IN VITRO ANTIPROLIFERATIVE EFFECTS OF CRUDE EXTRACTS OF *CARICA PAPAYA LINN* (CARICACEAE FAMILY) BLACK SEEDS AGAINST PROSTATE CANCER CELL LINES

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A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology

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ABSTRACT

Black seeds from papaya plants are utilized as traditional medicine in African and Asian cultures to improve the functioning of the male reproductive system and management of prostate cancer. This study analyzed the phytochemical composition, cytotoxicity and antiproliferative activity of *C. papaya* black seeds against the Prostate cancer cells and Vero cells. The phytochemical screening was performed by means of standard procedures. Methyl tetrazolium bromide (MTT) cell viability assay was employed in the evaluation of the cytotoxicity and antiproliferative activity of papaya crude extracts in the selected cell lines. Glycosides, alkaloids, terpenoids tannins, flavonoids and saponins were found in papaya seeds' crude extracts. The crude extracts were not toxic to Vero cells. All papaya seeds' extracts had antiproliferative activity towards prostate cancer cells. Ethyl acetate extract was found with higher antiproliferative activity, with inhibitory concentration (IC_{50}) of 3.64 µg/mL. Further scientific studies focusing on the isolation and characterization of active phytocompounds from crude extracts of papaya seeds are needed.

DECLARATION

I, Benson Mcheza Kateihwa do hereby declare to the Senate of the Nelson Mandela African Institution of Science and Technology that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institutions.

Benson Mcheza Kateihwa

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CERTIFICATION

The undersigned certify that they have read the dissertation title "*In vitro antiproliferative effects of Crude Extracts of Carica papaya black seeds against prostate cancer cell lines*" and it is recommended for examination in the fulfillment of the requirements for the degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology (NM-AIST).

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DEDICATION

This work is dedicated to my beloved family for their patience and tolerance for the entire duration of this master’s program.

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LIST OF SYMBOLS AND ABBREVIATIONS

^{0}C	Temperature in Celsius
Ab	Value of Absorbance for a Blank
Ac	Value of Absorbance for a Negative Control
ANOVA	Analysis of Variance
AR	Androgen Receptor
At	Absorbance Value of a Test Compound
CC_{50}	Concentration that Reduced the Normal Cell Viability by 50 Percent
COVID	Coronavirus Disease
DMEM	Dulbecco Modified Eagle Medium
FBS	Fetal Bovine Serum (FBS)
IC_{50}	Concentration that Reduced the Cancer Cell Viability by 50 Percent
KEMRI	Kenya Medical Research Institute
MS	Metabolic Syndrome
MTT	Methyl Tetrazolium Bromide
NM-AIST	Nelson Mandela African Institution of Science and Technology
S1	Aqueous Extract
S2	Ethyl Acetate Extract
S3	N-hexane Extract
S4	Ethanol Extract
S5	Methanol Extract
SEM	Mean ± Standard Error of Mean
Std	Standard Drug
TPRI	Tanzania Pesticide Research Institute
WHO	World Health Organization
µL	Micro Litre

CHAPTER ONE

INTRODUCTION

1.1 Background of the Problem

Prostatic carcinoma is a cancerous disease of the man's reproductive system. It is localized in prostate gland, and can be life-threatening when spreads to significant parts of the body such as bones and lymph nodes (Alotaibi *et al.*, 2017). It is the second most frequent cancer in males after lung cancer. Globally, bout 1.3 million cases and 358 989 deaths (3.8% of all men worldwide) due to prostate cancer were reported by World Health Organization (WHO) in the year 2018. It is anticipated that by 2035, the cancer incidences will rise to 24 million and in that case, the number of deaths from cancer will increase in the future (Bray *et al.*, 2018).

Prostate cancer is the most common cancer in Tanzania contributing 22.9% of all cancers in men (Olson *et al.*, 2020). It is the most frequent diagnosed cancer among men and the mortality rate due to prostate carcinoma increases from the age of 55 years and above (Lyimo *et al.*, 2020).

Some of the major contributing factors to prostate cancer include; age, race and genetics. Men who are 60 years old and above are prone to prostate cancer while males below 40 years are less prone to the same disease. People of African-American origin have a substantially higher probability of suffering from prostate carcinoma as compared to whites. It is reported that the fourth most frequent cause of death in African-American males is prostate cancer; about 19 percent of black men (1 in 5) are discovered with the cancer of the prostate and 5% of black men diagnosed with the same disease lose their life from it. A male who has a family member with prostate cancer is more likely to get it in comparison to person whose family members are free from prostate carcinoma (Alotaibi *et al.*, 2017). Moreover, a male who acquired the defective breast cancer gene from his parents is more presumably to develop the incurable prostatic carcinoma (Drudge-coates *et al*., 2017). Obesity and sex hormones also contribute to growth of prostate tumors. Reduced levels of testosterone are associated with metabolic syndrome, benign prostatic hyperplasia and obesity and may results into a prostate cancer (Alotaibi *et al.*, 2017).

Carica papaya L. (Caricaceae family) plant was selected in this study due to the reported phytochemical composition including saponins, terpenoids, carbohydrates, alkaloids, glycosides and flavonoids which attribute to anticancer effect (Ek *et al*., 2018). Furthermore, the plant species exhibits a combination of alkaline with potassium carbonate or borax; which produces potential results in treatment of cutaneous tubercles warts, eczema, sinuses corns and other skin hardness and also administered into tumors of indolent glandular to enhance their absorption (Aravind *et al.,* 2013). Papaya green fruits are useful in treatment of hypertension, constipation, dyspepsia, general debility, amenorrhea, stimulate reproductive organs and expel worms (Aravind *et al.*, 2013). Anticancer activities of various parts of papaya plant have been demonstrated to induce growth inhibition of liver cancer cells in some vitro laboratory experiments (Alotaibi *et al*., 2017).

Thus, several studies on medicinal properties of various parts of papaya plant (fruits, shoots, leaves, rinds, seeds, roots or latex) have been conducted (Otsuki *et al.,* 2010). Previous phytochemical investigations in *C. papaya* seeds in Tamil Nadu, India resulted in the isolation of carbohydrates, proteins, saponins, steroids, glycosides, amino acids and tannins, flavonoids and alkaloids (Ek *et al*., 2018).

The study of biochemistry found that 18.75 mg, 7.28 mg and 16.87 mg for carbohydrates, proteins and starch were contained in papaya, respectively (Ek *et al*., 2018). Papaya seeds' extract has excellent therapeutic and nutritional qualities that can be used to treat many diseases such as ringworm, psoriasis, cirrhosis of the liver and body maintenance (Anitha *et al*., 2018). Papaya phytochemical compounds support cardiovascular system, protect against strokes, heart attacks and help in preventing cancer of the colon (Ek *et al.,* 2018).

Papaya seeds have also been used in some Asian culture as a traditional medicine in male reproductive system. Papaya seeds have also been reported to impair the development of prostate cancer (Alotaibi *et al.,* 2017).

Moreover, papaya leaf extract is known to mediate type 1 T-helper (Th1) cell changes in the human immune system and provides the means to treat and prevent prostate cancer, allergic reactions and can further be applied as the immunoadjuvant in vaccine therapy (Otsuki *et al*., 2010).

The present study aimed to assess the cytotoxicity and antiproliferative activity of crude extracts of *Carica papaya* black seeds. To evaluate the potential effects of *C. papaya* seeds in normal cells and cancer cells, vero cells and prostate cancer cells were selected respectively and the MTT assay method was employed.

1.2 Statement of the Problem

Plant derived pharmaceutical products have not only been found to have phytoconstituents with antioxidant and immunomodulatory properties with the potential to inhibit/kill malignant cells but they are also safe and effective for cancer treatment (Salim *et al*., 2013). Several studies on papaya anticancer activity in various sections (fruits, shoots, leaves, rinds, seeds, roots or latex) show that papaya has anticancer action (Yogiraj *et al.*, 2014; Alotaibi *et al*., 2017). However, no studies have been conducted to compare the antiproliferative activities of papaya black seed extracts and standard anticancer medicines in treating prostate cancer cells, nor have cytotoxic effects been evaluated. In addition, the antiproliferative effect of bioactive chemicals extracted from papaya black seeds has not been studied. This study looked at the cytotoxicity and antiproliferative effects of crude extracts of papaya black seeds and doxorubicin in vero cells and prostate cancer cells.

1.3 Rationale of the Study

While prostate carcinoma disease causes a serious public health burden worldwide, its diagnosis and treatment methods remain quite challenging. Although chemotherapy, radiotherapy, hormone therapy and surgery have been the front line of the available management and treatment options for prostate cancer, they have been reported to have serious side effects and low survival benefits to patients (Islam *et al*., 2018). Erectile dysfunction, urinary dysfunction and intestinal dysfunction are among the major side effects to mention a few. Natural compounds from medicinal plants have been reported safe and effective for the treatment of cancer as such compounds can be tolerated by human tissues with less harm (Iqbal *et al.*, 2017). The biological activity and medicinal application of papaya for the treatment of cancer, dengue, acne, male infertility, ringworms, psoriasis and cardiovascular diseases (Yogiraj *et al*., 2014) has made it to be considered as a valuable nutraceutical plant. Therefore, due to increased treatment expenses and adverse effects of anticancer drugs, the need for innovative interventions, feasible and cost-effective drugs with precise efficiency and fewer side effects is imperative.

1.4 Research Objectives

1.4.1 General Objective

The present study mainly aimed to evaluate the biological properties of crude extracts of papaya black seeds in prostate cancer cells.

1.4.2 Specific Objectives

(i) Phytochemical analysis of natural compounds with anticancer activity from crude extracts of *Carica papaya* black seeds.

(ii) To assess the cytotoxic effects of crude extracts of *Carica papaya* black seeds against Vero cells.

(iii) To assess the antiproliferative effects of crude extracts of *Carica papaya* black seeds against prostate cancer cells.

1.5 Research Questions

(i) What natural compounds with anticancer activity are in papaya black seeds?

(ii) Can crude extracts of papaya black seeds induce cytotoxic effects in vero cells?

(iii) Can crude extracts of papaya black seeds induce antiproliferative effects in prostate cancer cells?

1.6 Study Significance

This study will contribute to the knowledge and efforts of discovering the alternative precise cancer treatment particularly prostate cancer that already exists. Moreover, it will pave the way for further scientific studies, which could lead to the discovery of cancer therapies with few or no adverse effects.

1.7 Delineation of the Study

This research aimed on the assessment of antiproliferative effects of crude extracts of papaya black seeds against the prostate cancer cells. It was the *in vitro* study which had two arms; the experimental arm and the control arm. The experimental arm consisted of five crude extracts of *Carica papaya* black seeds and the control arm had a negative control (media plus cells) and a positive control (doxorubicin). Seven serial dilutions of varied concentrations were prepared under the controlled environment for the crude extracts and doxorubicin. The Methyl tetrazolium bromide (MTT) assay was employed in evaluation of the cytotoxic and antiproliferative effects of crude extracts of papaya black seeds. The collected data were analyzed by R Software Version 4.3.1 and the study findings were reported accordingly.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview

Prostate cancer is a cancerous disorder in which aberrant cellular development spreads uncontrollably. It is commonly due to mutations, amplifications and deletions of androgen receptor (AR) genes; and structural change in the AR proteins have been postulated to cause androgen insensitivity (Zajac *et al.*, 2013). The stages of prostate cancer include the early or clinically localized prostate cancer; which is confined within prostate capsule. At this stage any appropriate treatment is either promising or possible. The second stage of the disease is the locally advanced cancer whereby the malignancy extends beyond prostate capsule, including seminal vesicles. The recommended treatment methods ere radiotherapy and androgen deprivation therapy (ADT). The third stage of the disease is the advanced prostate cancer which spreads to retroperitoneal lymph nodes or to bone and its management is done palliatively (Rawla *et al.*, 2019).

2.2 Disease Burden

Prostate cancer is the second most frequent cancer in males after lung cancer. Globally, bout 1.3 million cases and 358 989 deaths (3.8% of all men worldwide) due to prostate cancer were reported by World Health Organization (WHO) in the year 2018. It is anticipated that by 2035 the cancer incidences will rise to 24 million and in that case, the number of deaths from cancer will increase in the future (Bray *et al.*, 2018). In sub-Saharan Africa, it is reported yearly 51 945 cases, equivalent to 20.3% of all cancer cases, are contributed by prostate cancer. Prostate cancer is the most common cancer in Tanzania contributing 22.9% of all cancers in men (Olson *et al.*, 2020). It is the most frequent diagnosed cancer among men and the mortality rate due to prostate carcinoma increases from the age of 55 years and above (Lyimo *et al.*, 2020).

2.3 Prostate Cancer Treatment

Chemotherapy, radiotherapy, hormone therapy and surgery are traditional methods for management and treatment of prostate cancer in Tanzania and worldwide. Nonetheless, each of them has been reported to have serious side effects (Islam *et al*., 2018). Sexual dysfunction and urinary incontinence are the long-term adverse effects of radical prostatectomy. Major side effects include erectile dysfunction, urinary dysfunction and intestinal dysfunction for electron beam radiation therapy. Furthermore, androgen deprivation treatment results in sexual dysfunction, breast swelling and other possible long-term risks such as anemia and osteoporosis, for most patients. Most men suffer from distressing urinary symptoms, erectile dysfunction and frequent rectal bleeding for brachytherapy (Barqawi *et al*., 2012). The increased incidence of death and adverse effects of anticancer drugs are the main reasons that inspire scientists to look for new and more effective drugs with fewer side effects.

2.4 Medicinal Plants in Tanzania

About 80% of the Tanzania rural population depends on medicines from natural sources as remedies for many diseases, cancer inclusive (Matata *et al.*, 2018). Medicines from plants contribute so largely in primary health care to both urban and rural populations (Kitula *et al.*, 2007).

2.5 Medicinal Plants and Cancer

Plants with medicinal properties have been used for years; mainly by people in developing countries as alternative medical care (Rahman *et al*., 2018; Biochem *et al.*, 2016). Drugs from plants were preferred in treatment of cancer due to their safety and effectives.

2.6 Plant Phytochemicals and Cancer Prevention

Plants with medicinal properties such as C*arica papaya*, *Annona senegalensis*, *Moringa oleifera* and *Allophylus africanus* produce phytocompounds which are potential for treatment of various diseases caused by microbes and insects (Ajuru *et al*., 2017). Besides, they are non-toxic to healthy cells (Rahman *et al*., 2018). These plants are composed of phytocompounds such as alkaloids, tannins, flavonoids, terpenoids, glycosides and saponins. These phytocompounds possess antioxidant and good immunomodulatory properties which attribute to anticancer activity of these plants.

Alkaloids were reported to possess enormous pharmacological activities such as anticancer, antiasthma and antimalarial effects. It was also found with anti-hyperglycemic, analgesic, antibacterial properties. Tannins produce antioxidant and homeostatic effects. They exhibit also a reducing tendency for digestion of foods containing proteins. Plants natural enemies are fought by terpenoids. Also, terpenoids have medicinal effects against ulcers, malarial, cancer and microbes. The inflammation of the upper respiratory tract is healed by saponins; which are also reported to exhibit anti-fungal and anti-diabetic potential (Yessuf *et al*, 2015). Among main ailments, flavonoids exhibit anti-carcinogenic, antioxidant and anti-inflammatory (Ajuru *et al*., 2017).

2.7 Potentiality of *Carica papaya* to Cancer Treatment

Papaya (*Carica papaya* Linn) belongs to the Caricaceae family. It is typically grown in the neo-tropical regions and tropical regions between 32º South and North. Papaya is cultivated in subtropical and tropical countries including Nigeria, Indonesia, Brazil and India (Anitha *et al*., 2018).

Papaya is well regarded all over the world for its health and nutritional values. In most Asian countries, the properties of papaya fruit and other plant parts are also well recognized in the traditional medicinal system (Yogiraj *et al*., 2014). The biological activity and medicinal application of papaya has made considerable progress over the last few decades and is now considered as a valuable nutraceutical plant (Alotaibi *et al*., 2017). Papaya has outstanding medicinal properties for the treatment of cancer, dengue, acne, male infertility, ringworms, psoriasis and cardiovascular diseases (Yogiraj *et al*., 2014). Thus, several studies on medicinal properties of various parts of papaya plant (fruits, shoots, leaves, rinds, seeds, roots or latex) have been conducted (Otsuki *et al*., 2010).

Papaya seeds have also been used in some Asian culture as a traditional medicine in male reproductive system. Papaya seeds have also been reported to impair the development of prostate cancer (Alotaibi *et al*., 2017). Previous phytochemical investigations in *C. papaya* seeds in Tamil Nadu, India resulted in the isolation of carbohydrates, proteins, saponins, steroids, glycosides, amino acids and tannins, flavonoids and alkaloids (Ek *et al*., 2018).The study of biochemistry found that 18.75 mg, 7.28 mg and 16.87 mg for carbohydrates, proteins and starch were contained in papaya, respectively (Ek *et al*., 2018). Papaya seeds' extract has excellent therapeutic and nutritional qualities that can be used to treat many diseases such as ringworm, psoriasis, cirrhosis of the liver and body maintenance (Anitha *et al*., 2018). Papaya seeds phytochemical compounds support cardiovascular system, protect against strokes, heart attacks and help in preventing cancer of the colon (Ek *et al*., 2018).

Papaya leaves showed hepatoprotective, anti-inflammatory, anti-oxidant anti-dengue, anti-plasmodia, anti-cancer, anti-bacterial effects *in vivo* and *in vitro* experiments (Nugroho *et al.*, 2017). The roots of papaya plant have been the effective medicine for urinary bladder and kidney disorders (Anitha *et al*., 2018). In respiratory diseases treatment such as bronchitis and cough, the papaya root is chewed and swallowed (Anitha *et al.*, 2018). It also serves as an abortive, diuretic, anti-fungal and also prevents uterine irregular bleeding and piles (*Anitha et al.*, 2018). The roots are used as a herbal medicine to treat typhoid fever, wound infections gastroenteritis, urethritis and otitis media (Anitha *et al.*, 2018). It is further used to relieve stomach pain and discomfort and to treat heat-infectious pneumonia (Anitha *et al.*, 2018).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of Plant Materials

The ripe papaya fruits were harvested from a garden situated at Karangai village in Arusha, Tanzania, Arumeru District on 01 July, 2020. Prior to papaya fruits collection, the intended plant species (*Carica papaya Linn* (Caricaceae family) was identified by Mr. Simon Laizer, an independent botanist. The papaya plant was the indigenous variety whose voucher specimen No.01 was collected and then laid out and in the national herbarium at Tanzania Pesticide Research Institute (TPRI), Arusha on 05 July, 2020.

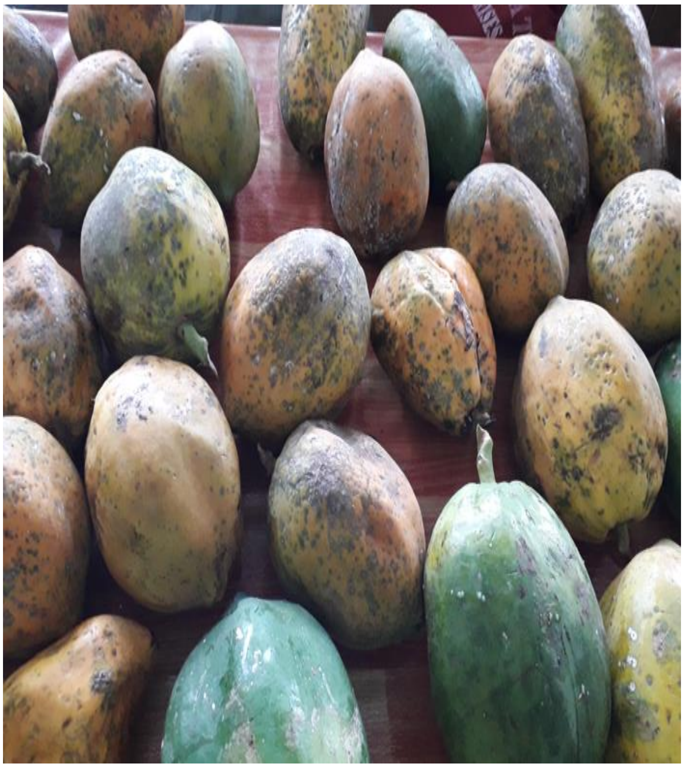


Figure 1: Collected papaya fruits

Figure 2: Dried papaya black seeds

3.2 Study Location

The extraction of plant materials and the phytochemical screening were performed at NM-AIST laboratory in Arusha, Tanzania. Evaluation of cytotoxicity and antiproliferative effects of crude extracts of papaya black seeds were accomplished in KEMRI laboratory in Nairobi, Kenya.

3.3 Preparation of Crude Extracts

Materials for extraction process included a grinder machine, butcher funnels, Whatman filter papers, flat and round bottom conical flasks, freeze dryer, methanol, distilled water, ethanol, n-hexane and ethyl acetate and they were provided by NM-AIST laboratory.

Using distilled water, papaya fruits were washed and cut into half to reach the seeds immediately after being dried at room temperature. The scrapped seeds were sorted and then scattered over a plastic tray and left to dry under sunscreen settings so as to obtain 1 kg as a constant weight after 14 days. Using grinder machine, then the seeds were thoroughly grinded.

Serial exhaustive extraction method was used in preparation of solvent extracts based on polarity of solvents (Nawaz *et al.*, 2015). The 150 g of powdered papaya black seeds was weighed by using beam balance and it was then placed in a flat-bottomed conical flask.

Thereafter, 1500 mL of n-hexane was added. The mixture was kept aside for 72 hours with occasional agitation in the environment of 25 °C and 60% humidity. Thereafter, Butcher funnel with Whatman No.1 filter paper was used to recover the filtrates. The rotary evaporator was employed in drying the filtrate at low pressure to obtain a concentrated sample. The weight of extracts was ascertained and stored in the refrigerator at 4 °C waiting for use (Nawaz *et al*., 2015). The successive extractions were carried out using the left-over residues by adopting the above procedure for ethyl acetate, ethanol and then methanol at room temperature.

Preparation of the aqueous extract was done by taking 150 g of papaya seeds powder and soaking into 500 mL of distilled water, then heated in a water bath at 60 °C for 6 hours. After cooling, the filtrate was obtained by using muslin gauze and then frozen for 24 hours. Lyophilization of the filtrate was achieved by usage of Modulyo Edwards freeze drying machine. Weight of lyophilized extract was verified and then kept at -20 °C in air tight bottle waiting for use.

3.4 Phytochemical Analysis

Standard procedures were used to test the five (5) crude extracts of papaya seeds for secondary metabolites (Ek *et al*., 2018). Flavonoids, alkaloids, tannins, terpenoids, glycosides and saponins were among the secondary metabolites studied as follows:

3.4.1 Analysis of Saponins

Analysis of saponins was done by adding 2 mL of distilled water into 2 mL of crude extract in a test tube, then the mixture was vigorously agitated for 30 seconds. Thereafter, the test tube was kept a side for one minute. The presence of saponins (Fig. 3) was indicated by formation of a persistent foam layer.

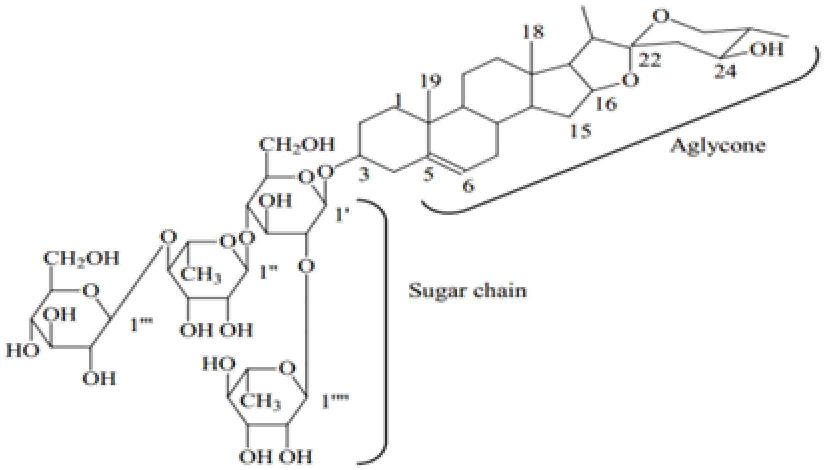


Figure 3: Saponins (Handali *et al*., 2015)

3.4.2 Analysis of Tannins

In a test tube containing 2 mL of crude extract, distilled water in 5 mL was added and boiled for one minute. Then, 2% $FeCl_3$ was added and the mixture was gently shaken. Formation of green precipitates was used as indicator for presence of tannins (Fig. 4).

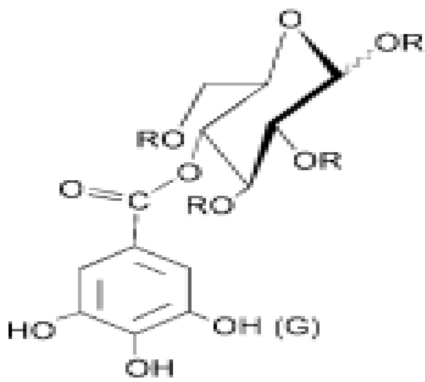


Figure 4: Tannins (Ree *et al*., 2001)

3.4.3 Analysis of Alkaloids

On a watch glass, 2 mL of crude extract was poured, then 1 percent hydrochloric acid (HCl) and followed by three drops of Mayer's reagent. The presence of alkaloids was confirmed by the production of white precipitate.

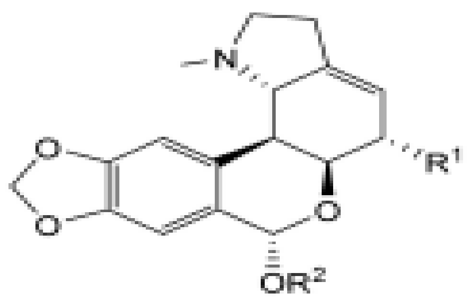


Figure 5: Alkaloids (Lucie *et al*., 2020)

3.4.4 Analysis of Flavonoids

In a test tube with 2 mL of crude extract, 5 mL of aqueous ammonia was added, and then 2 mL of concentrated sulfuric acid (Conc H_2SO_4) and the whole mixture was gently shaken for 30 seconds. Presence of flavonoids (Fig. 6) was indicated by intense yellow color formation.

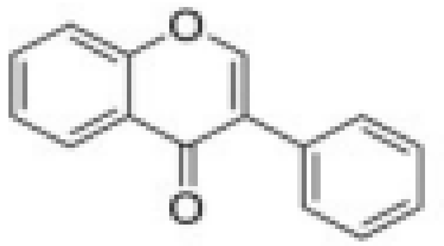


Figure 6: Isoflavonoids (Chandra *et al*., 2017)

3.4.5 Analysis of Terpenoids

In a test tube with 2 mL of crude extract, 2 mL of chloroform was added and vigorously shaken for about 30 seconds before being left to stand for 1 minute. Then, followed the addition of 2 mL of Conc H_2SO_4 and thereafter, the mixture was heated for 2 minutes. Grey color formation suggested the presence of terpenoids.

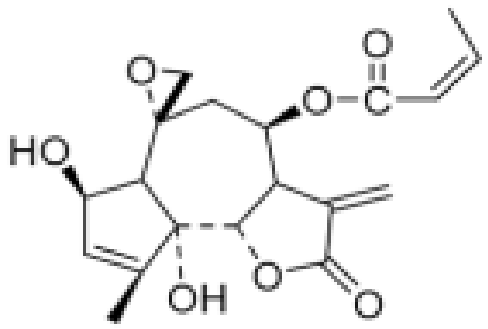


Figure 7: Terpenoids (Yang *et al*., 2020)

3.4.6 Analysis of Glycosides

In a test tube with 2 mL of crude extract, 2 mL of chloroform was added, then 2 mL of Conc H_2SO_4, and then the mixture was kept a side for one minute. The presence of glycosides (Fig. 8) was demonstrated by formation of brown color.

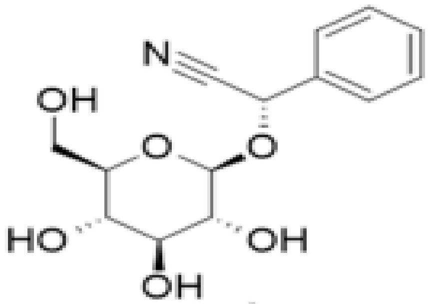


Figure 8: Glycosides (Hariono *et al*., 2021)

3.5 Preparation of Cell Culture

Materials for Culture media were 10% Fetal Bovine Serum (FBS), streptomycin 1%, Dulbecco Modified Eagle Medium (DMEM) and 1% amino acids and they were purchased from Keuta Technologies Ltd in Nairobi, Kenya. Prostate cancer cells (22VR1) and Vero cells (CCL81) were acquired from KEMRI laboratory.

Thawing of cells was done in a water bath at 37 °C with Dulbecco Modified Essential Medium (DMEM) supplemented with 100 µg/mL streptomycin to prevent bacterial growth and 10% Fetal Bovine Serum (FBS) as a cell growth promotor then incubated at 37 °C, 5% CO_2 and 95% humidity to attain confluence.

3.5.1 Preparation of Test Samples

Materials for test sample preparation were PBS, Dimethyl Sulfoxide and Eppendof tube; which were procured from purchased from Keuta Technologies Ltd in Nairobi, Kenya.

Analytical balance was used to weigh 10 mg of the crude extracts and doxorubicin and placed in a 1.5 mL Eppendorf tube and 100 µL of the solution of dimethyl sulfoxide (DMSO) was added and the whole content was swirled. Another 1.5 mL Eppendorf tube was used whereby a concentration of 1000 µg/mL was achieved by transferring into a tube 100 µL of the prepared mixture and then followed by adding 900 µL of PBS. The storage of test samples was under refrigeration at -20 °C until experimentation.

3.5.2 The Principle of MTT Bioassay

This is a colorimetric assay based on the enzymatic activity of mitochondria succinate dehydrogenase enzymes in living cells cleaving tetrazolium salt to form a blue formazan product (Twentyman *et al*., 1987). During MTT exposure, the percentage of cell viability is directly proportion to the amount of formazan produced by the enzymes and the percentage of cell growth inhibition is inversely proportional to the amount of formazan produced. This is measured in terms of absorbance by optical density spectrophotometer (Twentyman *et al*., 1987).

(i) Evaluation of Cytotoxicity

Materials for cytotoxicity assessment were multi-well plates, MTT dye, Phosphate-Buffered Saline (PBS), Dimethyl sulfoxide (DMSO) and automated microplate photometer; they were purchased from Keuta Technologies Ltd in Nairobi, Kenya. Vero cells were washed with saline phosphate after reaching the required confluence and then were harvested by trypsinization. Trypan blue dye exclusion method (cell density counting) was employed to measure the cell viability by the help of a hemocytometer (Ngule *et al*., 2018). Seeding of cells was done in 96-multi well plates by adding aliquot of 100 µl at a density of 2×10^5 cells/well and incubated for 24 hours in an environment of 95% humidity and 5% CO_2 at 37 °C.

After incubation for 24 hours, 15 µL of samples to be tested from serial dilution of seven different concentrations; 1000 µg/mL, 333.33 µg/mL, 11.11 µg/mL, 37.04 µg/mL, 12.35 µg/mL, 4.12 µg/mL and 1.37 µg/mL was added respectively starting from row H to B. Row A containing media and cells served as a negative control (Beauv *et al*., 2019). Doxorubicin, which is a commonly used cancer treatment drug was considered as a positive control (Tietbohl *et al*., 2017). It was placed in wells 10, 11 and 12 in 96-multi well plate. Further incubation was carried out for 48 hours at 37 °C and 5% CO_2.

The potential effect of tested samples was measured by the capacity of viable cells to reduce a yellow MTT dye to formazan which is a purple product (Reilly *et al.*, 1998). The 100 µL of the medium was drawn after 48 hours and to the remaining medium 10 µL of the MTT solution was added in each well and incubated for 4 hours at 37 °C in 5% CO_2. The surface media was then taken out from the plates and using 50 µL of 100% DMSO the formazan crystals were dissolved. The contents on the wells were shaken thoroughly followed by reading the absorbance at 540 nm with the wavelength of 720 nm as a reference using enzyme-linked immunoassay (ELISA) reader (Twentyman *et al*., 1987). The experiment was performed in triplicate.

(ii) Evaluation of Antiproliferative Activity

Materials for evaluation of antiproliferative effects in prostate cancer cells were multi-well plates, MTT dye, Phosphate-Buffered Saline (PBS), Dimethyl sulfoxide (DMSO) and automated microplate photometer; they were purchased from Keuta Technologies Ltd in Nairobi, Kenya.

Prostate cancer cells were washed with saline phosphate after reaching the required confluence and then were harvested by trypsinization. Trypan blue dye exclusion method (cell density counting) was employed to measure the cell viability by the help of a hemocytometer. Seeding of cells was done in 96-multi well plates by adding aliquot of 100 µl at a density of 2 x 10^5 cells/well and incubated for 24 hours in an environment of 95% humidity and 5% CO_2 at 37 °C. After incubation for 24 hours, 15 µL of samples to be tested from serial dilution of seven different concentrations; 1000 µg/mL, 333.33 µg/mL, 11.11 µg/mL, 37.04 µg/mL, 12.35 µg/mL, 4.12 µg/mL and 1.37 µg/mL was added respectively starting from row H to B. Row A containing media and cells served as a negative control (Beauv et *al.*, 2019). Doxorubicin, which is a commonly used cancer treatment drug was considered as a positive control (Tietbohl *et al*., 2017). It was placed in wells 10, 11 and 12 in 96-multi well plate. Further incubation was carried out for 48 hours at 37 °C and 5% CO_2.

The potential effect of tested samples was measured by the capacity of viable cells to reduce a yellow MTT dye to formazan which is a purple product (Reilly *et al*., 1998). The 100 µL of the medium was drawn after 48 hours and to the remaining medium 10 µL of the MTT solution was added in each well and incubated for 4 hours at 37 °C in 5% CO_2. The surface media was then taken out from the plates and using 50 µL of 100% DMSO the formazan crystals were dissolved. The contents on the wells were shaken thoroughly followed by reading the absorbance at 540 nm with the wavelength of 720 nm as a reference using enzyme-linked immunoassay (ELISA) reader (Twentyman *et al*., 1987). The experiment was performed in triplicate.

Figure 9: Cells appearance in 96 well plates on adding MTT reagent

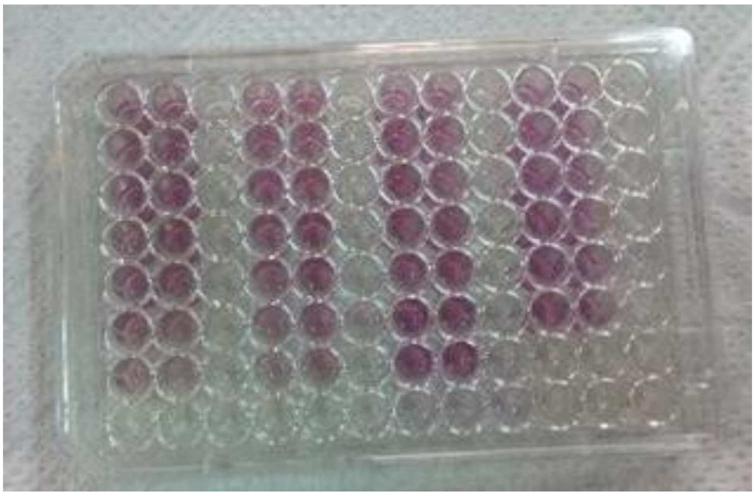


Figure 10: Cells appearance in 96 well plates on 2 hours incubation with MTT reagent

3.6 Calculation of Percentage Cell Growth Inhibition

The reduction percentage in cell growth was established via the formula indicated hereunder (Bézivin *et al*, 2003):

$100-(At-Ab)/(Ac-Ab)$ x 100= % cell growth reduction……………………………………… (1)

Where; Ab= value of absorbance for blank (media only), At= value of absorbance for sample tested (cells + extracts) and Ac= value of absorbance of negative control (cells plus media).

The CC_{50} and IC_{50} values were calculated via R Software Version 3.4.1 and they were used to express the effect of test samples on cells. The CC_{50} and IC_{50} are concentration of test sample which killed 50% of treated Vero cells and concentration of test sample which inhibited the growth of cancer cells by 50% respectively. Classification of antiproliferative activity was as follows: >1000 µg/mL, inactive, >100–1000 µg/mL, weakly active, >20–100 µg/mL, moderately active and ≤20 µg/mL, active (Baharum *et al*., 2014).

3.7 Data management and Analysis

All experimental raw data were recorded in the Microsoft Excel Sheets. Data transformation and analysis was performed by means of statistical packages in R Software Version 3.4.1 and excel data sheets. The statistical difference between treatments and controls was tested by One-way Analysis of Variance (ANOVA) ($p \leq 0.05$).

The percentage cancer cell growth inhibition and the percentage toxicity in Vero cells were calculated in terms of SEM (Mean ± Standard Error of Mean).

3.8 Ethics Consideration

Papaya fruits were harvested on garden owner's permission at Karangai village in Arumeru district. All standards and safety laboratory procedures were observed prior study commencement. There was no human or animal involved in this scientific research.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Preparation of Crude Extracts

4.1.1 Determination of Extract Percentage Yields

Five extracts were obtained from the powder of papaya black seeds after extraction as indicated in Table 1. The percentage yield varied from 2.9 to 9.1 depending on the type of extraction solvent. N-hexane extract was found with the lowest yield and aqueous extract was found with the highest yield. This could be due to high solubility of various phytocompounds in water (Dhanani *et al*., 2013).

Table 1: Percentage yield of *C. papaya* seeds extraction

Extraction Solvent	Sample Weight (g)	Extract Weight (g)	% Yield
Methanol	150	9.3	6.2
Ethyl acetate	150	6.0	4.0
n-hexane	150	4.4	2.9
Ethanol	150	10.4	6.9
Aqueous	150	13.6	9.1

4.1.2 Phytochemical Screening of Crude Extracts

All solvent extract fractions of papaya seeds were analyzed for secondary metabolites including flavonoids, saponins, glycosides, terpenoids, tannins and alkaloids as shown in Table 2. Flavonoids were present in all screened crude extracts; these phytocompounds were reported to possess antioxidant activity which attributes to antiproliferative potential against many cancer diseases by inducing apoptosis of cells followed by cell death (Tietbohl *et al*., 2017; Widyawati *et al.*, 2020). Presence of phytocompounds in plant extracts is determined by the polarity nature of particular compounds that are selectively soluble in solvents used (Ngo *et al.*, 2017).

Table 2: Phytocompounds present in solvent fractions of *C. papaya* seeds

Extract	Alkaloids	Saponins	Flavonoids	Glycosides	Terpenoids	Tannins
n-hexane Extract	+	-	+	_	+	+
Ethanol Extract	+	+	+	+	-	-
Ethyl acetate Extract	+	+	+	+	+	+
Methanol Extract	+	+	+	+	-	+
Aqueous Extract	+	+	+	+	-	+

(+) sign indicates the presence of phytocompounds and (-) indicates the absence of phytocompounds

Moreover, phytoconstituents such as saponins, terpenoids, alkaloids, flavonoids and glycosides have been reported to be effective antiproliferative compounds which are potential in convectional drugs development (Ulbricht *et al*., 2010).

4.2 Determination of Cytotoxicity

Results in Tables 3 and 4 and Fig. 5 and 6 are for toxicity evaluation of crude extracts and doxorubicin in vero cells. The details in Table 3 indicated the cell counts for all treatments with decreasing tendency from the highest (H) to the lowest (B) concentration. The findings in Table 4 showed that the crude extracts of papaya seeds were not toxic towards Vero cells (CC_{50} >23 µg/mL) as compared to doxorubicin, the positive control with CC_{50} of 15 µg/mL. Generally, the cytotoxicity of the tested samples was close to other indicated by similar letter A in a bar graph in Fig. 4 and overlapping curves in Fig. 4. This is the first study to the best our knowledge to report on the toxicity of crude exacts of papaya black seeds in normal cells. However, the toxic effects of doxorubicin on normal cells were also reported in previous studies (Wang *et al.*, 2004).

Table 3: Vero cell counts after treatment with papaya seeds' extracts and doxorubicin (Cell Counts (µg/mL) for Vero cells _ CCL81)

Rows	Conc. (µg/m)	Aqueous Extract	Ethyl acetate Extract	n-hexane Extract	Ethanol Extract	Methanol Extract	Doxorubicin
A	0.00	0.95	0.92	0.95	0.92	0.89	0.84
B	1.37	0.80	0.74	0.78	0.83	0.71	0.72
C	4.12	0.63	0.63	0.71	0.73	0.48	0.58
D	12.35	0.54	0.59	0.57	0.58	0.48	0.34
E	37.04	0.47	0.41	0.41	0.47	0.38	0.27
F	111.11	0.36	0.33	0.30	0.25	0.32	0.23
G	333.33	0.25	0.23	0.25	0.12	0.27	0.18
H	1000.00	0.13	0.10	0.14	0.05	0.25	0.07

Table 4: Cytotoxic concentrations (CC_{50}) of papaya seeds' extracts and doxorubicin in Vero cells

Variable	Aqueous Extract	Ethyl acetate Extract	n-hexane Extract	Ethanol Extract	Methanol Extract	Doxorubicin
%Toxicity (Mean ± se)	53.6 ± 9.5	54.5± 9.8	53.5 ± 9.7	53.5± 12.8	58.1±7.5	59.4 ± 10.3
CC_{50} (CI 95%)	25.3 (17.7-35.6)	23.2 (16.4-32.3)	25.8 (18.3-36.1)	27.1 (21.2-34.6)	12.7 (3.09-33.1)	15.0 (6.56-29.4)
χ^2	10.34*	11.75*	19.1**	12.62*	12.6*	13.5*

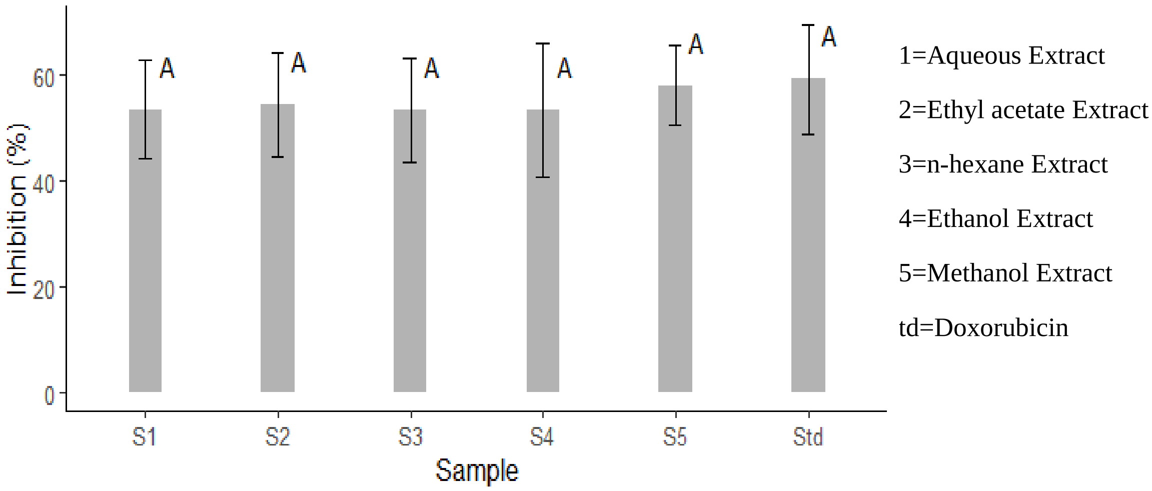


Figure 11: Percentage toxicities of crude extracts and doxorubicin in Vero cells

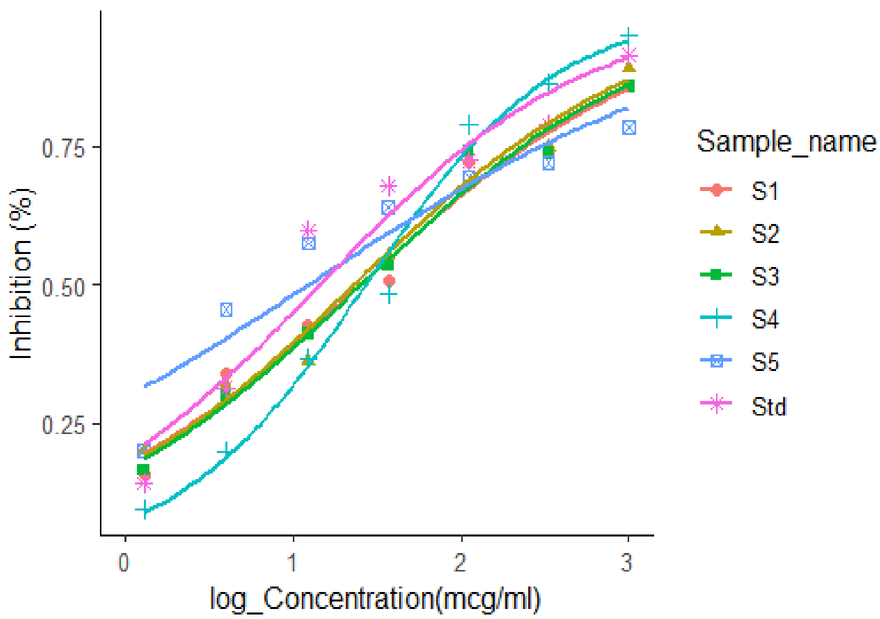


Figure 12: Dose response curves for tested samples in Vero cells

4.3 Determination of Antiproliferative Activity

Tables 5 and 6 and Fig. 7 and 8 are the results obtained from antiproliferative studies on prostate cancer cells. The plant extracts seemed to possess strong anticancer potential as compared to the control. Ethyl acetate extract had the IC_{50} value of 3.64 µg/mL (Table 5) which induced higher inhibitory growth effect on cancer cells. This could be attributed to the phytocompounds available in the crude extracts (Ek *et al*., 2018).

Table 5: Inhibitory concentration (IC_{50}) of crude extracts and doxorubicin in Prostate cancer cells

Variable	Aqueous Extract	Ethyl acetate Extract	n-hexane Extract	Ethanol Extract	Methanol Extract	Doxorubicin
% Inhibition (Mean ± se)	56.2^{A} ± 9.9	70.5±7.9	53.5^{A}± 10.1	56.3±9.3	51.8^{A} ± 9.6	49.6^{A} ± 10.7
IC_{50}	19.9 (14.2-27.3)	3.64 (2.06-5.69)	25.89 (18.6-35.4)	18.9 (13.0-26.6)	30.1 (21.4-42.1)	37.4 (7.94-179.0)
χ^2	12.26*	7.93*	9.14*	14.56*	12.6*	13.5*

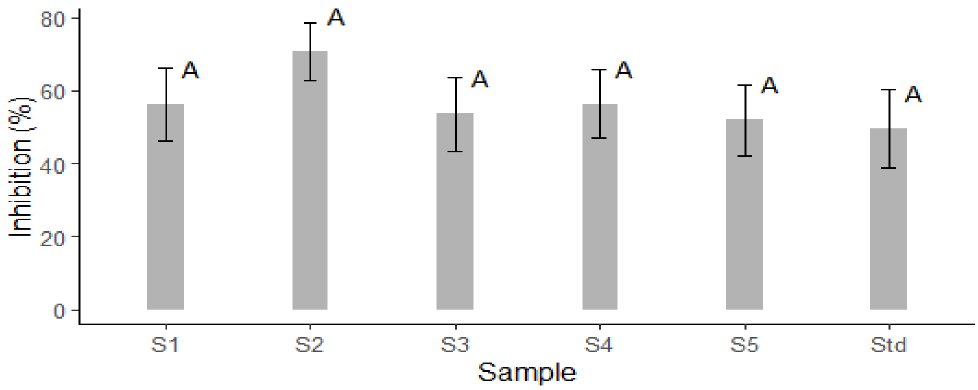
Aqueous extract was also found to possess relatively higher antiproliferative activity towards cancer cells as compared to the control with the IC_{50} value of 19.9 µg/mL (Table 5). Water is the most commonly used solvent by herbalists for the extraction of medicinal plants (Abebe *et al*., 2017). Our study findings have shown that water could also be a suitable solvent for extraction of anticancer compounds from papaya black seeds as described in section 4.1.1.

Cell growth inhibition was found to be dependent to sample concentration, thus the change in sample concentration from 1000 µg/mL to 1.37 µg/mL caused low antiproliferation of cancer cells (Table 6). The rate of cell proliferation was higher at minimum sample concentration and lower at maximum sample concentration from row B towards H respectively (Table 6). Current results are supported by the previous study findings of anticancer activity of 24 plants which indicated that anticancer potency of the tested plant extracts was dose dependent (Fadeyi *et al*., 2013).

Antiproliferative potential of papaya black seeds in prostate cancer cells was also reported in previous studies (Alotaibi *et al*., 2017) and it was attributed to the antioxidation character of papaya seeds towards selected cancerous cells (Zhou *et al*., 2011). The bar graphs in Fig. 7 and the dose response curves in Fig. 8 showed the antiproliferative activity for the tested samples (S1 – Std) which was relatively close to each other (letter A). However, ethyl acetate (S2) had the highest antiproliferative effect.

Table 6: Prostate cancer cell counts after treatment with crude extracts and doxorubicin (22VR1_Cell Counts (µg/mL)

Rows	Conc. (µg/m)	Aqueous Extract	Ethyl acetate Extract	n-hexane Extract	Ethanol Extract	Methanol Extract	Doxorubicin
A	0.00	0.94	0.87	0.91	1.13	0.93	1.12
B	1.37	0.77	0.57	0.76	0.87	0.76	0.93
C	4.12	0.62	0.41	0.65	0.75	0.65	0.62
D	12.35	0.51	0.30	0.55	0.60	0.55	0.58
E	37.04	0.41	0.20	0.40	0.50	0.46	0.42
F	111.11	0.30	0.14	0.29	0.40	0.39	0.35
G	333.33	0.20	0.12	0.24	0.25	0.23	0.24
H	1000.00	0.06	0.05	0.08	0.08	0.09	0.13



S1=Aqueous Extract

S2=Ethyl acetate Extract

S3=n-hexane Extract

S4=Ethanol

S5= Methanol

Std= Doxorubicin

Figure 13: Percentage cell growth inhibitions of crude extracts and doxorubicin in Prostate cancer cells

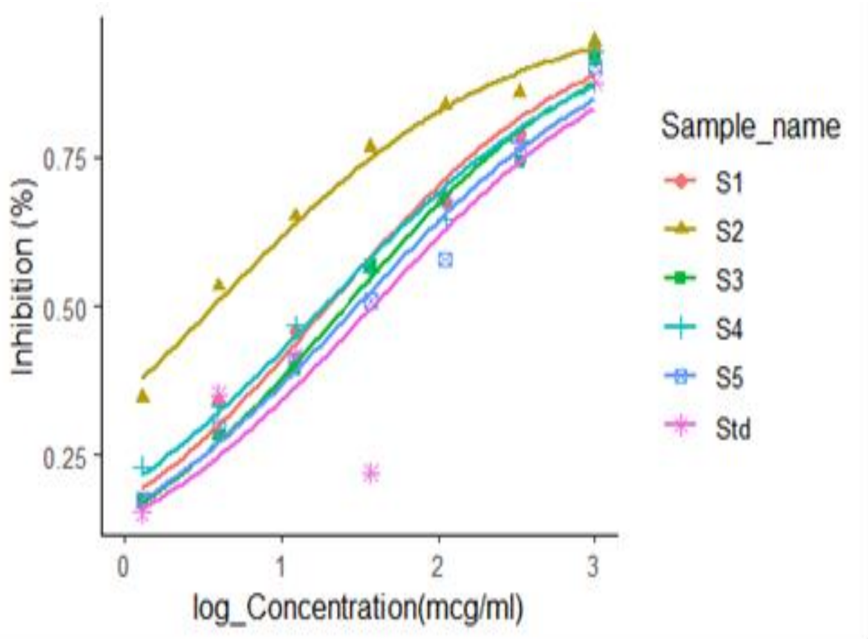


Figure 14: Dose response curves for test samples in prostate cancer cells

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This research project intended to analyze the phytochemicals, cytotoxicity and antiproliferative effects of *Carica papaya* black seeds' extracts. The study findings have revealed that the evaluated papaya seeds' extracts contain phytocompounds such as flavonoids, saponins, tannins, glycosides, terpenoids and alkaloids. Furthermore, cytotoxicity results have indicated the papaya seeds' extracts to be less toxic to vero cells against doxorubicin. In addition, crude extracts showed significant antiproliferative effects towards the prostate cancer cells against doxorubicin. The anticancer activity of the crude extracts was found to be concentration dependent. Generally, the research findings may validate the traditional use of *C. papaya L*. black seed extracts in the prostate cancer management and treatment. However, the execution of this research project did not align with the approved work plan due to COVID-19 pandemic.

5.2 Recommendations

Further research involving the isolation, characterization, optimization and formulation of active compounds from crude extracts is required. However, in robust laboratory settings, Supercritical Fluid Extraction is recommended in future studies. Moreover, the animal studies that could predict so better the anticancer potential of papaya seeds' extracts are also important. It is also recommended that the mechanism of action of papaya seed extracts and phytochemicals be studied.

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RESEARCH OUTPUTS

(i) Publication

Kateihwa, B., Swai, H., Gathirwa, J., & Sauli, E. (2022). *In vitro* antiproliferative potential of crude extracts from *Carica papaya L.* (Caricaceae) black seeds against prostate cancer cell lines'. *Journal of Medicinal Plants Research, 16*(4), 141-147

(ii) Poster Presentation

In Vitro Antiproliferative Effects of Crude Extracts of *Carica Papaya Linn* (Caricaceae Family) Black Seeds against Prostate Cancer Cell Lines