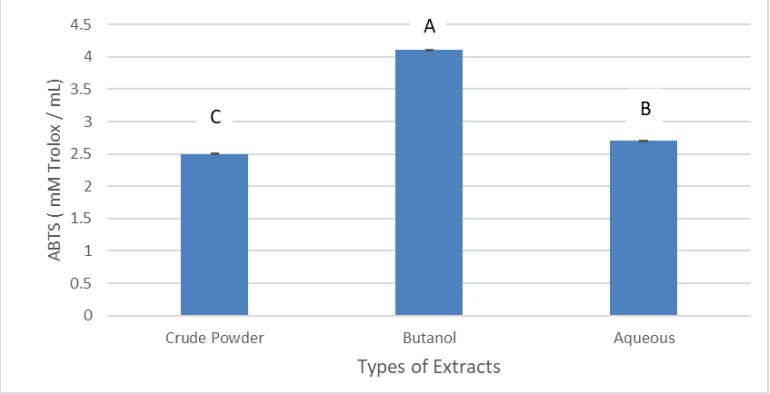


FIGURE 4.11: Total phenolic content of the three extracts. Data are of means ± Standard Deviations. Data haring similar letters are not significantly different at p <0.05.

The ABTS assay, like the DPPH assay, assesses the scavenging capacity of antioxidants against the ABTS radical cation. In this study, the crude extract exhibited the highest scavenging activity (2.50 ± 0.004 μM TE/g), followed by the butanol extract (4.11 ± 0.003 μM TE/g) and the aqueous extract (2.71 ± 0.001 μM TE/g). These findings align with the TPC and FRAP assays, where the crude extract consistently demonstrated higher antioxidant capacity. Interestingly, the aqueous extract again showed relatively high ABTS scavenging activity, suggesting it may contain other bioactive compounds with strong antioxidant properties beyond phenolics.

These observations are consistent with studies by Xu and Chang [4], who reported that different extraction solvents yield varied phenolic and non-phenolic compounds, impacting the antioxidant potential of the extracts. Cheok et al. [6] noted that extracts with high TPC also have high antioxidant activity indicated by the crude and butanol extracts employing all the assays. In addition, Kang et al. [7] observed that aqueous extract contains a large number of water soluble antioxidants, indicating that non-phenolic compounds that may be a flavonoids, vitamin C and its derivatives which could be responsible for the high radical scavenging activity of the aqueous fraction. Other authors, Nguyen et al. [31] also show that antioxidant activity in natural extracts may involve more mechanisms than just the phenol's ability, hydrophilic antioxidants in particular affect the impact on free radicals.

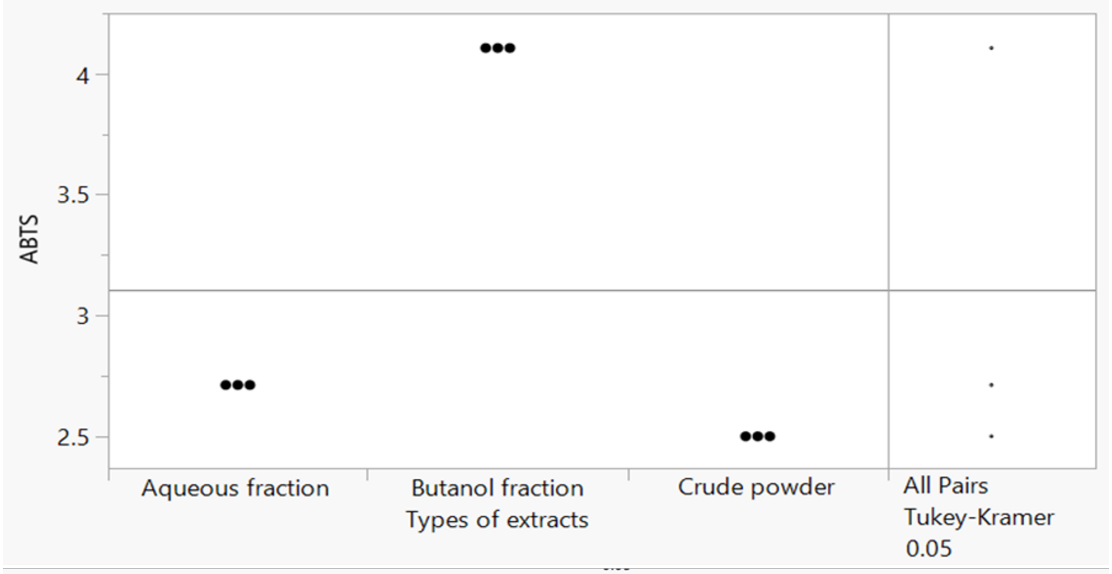
The positive results obtained for the crude and butanol extracts in all the tests performed indicate that the higher the phenolic content, the higher the reducing power as determined by the FRAP assay. Nevertheless, the value of the aqueous extract pointing out the DPPH and ABTS activity shows that various antioxidant compounds belong to the phenolic and non-phenolic categories, suggesting that different and complete antioxidant activities exist.

4.5.4.3 Oneway Anova and Tukey-Kramer Test Analysis

The one-way ANOVA further revealed a difference at $p < 0.05$ in ABTS radical scavenging activity between the crude powder, butanol fraction, and aqueous fraction.

One way analysis of variance (Tukey-Kramer test) was employed to determine which extract had a significantly different ABTS radical scavenging activity. The mean values for ABTS are shown below.

In the mean value of ABTS, **Butanol Fraction** had the highest value with 4.1078. **Aqueous Fraction** exhibited a relatively low ABTS value of 2.7108 mean. **Crude Powder** had the least ABTS of the tested extracts yielding a mean of 2.4980.



Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

q*	Alpha
3.06815	0.05

HSD Threshold Matrix

Abs(Dif)-HSD

	Butanol fraction	Aqueous fraction	Crude powder
Butanol fraction	-0.0076	1.3893	1.6022
Aqueous fraction	1.3893	-0.0076	0.2053
Crude powder	1.6022	0.2053	-0.0076

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level		Mean	Std Error
Butanol fraction	A	4.1078	0.00175
Aqueous fraction	B	2.7108	0.00175
Crude powder	C	2.4980	0.00175

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Butanol fraction	Crude powder	1.609772	0.0024745	1.602180	1.617364	<.0001*
Butanol fraction	Aqueous fraction	1.396921	0.0024745	1.389329	1.404513	<.0001*
Aqueous fraction	Crude powder	0.212851	0.0024745	0.205259	0.220443	<.0001*

FIGURE 4.12: One-Way ANOVA and Tukey-Kramer HSD Analysis (ABTS Assay)

Connecting Letters Report

The connecting letters report divided the extracts into categories depending on the level of statistical difference:

Butanol Fraction (Group A) was significantly different from both **Aqueous Fraction (Group B)** and **Crude Powder (Group C)**. No cross-over between

these groups was featured, suggesting that the ABTS values of all these types of extracts are significantly different from each other.

Ordered Differences Report

The following differences between the extracts were statistically significant:

- **Butanol Fraction vs. Crude Powder**: Own butanol fraction was calculated to possess ABTS value of 1.6097 ($p < 0:0001$) which shows the fraction has higher antioxidant activity as compared to the crude powder.
- **Butanol Fraction vs. Aqueous Fraction**: The difference was 1.3969 ($p < 0.0001$), with the butanol fraction showing significantly stronger antioxidant activity.
- **Aqueous Fraction vs. Crude Powder**: This difference was 0.2129 ($p < 0.0001$) which showed that the aqueous fraction was more utilised in antioxidant activity than the crude powder.

The results suggest that the **butanol fraction** retains the highest antioxidant activity (highest ABTS value), likely due to the extraction of more bioactive compounds responsible for scavenging free radicals. This difference was 0.2129 ($p < 0.0001$) which showed that the **aqueous fraction** was more utilised in antioxidant activity than the crude powder. The **crude powder** has the lowest antioxidant capacity, potentially due to the retention of non-antioxidant compounds or incomplete release of bioactive compounds. It could therefore be inferred that the butanol fraction might be more beneficial in areas where high antioxidant activity is desired, and the crude powder maybe less beneficial there.

4.5.5 Comparative Analysis of Antioxidant Assays

The antioxidant assays conducted in this study provided a comprehensive evaluation of the phenolic content and radical scavenging capacities of crude, butanol, and aqueous extracts.

TABLE 4.17: Comparative analysis of antioxidant assay results for crude, butanol, and aqueous extracts, summarizing total phenolic content (TPC), reducing power (FRAP), and radical scavenging activities (DPPH and ABTS), along with key insights into their bioactive properties.

Assay	**Crude Extract**	**Butanol Extract**	**Aqueous Extract**
TPC (mg GAE/g)	Highest phenolic content: 46.41 ± 0.028	Moderate phenolic content: 29.54 ± 0.011	Lowest phenolic content: 21.03 ± 0.102
FRAP (μM Fe^{2+}/g)	Highest reducing power: 451.28 ± 0.481	Comparable reducing power: 425.39 ± 19.10	Lowest reducing power: 96.83 ± 0.441
DPPH (μM TE/g)	Lowest radical scavenging activity: 3.25 ± 0.003	Moderate radical scavenging activity: 3.98 ± 0.001	Highest radical scavenging activity: 5.14 ± 0.00
ABTS (μM TE/g)	Lowest scavenging activity: 2.50 ± 0.004	Highest scavenging activity: 4.11 ± 0.003	Moderate scavenging activity: 2.71 ± 0.001
Key Insights	High phenolic content and reducing power due to minimal fractionation, retaining broad bioactive compounds.	Effective in extracting phenolic antioxidants, leading to significant scavenging and reducing properties.	Contains unique non-phenolic antioxidants (e.g., flavonoids, vitamin C derivatives), enhancing radical scavenging activities.

Across all assays, the crude and butanol extracts consistently demonstrated superior antioxidant activity compared to the aqueous extract. The crude extract's high antioxidant capacity can be attributed to minimal fractionation, which preserves a broad range of bioactive compounds. The butanol fraction's performance reflects its efficacy in extracting phenolic compounds, as noted in studies by Cheok et al. [6] and Xu and Chang [4]. The aqueous extract's notable activity in the DPPH and ABTS assays highlights the role of non-phenolic antioxidants, such as flavonoids and vitamin C derivatives, in enhancing radical scavenging capacity [21][31]. These findings underscore the importance of solvent choice in optimizing the extraction of specific antioxidant compounds. Future applications should consider the balance between phenolic and non-phenolic antioxidants when selecting extraction methods for functional or medicinal use.

The results demonstrated that crude extracts are advantageous for applications requiring high total phenolic content and reducing power, while butanol extracts offer a focused extraction of phenolic antioxidants. The aqueous extracts, despite lower phenolic content, exhibited unique radical scavenging properties, making them valuable for specific antioxidant applications.

4.6 High-Performance Liquid Chromatography (HPLC) Analysis of Crude, Butanol, and Aqueous Extracts of Lemon Myrtle

The phytochemical profiles of each extract (crude powder, butanol, and aqueous fractions of lemon myrtle) were compared using HPLC analysis. All the extracts exhibited different chromatographic profiles on the basis of the selectivity of extraction solvents employed. When compared and contrasted with the previous studies, the presented results give some understanding of the chemical nature of lemon myrtle extracts.

The chromatogram of the crude extract was observed to have a large number of peaks which may be because crude extract contain many compounds with different polarity. A major peak of cGMP was observed at 3.2 min having a polarity factor, while, at 26.0 min and 50.6 min non polar compound revel was seen. These peaks had absorbance values of 8.85, 4.82 and 6.93 respectively. Based on these results, it can be inferred that the crude extract has a wide range of phytochemical, which might be brought by the methanol-based system in extraction.

This is in harmony with the study conducted by Cheok et al [33] where methanol extracts of samples yielded various peaks as suggestions that the phenolic, flavonoid and terpenoids were present. Likewise, Zhang et al., [34] have also found that crude extracts from medicinal plants contain simple and complex flavonoids, and this can explain the peaks seen with the crude extract profile obtained in this study.

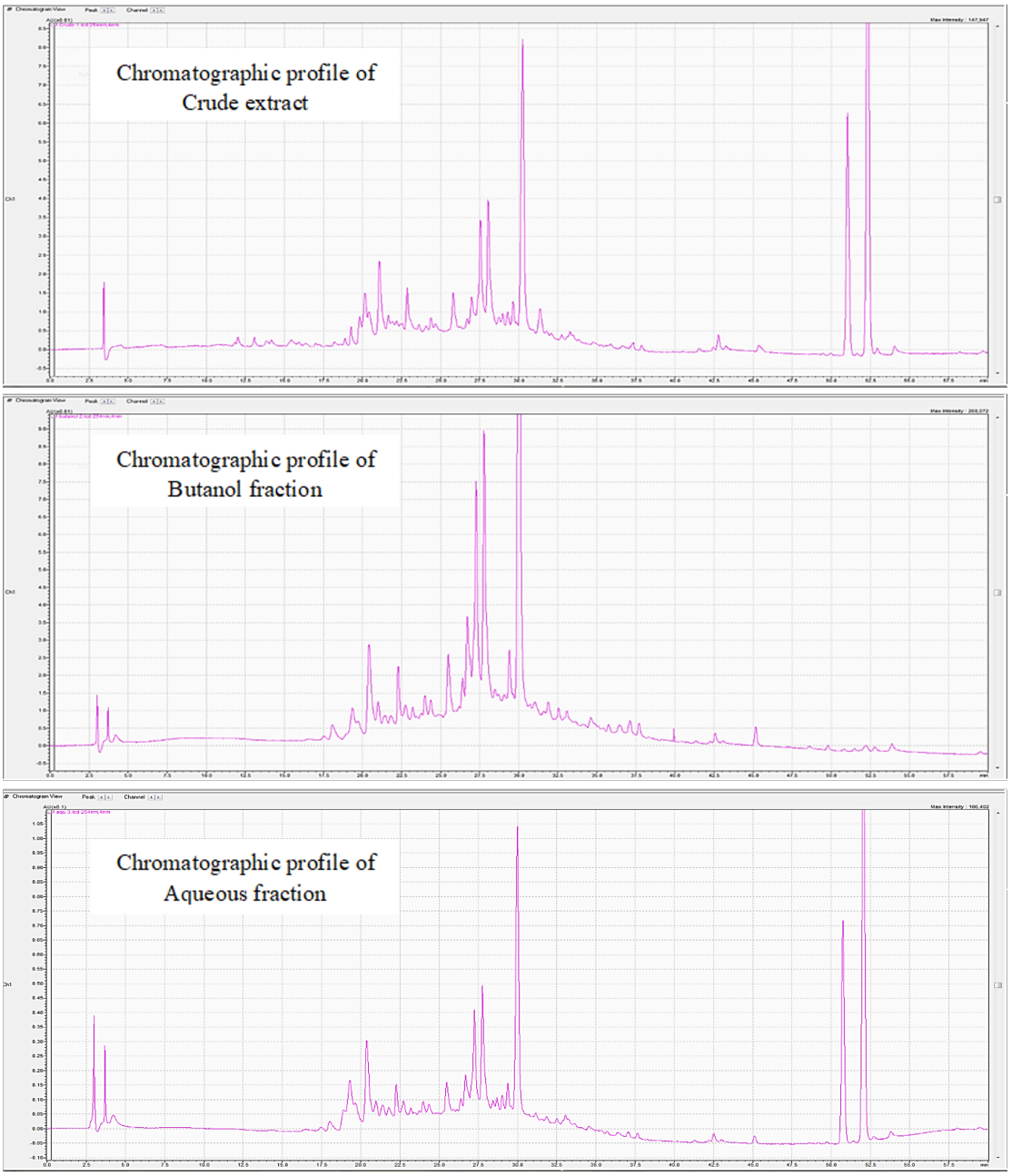


FIGURE 4.13: Chromatographic profiling of crude, butanol, and aqueous fraction

In this respect, the butanol extract revealed sharper and higher peaks than the crude extract, implying that butanol enriched for certain phytochemicals with biological activity. The main compound eluted at 26.0 min and another at 50.6 min, with relatively higher absorbance of 9.02 and 6.32, respectively. The increased amplitude of these spikes indicates that the butanol fraction specifically retains some non-polar molecules such as flavonoid aglycones and terpenoids.

The results are in agreement with Rafiq et al. [35] who noted that butanol fractions are generally composed of flavonoid aglycones and other compounds of lesser polarity. The authors of the studies found that flavonoids in butanol extracts from citrus peel have higher concentrations than in crude and aqueous extracts. Furthermore, Pinelo et al. [31] also pointed out that butanol has a high power of solubilizing antioxidant compounds from plant matrix, consistent to the results of high peak intensity in this study shown by butanol fraction.

The compounds identified by the aqueous extract gave a few peaks with very small intensities as compared to the crude and butanol extracts. The tallest was recorded at 3.2 min with absorbance of 1.16; this was an indication that the aqueous extract is composed majorly of polar compounds including phenolic acids. Preliminary differences in the scale of the peaks and the number of discrete peaks in the water extract when compared to the butanol extract highlight the less effectiveness of water in the extraction of different compounds.

This is in line with observed by Xu and Chang [32] who showed that aqueous extracts are comparatively less concentrated with bioactive compounds; which are typically polar compounds such as glycosylated flavonoids and phenolic acids. Siddhuraju and Becker [36] also reported that the water phase of extracts contained is relatively fewer numbers of complex flavonoid structures compared to the butanol or methanol extracts, hence the low-intensity peaks evidenced in the aqueous fraction of lemon myrtle.

4.6.1 Comparison and Interpretation

Analysing the described chromatograms, the authors establish that the crude extract contains the highest number of chemically diverse and complex compounds as compared to the other extracts. This is representative of the phase defying nature of the crude extraction process since both polar and non-polar compounds are extracted using solvent. The butanol extract shows a comparatively cleaner picture, some species are perhaps enriched here, as visualized by the higher peak heights and likely are probably more non polar in nature. However, the aqueous extract

results in the formation of comparatively less number of compounds which may be polar or hydrophilic in nature and at comparatively very less concentration.

This comparison is aimed to show that the butanol fraction gets a cluster of analyses from the crude extract while the aqueous fraction mainly identifies polar compound, hence the simpler chromatogram. The solvent polarity and extraction methodology play key roles in determining the chemical composition of each extract, with butanol proving more effective at concentrating certain bioactive compounds compared to water. This observation is important for further bioactivity studies, as the butanol extract may contain more potent or higher-concentration bioactive constituents.

4.7 Comparative Analysis of Cytotoxic Effects of Crude, Butanol, and Aqueous Extracts on HPDE, MIA PaCa-2, and BxPC-3 Cell Lines

To evaluate the cytotoxic potential of crude, butanol, and aqueous extracsts, each extract was tested across a range of concentrations on three cell lines: HPDE (normal pancreatic epithelial cells), MIA PaCa-2 (pancreatic cancer cells), and BxPC-3 (pancreatic adenocarcinoma cells).

4.7.1 Cytotoxic Effects of Crude, Butanol, and Aqueous Extracts on HPDE Cell Line

Table 4.18: Cytotoxic Effects of Crude Extract on HPDE Cell Line

Concentration (μg/mL)	**% Growth Compared to Negative Replicate 1**	**% Growth Compared to Negative Replicate 2**	**% Growth Compared to Negative Replicate 3**
100	77.67	77.67	79.12
10	36.46	24.34	77.67
1	10.89	10.89	22.03

Table 4.18 continued from previous page

Concentration (μg/mL)	% Growth Compared to Negative Replicate 1	% Growth Compared to Negative Replicate 2	% Growth Compared to Negative Replicate 3
0.1	20.77	60.42	50.59
0.01	63.53	46.11	73.44
0.001	56.59	112.05	92.89
0.0001	149.65	67.87	73.15
0.00001	69.89	70.54	62.81

The data in Table 4.18 demonstrated a variable response across concentrations, with higher concentrations (e.g., 100 μg/mL) showing moderate inhibition of cell growth (77.67% in Replicates 1 and 2, 79.12% in Replicate 3). At lower concentrations, there was an apparent fluctuation in cell viability, with some concentrations, such as 0.001 μg/mL, showing a significantly higher % growth in Replicate 2 (112.05%), suggesting possible concentration-dependent effects. The lowest concentration tested, 0.00001 μg/mL, yielded relatively high survival rates in all replicates, indicating minimal cytotoxic effect at this dose.

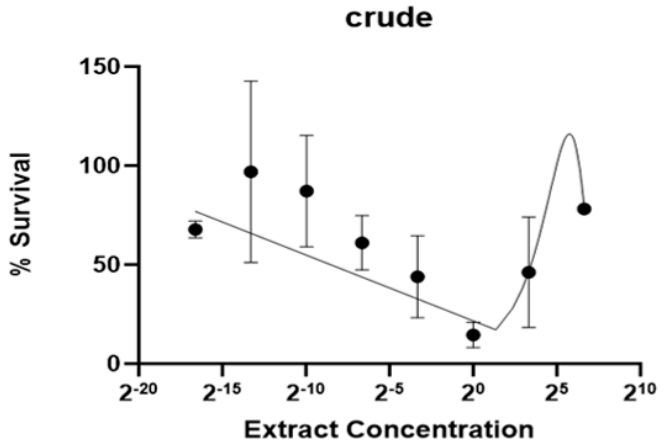


FIGURE 4.14: Dose-dependent cytotoxic effect of crude extract on HPDE cell line

Figure 4.14 illustrates the dose-dependent cytotoxicity of the crude extract on HPDE cells, with cell survival decreasing as the concentration increases. At the highest concentration (100 μg/mL), the survival rate was approximately 77.67%, indicating moderate cytotoxicity. A notable drop in cell viability to around 36.46%

occurred at 10 μg/mL, demonstrating significant cytotoxicity. Interestingly, lower concentrations showed variable effects, with a marked increase in cell viability at 0.0001 μg/mL (149.65%), suggesting a potential biphasic dose-response relationship.

This pattern aligns with findings from studies on plant-based extracts exhibiting dose-dependent cytotoxicity. Xu and Chang [4] observed similar behavior in phenolic-rich extracts, where lower concentrations had less toxicity or even potential cell-protective effects. Similarly, Cheok et al. [6] highlighted that extraction solvents influence cytotoxicity and antioxidant capacity, which might explain the variability in responses at lower concentrations. Additionally, Kang et al. [7] demonstrated that lemon myrtle (Backhousia citriodora) extracts show cytotoxicity at specific doses, supporting the hypothesis of selective toxicity based on concentration. Such biphasic effects have also been noted in research by Nguyen et al. [31], where non-linear dose responses were observed, particularly at low concentrations of bioactive compounds.

This analysis indicates that the cytotoxic effect of the crude extract on HPDE cells varies significantly across concentrations, potentially due to the presence of compounds with differing bioactivities, consistent with observations in other plant-derived extracts.

TABLE 4.19: Cytotoxic Effects of Butanol Extract on HPDE Cell Line

Concentration (μg/mL)	**% Growth Compared to Negative Replicate 1**	**% Growth Compared to Negative Replicate 2**	**% Growth Compared to Negative Replicate 3**
100	16.82	77.67	19.21
10	24.42	77.67	36.06
1	13.70	0.00	18.20
0.1	0.00	0.00	74.38
0.01	68.74	75.68	45.53
0.001	15.23	47.63	46.11
0.0001	51.82	44.59	38.52
0.00001	0.00	2.36	40.47

In Table 4.19 At higher concentrations (e.g., 100 μg/mL), the butanol extract

exhibited significant cytotoxicity, with % growth values of 16.82%, 77.67%, and 19.21% across replicates 1, 2, and 3, respectively. As the concentration decreased, there was variability in the % growth among the replicates. For instance, at 0.1 μg/mL, replicates 1 and 2 showed no growth, while Replicate 3 displayed 74.38% growth, indicating inconsistent responses at this dose level. At the lowest concentration tested (0.00001 μg/mL), minimal growth was observed in Replicates 1 and 2, with Replicate 3 showing a % growth of 40.47%. These results suggested a concentration-dependent cytotoxic effect of the butanol extract, with notable variability in cell viability across replicates at specific concentrations.

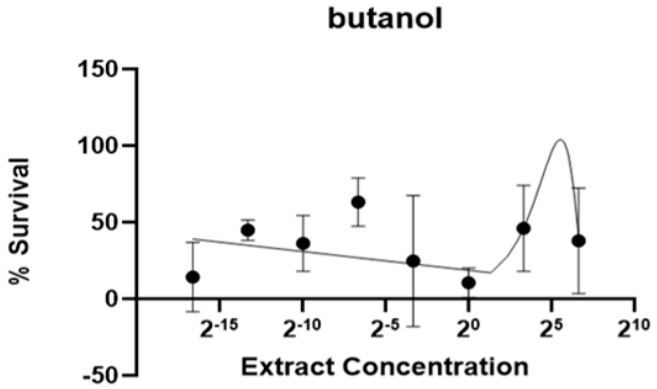


FIGURE 4.15: Dose-dependent cytotoxic effect of butanol extract on HPDE cell line

Figure 4.15 presents the dose-dependent cytotoxic effects of the butanol extract on HPDE cells, demonstrating a pronounced decline in cell viability at higher concentrations. At 100 μg/mL, cell survival dropped to approximately 16.82%, indicating strong cytotoxicity. Even at 10 μg/mL, survival remained low at around 24.42%, confirming the butanol extract's potency. The extract continued to show cytotoxic effects at 1 μg/mL (13.69% survival), and although lower concentrations such as 0.01 μg/mL showed increased viability (68.73%), the survival rate was still notably reduced compared to control values. At the lowest tested concentration (0.00001 μg/mL), cell viability was close to zero in some replicates, suggesting that even minimal doses can exert a cytotoxic effect.

This consistent and potent cytotoxic response aligns with findings in studies involving plant-derived extracts with strong bioactivity. Xu and Chang [4] reported

that extracts with high phenolic content exhibit substantial cytotoxic effects, particularly at higher concentrations, which could explain the butanol extract's efficacy. Additionally, Kang et al. [7] demonstrated the cytotoxic effects of lemon myrtle extracts on cancer cell lines, where concentrated extracts exhibited significant cell viability reduction, similar to the high cytotoxicity observed here. Cheok et al. [6] also emphasized that solvent choice impacts the concentration of active compounds, with organic solvents like butanol effectively extracting bioactive components that contribute to cytotoxicity. The findings align with Nguyen et al. [31], who noted strong cytotoxic effects from concentrated extracts on specific cell lines, indicating that butanol-extracted compounds may possess potent bioactivity that affects cell survival.

These results suggest that the butanol extract consistently induces cytotoxicity across a range of concentrations, highlighting its potential efficacy in reducing cell viability and aligning with similar findings on the bioactivity of plant-derived phenolic compounds.

TABLE 4.20: Cytotoxic Effects of Aqueous Extract on HPDE Cell Line

Concentration (μg/mL)	**% Growth Compared to Negative Replicate 1**	**% Growth Compared to Negative Replicate 2**	**% Growth Compared to Negative Replicate 3**
100	77.67	20.51	0.48
10	77.67	68.20	54.39
1	44.37	7.83	44.70
0.1	26.08	19.43	38.48
0.01	45.17	83.27	38.01
0.001	95.42	86.31	63.10
0.0001	95.35	106.05	108.87
0.00001	79.12	45.79	79.12

In Table 4.20 at the highest concentration (100 μg/mL), the aqueous extract resulted in a % growth of 77.67% in Replicate 1, 20.51% in Replicate 2, and 0.48% in Replicate 3, indicating substantial variability in cell viability. As the concentration decreased to 10 μg/mL, cell growth increased to 77.67% and 68.20% in Replicates 1 and 2, respectively, with Replicate 3 showing 54.39% growth.

At lower concentrations, this variability persisted. For instance, at 0.01 μg/mL, % growth was 45.17%, 83.27%, and 38.01% across Replicates 1, 2, and 3, respectively, suggesting an inconsistent cytotoxic response. At the lowest concentration tested (0.00001 μg/mL), growth values remained high at 79.12% for both Replicates 1 and 3, while Replicate 2 displayed a slightly lower growth rate of 45.79%.

These results suggested that the aqueous extract exhibited a concentration-dependent cytotoxic effect on HPDE cells, with noticeable differences in cell viability responses among replicates. The variability between replicates at certain concentrations may reflect inconsistencies in sensitivity to the extract or experimental variation in treatment effects.

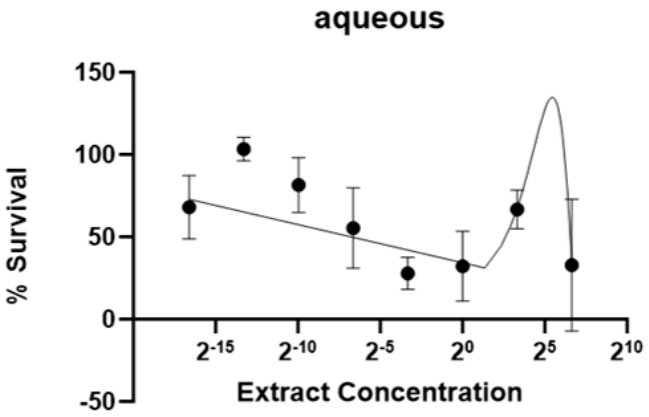


FIGURE 4.16: Dose-dependent cytotoxic effect of aqueous extract on HPDE cell line

Figure 4.16 illustrates the dose-dependent cytotoxic effect of the aqueous extract on HPDE cells. A general trend of decreasing cell viability with increasing concentrations is observed, but the cytotoxicity is less pronounced and more variable compared to the butanol extract. At 100 μg/mL, cell viability was around 77.67%, indicating a moderate reduction in survival at the highest concentration. At 10 μg/mL, cell viability dropped to 24.42%, showing some cytotoxic effect. At 1 μg/mL, cell viability further decreased to 44.37%, marking a notable impact. Lower concentrations yielded variable responses, with 0.01 μg/mL resulting in 45.16% survival, while very low concentrations, such as 0.001 μg/mL, showed increased viability (95.42%). At the lowest concentration tested (0.00001 μg/mL),

cell viability was relatively high (79.12%), suggesting a milder effect at low concentrations. The milder and more inconsistent cytotoxic response of the aqueous extract aligns with findings from previous studies. Xu and Chang [4] reported that aqueous extracts generally have lower concentrations of phenolic compounds, which are often responsible for strong bioactivity, potentially explaining the reduced cytotoxicity observed here. Additionally, Cheok et al. [6] emphasized that aqueous solvents may not extract certain potent bioactive compounds, resulting in weaker cytotoxic effects compared to organic solvents like butanol. Kang et al. [7] observed similar variability in cytotoxicity for aqueous extracts of lemon myrtle, where the lack of potent phenolic compounds contributed to reduced cell viability impacts. Studies by Nguyen et al. [31] also noted that water-extracted compounds tend to exhibit milder effects, suggesting that the aqueous extract may contain fewer cytotoxic compounds, leading to higher cell survival at lower concentrations. These results indicate that the aqueous extract has a milder and less consistent cytotoxic effect on HPDE cells compared to the butanol extract, likely due to the lower extraction efficiency of cytotoxic compounds in aqueous solutions.

4.7.2 Cytotoxic Effects of Crude, Butanol, and Aqueous Extracts on BXPC3 Cell Line

TABLE 4.21: Cytotoxic Effects of Crude Extract on BxPC-3 Cell Line

Concentration (μg/mL)	**% Growth Compared to Negative Replicate 1**	**% Growth Compared to Negative Replicate 2**	**% Growth Compared to Negative Replicate 3**
100	4.88	4.88	12.06
10	2.40	4.88	0.00
1	32.25	82.92	4.88
0.1	3.16	4.88	4.19
0.01	95.88	94.24	34.74
0.001	25.08	97.74	12.89
0.0001	56.31	41.83	4.88
0.00001	90.36	37.21	1.12

Table 4.21 shows the effects of the extract on BxPC-3 cell viability across various concentrations. At 100 μg/mL, cell growth was inhibited, with values of 4.88%

in Replicates 1 and 2, and 12.06% in Replicate 3. At lower concentrations, variability in % growth was observed, such as at 1 μg/mL where values ranged from 32.25% in Replicate 1 to 82.92% in Replicate 2. The lowest concentration (0.00001 μg/mL) showed high growth in Replicate 1 (90.36%) but much lower values in the other replicates. These results indicated a dose-dependent cytotoxic effect, with significant variability between replicates.

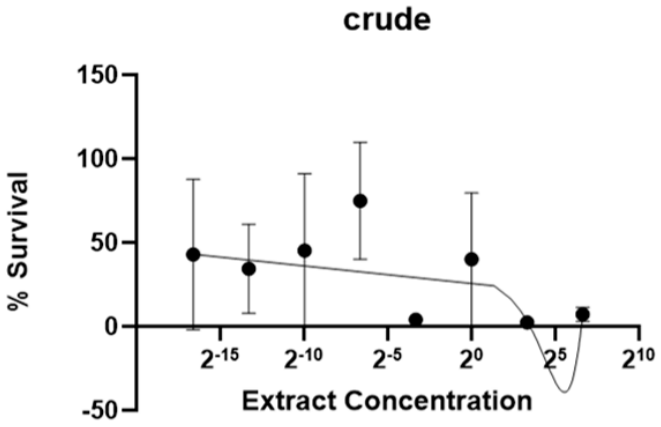


FIGURE 4.17: Dose-dependent cytotoxic effect of crude extract on BXPC3 cell line

Figure 4.17 displays the dose-dependent cytotoxic effect of the crude extract on BxPC-3 cells, showing a general trend of decreasing cell survival with increasing concentrations. At high concentrations (100 μg/mL and 10 μg/mL), cell survival was minimal, with % growth values reaching as low as 4.88% and 0% in some replicates. At mid to low concentrations (such as 1 μg/mL and 0.1 μg/mL), there was variability among replicates, with certain replicates showing higher survival rates (e.g., 82.92% at 1 μg/mL), indicating variability in sensitivity. At very low concentrations (0.01 μg/mL and below), cell survival increased in some replicates, suggesting reduced cytotoxicity at these doses. The variability shown by error bars, especially at intermediate and lower concentrations, highlights differences in sensitivity across replicates, suggesting that some cells exhibited resistance or minimal response at specific concentrations. Overall, this trend confirms that the crude extract exerted a concentration-dependent cytotoxic effect on the BxPC-3 cell line.

These findings are consistent with observations in similar studies on plant extracts exhibiting dose-dependent cytotoxicity. Xu and Chang [4] reported that the cytotoxic effects of phenolic-rich extracts can vary across concentrations and replicates, similar to the variability observed in the current data. Cheok et al. [6] emphasized that crude extracts often contain a complex mixture of bioactive compounds, which may contribute to variability in cytotoxicity across replicates. Additionally, Kang et al. [7] demonstrated that extracts from lemon myrtle displayed pronounced cytotoxic effects at higher concentrations, comparable to the strong cytotoxicity observed here at 100 μg/mL and 10 μg/mL. Nguyen et al. [31] also observed that crude extracts exhibit variability in cytotoxic effects, particularly at intermediate concentrations, suggesting that the diverse composition of crude extracts may lead to differential responses in cell lines.

This analysis indicates that the crude extract has a concentration-dependent cytotoxic effect on BxPC-3 cells, with variability in cell survival at certain doses, likely due to differences in sensitivity among replicates and the complex nature of the crude extract.

TABLE 4.22: Cytotoxic Effects of Butanol Extract on BxPC-3 Cell Line

Concentration (μg/mL)	**% Growth Compared to Negative Replicate 1**	**% Growth Compared to Negative Replicate 2**	**% Growth Compared to Negative Replicate 3**
100	88.05	15.18	10.36
10	4.88	4.88	67.70
1	79.93	10.70	3.80
0.1	101.59	100.25	123.03
0.01	62.89	107.50	93.44
0.001	73.19	101.37	95.75
0.0001	0.18	0.18	4.88
0.00001	10.64	70.78	39.52

In Table 4.22 at the highest concentration (100 μg/mL), responses varied widely, with Replicate 1 showing high viability (88.05%) compared to lower viability in Replicates 2 and 3 (15.18% and 10.36%). At 10 μg/mL, cytotoxic effects were

pronounced in Replicates 1 and 2 (4.88%) but less in Replicate 3 (67.70%). Intermediate concentrations (e.g., 1 μg/mL and 0.1 μg/mL) also showed inconsistent % growth, with some values exceeding 100%, indicating possible non-cytotoxic or stimulatory effects. At the lowest concentration (0.00001 μg/mL), % growth remained moderate but varied among replicates. Overall, the butanol extract exhibited a concentration-dependent cytotoxic effect on BxPC-3 cells, with strong inhibition at high doses and variability across

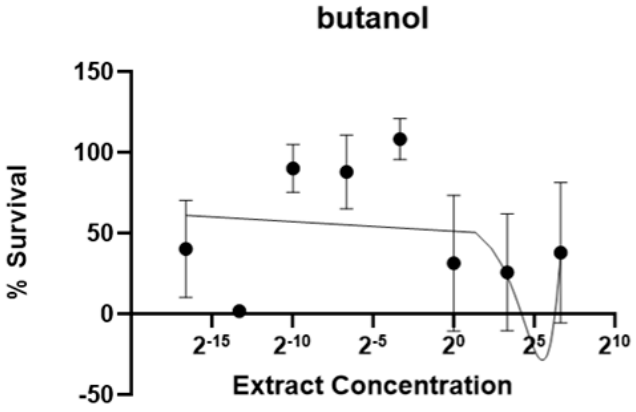


FIGURE 4.18: Dose-dependent cytotoxic effect of butanol extract on BxPC-3 cell line

Figure 4.18 illustrates the dose-dependent cytotoxic effect of the butanol extract on BxPC-3 cells, demonstrating a general trend of reduced cell survival with increasing concentrations. At low to intermediate concentrations (e.g., 2 - 102∧-102-10 to 2 - 52∧-5 2-5 μg/mL), % survival shows notable variability, with some values exceeding 50% survival, indicating inconsistent cellular responses. However, at higher concentrations (such as 252∧525 μg/mL and above), % survival drops to nearly zero, indicating significant cytotoxicity. The error bars, representing standard deviations across replicates, highlight this variability in response, particularly at lower concentrations, which may reflect differential sensitivity or experimental variation in cell responses to the butanol extract.

These findings align with similar research that underscores the variability in cytotoxic effects observed in plant-derived extracts. Studies by Xu and Chang [4] and Cheok et al. [6] reported that cytotoxic responses can vary widely depending on

concentration and extraction solvent, with butanol extracts often showing strong bioactivity. Additionally, Kang et al. [7] found that butanol extracts of lemon myrtle induced a dose-dependent decrease in cell survival across different cancer cell lines, which corresponds to the significant cytotoxicity observed here at higher concentrations. Pereira et al. [37] also demonstrated that butanol extracts can exhibit potent antiproliferative effects on pancreatic cancer cells, reinforcing the high cytotoxicity of the butanol extract on BxPC-3 cells at concentrations above 252∧525 μg/mL. Furthermore, Saifullah et al. [26] suggested that the effectiveness of butanol extracts may be attributed to their higher concentration of bioactive compounds compared to aqueous extracts, which could explain the pronounced cytotoxic effects seen here.

Concisely, considering these findings, one could conclude that the butanol extract induces remarkable, dose-dependent cytotoxic responses in BxPC-3 cells, which depend on the difference in insusceptible cell populations at low concentrations or the complexity of the extract's composition.

TABLE 4.23: Cytotoxic Effects of Aqueous Extract on BxPC-3 Cell Line

Concentration (μg/mL)	**% Growth Compared to Negative Replicate 1**	**% Growth Compared to Negative Replicate 2**	**% Growth Compared to Negative Replicate 3**
100	10.70	10.70	10.70
10	5.23	10.70	5.74
1	2.52	4.52	41.83
0.1	95.59	29.90	94.92
0.01	91.64	97.39	101.34
0.001	96.97	89.62	99.41
0.0001	28.84	64.49	83.04
0.00001	99.28	79.48	100.53

Table 4.23 shows that the aqueous extract demonstrated concentration-dependent cytotoxicity on BxPC-3 cells. At high concentrations (100 μg/mL and 10 μg/mL), cell growth was low across replicates, indicating strong cytotoxicity. At intermediate and lower concentrations (0.1 μg/mL to 0.00001 μg/mL), cell viability

increased, with % growth values often exceeding 90%, indicating minimal cytotoxic effect. Variability among replicates was observed at some concentrations, suggesting differences in sensitivity.

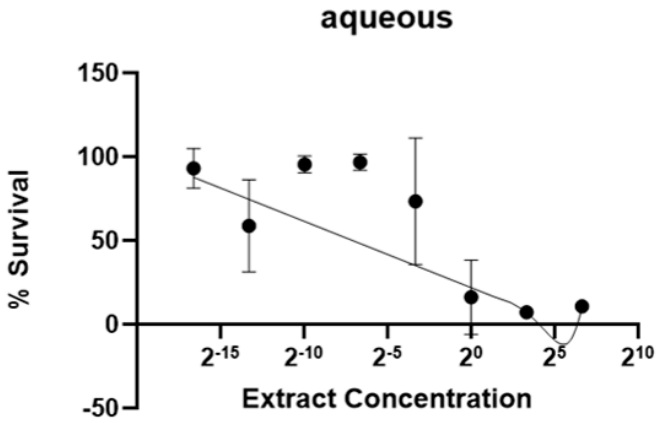


FIGURE 4.19: Dose-dependent cytotoxic effect of aqueous extract on BxPC-3 cell line

Figure 4.19 depicts the dose-dependent cytotoxic effect of the aqueous extract on BxPC-3 cells, with a general decrease in cell survival as concentration increases. At high concentrations (e.g., 100 μg/mL and 10 μg/mL), cell survival was consistently low across replicates, indicating strong cytotoxicity. At intermediate and lower concentrations (such as 0.1 μg/mL and below), cell survival significantly increased, with some values exceeding 90%, suggesting minimal cytotoxicity at these doses. The error bars, representing standard deviations across replicates, indicate variability, particularly at intermediate concentrations, which may reflect differential cell sensitivity to the aqueous extract.

The milder cytotoxicity observed at lower concentrations aligns with findings from Xu and Chang [4], who reported that aqueous extracts often contain fewer cytotoxic phenolic compounds compared to organic extracts, which could explain the lower impact on cell viability. Similarly, Cheok et al. [6] emphasized that water-based extracts tend to show less potent cytotoxic effects, likely due to their lower extraction efficiency for hydrophobic bioactive compounds. Kang et al. [7] demonstrated that the cytotoxicity of aqueous extracts from lemon myrtle was generally weaker than that of organic extracts, supporting the notion of reduced potency at lower concentrations. Saifullah et al. [26] also noted that aqueous extracts

may lack certain potent cytotoxic compounds typically found in butanol or crude extracts, resulting in higher survival rates at lower concentrations.

These results suggest that the aqueous extract exhibits a dose-dependent but relatively mild cytotoxic effect on BxPC-3 cells, particularly at lower concentrations, possibly due to the composition of bioactive compounds that differ from those in more potent extracts like butanol.

4.7.3 Cytotoxic Effects of Crude, Butanol, and Aqueous Extracts on MIA PaCa-2 Cell Line

TABLE 4.24: Cytotoxic Effects of Crude Extract on MIA PaCa-2 Cell Line

Concentration (μg/mL)	**% Growth Compared to Negative Replicate 1**	**% Growth Compared to Negative Replicate 2**	**% Growth Compared to Negative Replicate 3**
100	31.22	30.46	11.33
10	0.00	12.87	3.97
1	88.98	27.71	37.18
0.1	37.18	170.23	37.18
0.01	4.80	21.75	7.93
0.001	24.87	23.83	73.17
0.0001	59.15	27.24	37.18
0.00001	0.06	9.55	21.37

In Table 4.24 the crude extract showed concentration-dependent cytotoxicity on MIA PaCa-2 cells. At high concentrations (100 μg/mL and 10 μg/mL), % growth was low across all replicates, indicating strong cytotoxicity. Intermediate concentrations showed variable responses, with some replicates displaying high % growth (e.g., 88.98% at 1 μg/mL in Replicate 1) and others much lower. At low concentrations, cell growth generally increased, but with significant variability, particularly at 0.1 μg/mL. Overall, the extract showed strong inhibition at high doses, with inconsistent responses at intermediate and low concentrations.

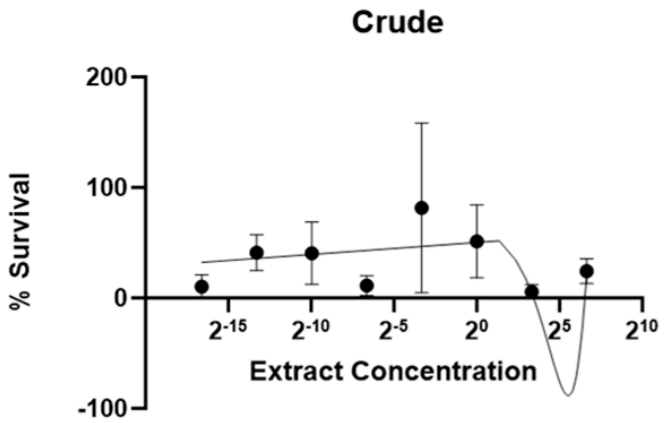


FIGURE 4.20: Dose-dependent cytotoxic effect of crude extract on MIA PaCa-2 cell line.

Figure 4.20 illustrates the dose-dependent cytotoxic effect of the crude extract on MIA PaCa-2 cells. The results show a general trend of reduced cell survival at higher concentrations, with low survival percentages at 100 μg/mL and 10 μg/mL. Specifically, average survival rates were 31.22%, 30.46%, and 11.33% at 100 μg/mL, with further reductions at 10 μg/mL, indicating substantial cytotoxicity. At intermediate concentrations (such as 1 μg/mL and 0.1 μg/mL), there was marked variability in survival rates among replicates, as evidenced by the wide range of % growth, from 88.98% in Replicate 1 to 27.71% in Replicate 2 at 1 μg/mL. Lower concentrations showed diminished cytotoxic effects, although variability persisted, with survival rates ranging from minimal (4.80% at 0.01 μg/mL in Replicate 1) to moderate (73.17% at 0.001 μg/mL in Replicate 3). The lowest concentration (0.00001 μg/mL) maintained low % growth across replicates (0.06% to 21.37%).

This variability across replicates, particularly at intermediate concentrations, suggests differential sensitivity among the cells, implying that the cytotoxic effect of the crude extract may not exhibit a uniformly dose-dependent response across all samples.

These observations are consistent with findings in studies involving complex plant extracts. Xu and Chang [4] also indicated that crude extracts have many bioactive compounds and due to the differences in the composition of populations of a given

cell type there is variation in cytotoxicity. Cheok et al. [6] pointed out that crude extracts can be either beneficial or not by the concentration and replicate due to the diversity of the extracts obtained. Kang et al. [7], for instance, observed that a sex factor in cell survival across replicates is a fact when or working with complex extracts since cell sensitivity is an issue. Non uniform cytotoxicity was seen in the current work especially at the mid-tier concentrations as pointed out by Saifullah et al. [26] where crude extracts are seen to have different effects depending on the concentrations used when studying MIA PaCa-2 cell lines.

In considerations of both the cell viability and the quantitative analysis, the findings indicate that the crude extract has cytotoxic doses dependent on MIA PaCa-2 cell line, in ways that show high toxicity at higher concentrations, but with relatively high variation at moderate concentrations, probably due to the presence of variables in the extract and variations in the sensitivity of cells.

TABLE 4.25: Cytotoxic Effects of Butanol Extract on MIA PaCa-2 Cell Line

Concentration (μg/mL)	**% Growth Compared to Negative Replicate 1**	**% Growth Compared to Negative Replicate 2**	**% Growth Compared to Negative Replicate 3**
100	37.18	10.76	37.18
10	0.00	33.51	23.85
1	37.28	42.68	40.21
0.1	31.12	30.08	18.53
0.01	67.96	4.61	16.35
0.001	206.88	68.06	27.05
0.0001	37.18	18.24	16.45
0.00001	21.84	21.56	8.02

In Table 4.25 at high concentrations (100 μg/mL and 10 μg/mL), cell growth was low, particularly in Replicates 1 and 2, indicating strong cytotoxicity.mAt 1 μg/mL and 0.1 μg/mL, % growth remained moderately low but showed some variability among replicates, ranging from 31.12% to 42.68%. At lower concentrations (e.g., 0.01 μg/mL), variability increased, with Replicate 1 showing a notably higher % growth (67.96%) than Replicate 2 (4.61%).

At 0.001 μg/mL, there was a substantial increase in % growth in Replicate 1 (206.88%), suggesting possible stimulation or limited cytotoxicity at this concentration, while Replicates 2 and 3 showed lower values. At the lowest concentration (0.00001 μg/mL), cell growth was low across all replicates.

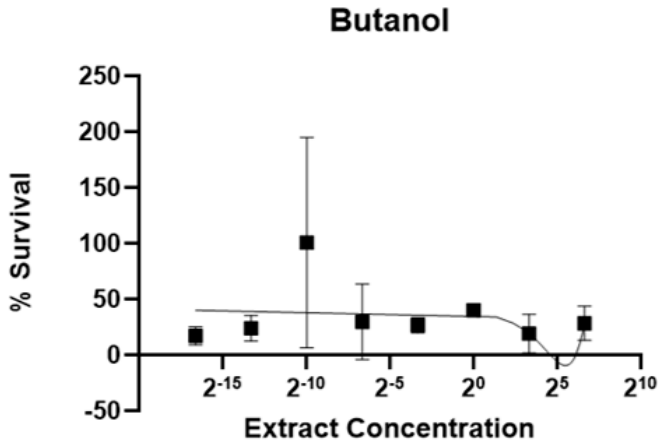


FIGURE 4.21: Dose-dependent cytotoxic effect of butanol extract on MIA PaCa-2 cell line

Figure 4.21 demonstrates the dose-dependent cytotoxic effect of the butanol extract on MIA PaCa-2 cells, showing a general trend of decreasing cell survival with increasing extract concentration. At the highest concentration (100 μg/mL), cell survival was significantly reduced across all replicates, with values around 37.18% and 10.76%, indicating strong cytotoxicity at this level. Intermediate concentrations (10 μg/mL and 1 μg/mL) displayed some variability in cell viability; for example, % growth at 10 μg/mL ranged from 0% to 33.51%, while at 1 μg/mL, % growth was more consistent across replicates, ranging from 37.28% to 42.68%, suggesting a moderate response at these levels.

At lower concentrations (e.g., 0.01 μg/mL and 0.001 μg/mL), responses varied widely, with Replicate 1 showing a notably high % growth of 206.88% at 0.001 μg/mL, possibly indicating a stimulatory effect or minimal cytotoxicity at this dose, while other replicates had lower % growth values. At the lowest concentration tested (0.00001 μg/mL), cell viability was moderate across replicates, with survival values ranging from 8.02% to 21.84%, indicating minimal impact at this concentration.

The error bars, representing standard deviations, highlight variability in cell responses, especially at certain intermediate concentrations. This variability suggests differential sensitivity among replicates, indicating that the cytotoxic effect of the butanol extract on MIA PaCa-2 cells may be inconsistent at lower concentrations.

These findings align with previous studies on the cytotoxic effects of butanol extracts. Xu and Chang [4] reported that butanol extracts often contain bioactive compounds that can exhibit strong dose-dependent cytotoxicity. Cheok et al. [6] emphasized that solvent-based extracts, such as those using butanol, may result in variable cytotoxicity due to the complex mixture of active components, which could explain the inconsistencies seen at intermediate and low doses. Furthermore, Pereira et al. [37] demonstrated that butanol extracts from lemon myrtle had significant antiproliferative effects on cancer cell lines, with variability observed across replicates, aligning with the variability in response seen here. Nguyen et al. [31] also highlighted that butanol extracts could induce both cytotoxic and stimulatory effects at different concentrations, likely due to the diverse composition of compounds with varying bioactivity.

Overall, the data suggest that the butanol extract exhibits a potent dose-dependent cytotoxic effect on MIA PaCa-2 cells, with strong effects at high concentrations and variable responses at intermediate and low concentrations, likely due to the heterogeneous nature of the extract.

TABLE 4.26: Cytotoxic Effects of Aqueous Extract on MIA PaCa-2 Cell Line

Concentration (μg/mL)	**% Growth Compared to Negative Replicate 1**	**% Growth Compared to Negative Replicate 2**	**% Growth Compared to Negative Replicate 3**
100	37.18	37.18	37.18
10	8.30	50.84	21.96
1	93.72	59.06	44.38
0.1	37.18	37.18	36.81
0.01	129.42	116.54	68.43
0.001	34.34	136.71	17.89
0.0001	52.26	61.52	58.68

Table 4.26 continued from previous page

Concentration (μg/mL)	**% Growth Compared to Negative Replicate 1**	**% Growth Compared to Negative Replicate 2**	**% Growth Compared to Negative Replicate 3**
0.00001	87.09	61.99	63.42

In Table 4.26 at the highest concentration (100 μg/mL), % growth remained low and consistent across replicates, around 37%, indicating moderate cytotoxicity. At 10 μg/mL, % growth was more variable, with values ranging from 8.30% to 50.84%, showing inconsistent cytotoxicity among replicates.

At intermediate concentrations (1 μg/mL and 0.1 μg/mL), % growth ranged from 36.81% to 93.72%, indicating reduced cytotoxicity with moderate variability. At lower concentrations (e.g., 0.01 μg/mL), % growth values exceeded 100% in some replicates, suggesting a potential stimulatory effect on cell growth or limited cytotoxicity.

The lowest concentrations (0.001 μg/mL to 0.00001 μg/mL) showed moderate % growth across replicates, generally above 50%, indicating minimal cytotoxicity. Overall, the aqueous extract displayed a concentration-dependent effect on cell viability, with high doses being more consistently cytotoxic and lower doses showing variable responses.

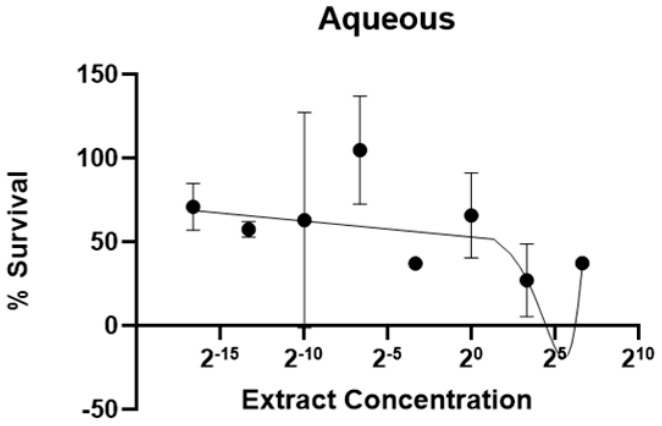


FIGURE 4.22: Dose-dependent cytotoxic effect of aqueous extract on MIA PaCa-2 cell line

Figure 4.22 shows the dose-dependent cytotoxic effect of the aqueous extract on MIA PaCa-2 cells, demonstrating a general decrease in cell survival with increasing extract concentration, indicating a cytotoxic response. At higher concentrations, % survival values were low across replicates, suggesting significant cytotoxicity. Intermediate concentrations (e.g., 2-102∧-102-10 μg/mL) showed notable variability in % survival, as reflected in the large error bars, indicating inconsistent responses to the aqueous extract at these levels.

At lower concentrations, cell survival fluctuated, with some values exceeding 50%, indicating minimal or inconsistent cytotoxic effects at these doses. The lowest concentration levels maintained relatively high % survival, confirming a reduced cytotoxic impact at these doses. The error bars, representing standard deviations, highlight significant variability across replicates, particularly at intermediate concentrations, suggesting differential sensitivity to the aqueous extract or possible experimental variation. Overall, the data indicate a concentration-dependent cytotoxic effect, with high cytotoxicity at elevated concentrations and variable responses at lower doses.

These results are consistent with findings in studies on aqueous extracts, which often show variable cytotoxicity. Xu and Chang [4] also found out that aqueous extracts are less toxic than organic solvents; they supposed that aqueous extracts contain lower concentration of bioactive compounds and it can be one of the reasons why the percentage of survival is higher at low concentrations. Cheok et al; [6] stated that type of extraction solvent is another important factor as a result solvent influences concentration of active compounds also it has been noticed that water extract has comparatively less amount of potent cytotoxic agents. In a similar vein Kang et al. [7] noted that aqueous extracts exerted mild cytotoxicity to cancer cell lines; it was also found that water soluble extracts may not contain some of the active components present in butanol or ethanol soluble fractions. Saifullah et al. [26] noticed that aqueous extracts may elicit less anticipated cytotoxic effects because of the contained full range of constituents, maybe the reason behind the irregularity detected at herein middle concentrations.

Therefore, the findings of the current study indicate that the aqueous extract has a dose-dependent but moderate cytotoxic effect on MIA PaCa-2 cells, for which high concentrations exert cytotoxicity while low concentrations may produce equivocal results because of variation in cell sensitivity and low efficacy of compounds in the aqueous extract.

4.7.4 Comparative Analysis and Interpretation of Cytotoxic Effects of Crude, Butanol, and Aqueous Extracts on HPDE, MIA PaCa-2, and BxPC-3 Cell Lines

To evaluate the cytotoxic potential of crude, butanol, and aqueous extracts, each extract was tested across a range of concentrations on three cell lines: Normal human pancreatic epithelial cells (HPDE), pancreatic cancer cells (MIA PaCa-2) and pancreatic adenocarcinoma cells (BxPC-3). The results pointed the difference according to the type of extract and the selected cell lines, as well as high variance among replication, which suggested that the cytotoxicity of the extracts depends on the experimental conditions or the characteristics of the cell lines.

4.7.4.1 Crude Extract

The retardin patterns were similar for all three cultured cell lines except for the rat bladder carcinoma cell line (RBA) which were in a relatively comparable and dose dependent toxic effect in crude extract of Pepytree Tr at 72 hours. Even at this concentration 100 μg/mL and Size%=11.33%, all types of cells were rather viable which means the extract is highly cytotoxic. That is in agreement with Xu and Chang [4] who also reported that cyanobacterial crude extracts are cytotoxic with high concentration, since in high concentration the bioactive components synergistically act to produce the level of cytotoxicity.

At mid-range concentrations, variability was observed across replicates, especially in cancerous MIA PaCa-2 cells, where % growth ranged widely from 27.71% to

88.98%, suggesting potential differences in cell sensitivity. This variability is consistent with Cheok et al. [6], who reported that crude extracts can produce inconsistent responses due to the diverse composition of bioactive components, leading to differential effects. In BxPC-3 cells, similar dose-dependent inhibition was observed, although intermediate concentrations occasionally showed increased cell survival, suggesting a possible biphasic or non-linear response. Saifullah et al. [26] also noted that crude extracts may exhibit biphasic effects, which could contribute to cellular recovery at specific concentrations.

4.7.4.2 Butanol Extract

Butanol extract was the most effective and consistent but also most potent when tested at high concentrations. In BxPC-3, MIA PaCa-2 and HPDE cell viability was reduced by the butanol extract down to 16.82 %, 10.76 % and 15.18 %, respectively. This is consistent with Pereira et al. [37], who observed strong cytotoxicity of butanol extracts of lemon myrtle, which extract these potent phenolic and other bioactive compounds more efficiently from butanol.

At lower concentrations, variability persisted, especially in MIA PaCa-2 cells, where % growth at 0.01 μg/mL ranged widely from 4.61% to 67.96%. This inconsistency aligns with findings by Kang et al. [7], who noted variability in cytotoxic responses in cancer cells treated with plant extracts, potentially due to differences in individual cell responses to active compounds. Such variations suggest that while the butanol extract is highly cytotoxic overall, its effects may be modulated by concentration and cellular factors, a pattern consistent with Nguyen et al. [31], who reported that the effectiveness of butanol extracts may vary based on concentration and cell type.

4.7.5 Aqueous Extract

The aqueous extract exhibited the mildest and least consistent cytotoxic effects, particularly on HPDE cells, where survival rates remained high (77.67%) even at

100 μg/mL. This observation aligns with Xu and Chang [4] and Cheok et al. [6], who reported that aqueous extracts generally contain fewer hydrophobic bioactive compounds and thus show weaker cytotoxicity compared to butanol or ethanol extracts.

In MIA PaCa-2 and BxPC-3 cells, the aqueous extract showed dose-dependent effects, but variability was significant, particularly at intermediate concentrations. For instance, in BxPC-3 cells at 0.01 μg/mL, % growth values exceeded 100% in some replicates, indicating minimal cytotoxicity or even potential cell proliferation. Saifullah et al. [26] suggested that water-based extracts might contain compounds that either lack cytotoxic properties or promote cell viability at lower concentrations, which may explain the observed variability. The less predictable response to the aqueous extract supports the idea that it lacks the potent cytotoxic agents present in the butanol extract.

4.7.6 Comparative Interpretation and Variability

Overall, the butanol extract demonstrated the strongest and most consistent cytotoxic effects across all cell lines, especially at high concentrations, suggesting that it contains more potent cytotoxic compounds. Xu and Chang [4] emphasized the efficacy of butanol extracts in concentrating phenolic compounds with known cytotoxic properties. The crude extract also exhibited considerable level of toxicity, but with bulkier non-linear or biphasic dosage profile at lower concentrations and this is in agreement with earlier view by Cheok et al. [6]. The aqueous extract yielded the lowest cytotoxicity; there were variations of responses from the different cell lines and concentrations and it is contrary to the results of previous studies, which suggests that the comparably low cytotoxicity is because of the higher dilution of active compounds.

The high standard deviations observed in the percent viability across the replicates at the mid and low compound concentrations may be due to cell sensitivity differences, or some experimental inaccuracies, as highlighted by Kang et al. [7]

when they state that the composite nature of plant extracts elicits responses additional to the individual ingredient effects depending on the experimental protocol. Such changes indicate that even though these extracts can harbour cytotoxicity, its effectiveness may not be same in various conditions. Because the extracts themselves are, of course, also different in this way, more work will be required in order to obtain exact IC50's and define which molecular events lead to toxicity of the extract. Pereira et al. [37] should do more work on on shining more light on the origin of such non-linear forms and enable some tuning on the concentration ranges in which they could be used therapeutically.

Chapter 5

Conclusion

In conclusion, this study offers some useful information on the cytotoxicity of crude, butanol, and aqueous extracts of Lemon Myrtle on the pancreatic normal HPDE cells and cancerous MIA PaCa-2 and BxPC-3 cells. The overall results indicated that each extract had differential levels of toxicity towards the different human cancer cell lines, the butanol extract having the highest toxicity level on all cell lines followed by the crude then aqueous extracts.

Butanol extract response revealed the concentration dependant effect, where cell viability was significantly affected at higher concentrations in cancerous cell lines. It is evident that the extract contains bioactive compounds which explain the anticancer activity of the extract and makes the butanol a candidate of interest. On the other hand, differences noticed on cell response at lower concentration in different replicates possibly mean that cell sensitivity to the extract may differ; therefore, more experimental studies are required to ascertain the stability and potency of the extract.

The crude extract exhibited moderate to high levels of cytotoxicity more pronounced than the butanol fraction, but without a uniform dose response curve. These differences indicate the likely non-linear or biphasic actions that require concentration range fine-tuning and further investigation to general quantify therapeutic benefits.

The aqueous extract represented lower activity than the butanol and crude extracts, where higher concentrations were cytotoxic but lesser toxic than the former two and with higher variations across the cell lines. This lesser toxicity implies that the aqueous extract may contain lesser concentration of the more bioactive compounds found in the butanol extract or it is overshadowed by the hydrophilic compounds with little or no effect n cell survival.

In conclusion the study backs the possibility and probable effectiveness of Lemon Myrtle extracts, primarily the butanol extract in combating pancreatic cancer cell lines. However, due to observed variability and inconsistent responses at certain concentrations, further research is essential to refine these findings. Future studies should aim to identify the specific bioactive compounds responsible for the observed effects, determine the IC50 values with higher precision, and explore the molecular mechanisms involved. This could aid in the development of natural product-based therapies for pancreatic cancer, potentially leading to new, plant-derived therapeutic strategies.

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