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Research Paper

Effects of Aqueous and Methanolic Extracts of Unripe *Carica Papaya* Seed Extract on Mcf-7 Breast Cancer Cell Lines

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ABSTRACT
BACKGROUND: *The global incidence of breast cancer has been on the rise over the past decade. Due to high mortality rate from breast cancer, the side effect of orthodox medicine has led to the screening of several medicinal plants with potential anticancer activity.*
AIM:*The aim of this study is to evaluate the effect of aqueous and methanolic extracts of unripe Carica papaya seeds extract on breast cancer cell lines.*
METHOD:*Unripe papaya fruits were purchased sliced and the seeds were removed, washed with distilled H_2O and air-dried. The extract was processed using cold percolation method to obtain the methanolic and aqueous extracts respectively. Phytochemical analysis was performed to determine the active components. GCMS analysis was done to determine the phytocompounds. The effect of both extracts was determined by evaluating their cytotoxicity on MCF-7 breast cancer cell lines using cell titre glow luminescent cell viability assay.*
RESULTS:*The preliminary photochemical screening showed that aqueous and methanolic extracts of unripe seeds of Carica papaya contains active constituents such as Phenol and Saponins, among others, which were present in excess and are known from other similar studies for their anti-cancer and anti-inflammatory activity. However, the aqueous extract of the unripe Papaya seeds were found to be more effective on the breast cancer cells (MCF-7) at a higher concentration (400µg/ml) with a higher cytotoxicity activity and lower cell viability on the cells than the methanolic crude extract of the seeds. It was also found that the aqueous and methanolic extracts of papaya (ripe and unripe) were more effective at higher concentration when compared to controls. Results show a dose dependant inhibition of the MCf-7 cancer cells.*
CONCLUSION:*In conclusion, the results obtained from this study indicates that aqueous and methanolic unripe papaya seeds extract specifically reduced cell viability of human breast cancer cells MCF-7. Thus, Carica papaya extract could be helpful in cancer prevention and treatment. Therefore, papaya seed has anti-proliferative potentials and apoptotic induction on breast cancer cells (MCF-7).*
KEY WORDS:*MCF-7, Carica papaya, breast cancer cells, H_2O, anticancer activity.*

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I. INTRODUCTION

The papaya (*C. papaya*) belongs to the order *Brassicales* and family *Caricaceae. Carica papaya* is a herbaceous succulent plant popularly known as Papaya.It originated from Central America, and is now grown in all tropical countries and many subtropical regions of the world. It lives for about 5–10 years, and normally grows with a single unbranched trunk (Morton, 1987). A study conducted has documented that papaya possessed powerful anticancer properties and its impact on numerous lab-grown tumours. Medicinal uses of papaya seeds are carminative, anti-fertility agent in males, counter irritant, as a paste in the treatment of ringworm, liver cirrhosis and abortificient. Seed juice paste is used as antihelmintic and in stimulation of

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menstruation or abortion, studies has shown that *Carica papaya* seeds possessed effective antiheliminitic properties against nematodes found in animals (Chota, 2010).

Chinoy*et al*. (2006) proved the anti-fertility, anti-implantation and abortifacient properties of extracts from papaya seeds. It has been established in males that the seeds of C*arica papaya* has potential anti-fertility drugs (Lohiya*et al*., 2005).

Breast cancer is cancer that develops from breast tissue. Signs of breast cancer may include a lump in the breast, a change in breast shape, dimpling of the skin, and fluid coming from the nipples, a newly inverted nipple, or a red or scaly patch of skin. In those with distant spread of the disease, there may be bone pain, swollen lymph nodes, shortness of breath or yellow Skin (Saunders *et al.*, 2001).

There are more than 18 other sub-types of breast cancer, some cancers such as ducted carcinoma in-situ, develop from pre-invasive lesions (WHO, 2014). The diagnosis of the breast cancer is confirmed by taking a biopsy of the concerning lump. Once the diagnosis is made, further test are carried out to determine the spread of the disease beyond the breast and the type of treatments it may respond to. The balance of benefits versus harm of breast cancer screening is controversial. In 2013, a cochrane review stated that it is unclear if mammographic screening does more good than harm (Gotzsche and Jorgensen, 2013).

II. MATERIALS AND METHODS

Sample Source and Collection

Unripe fruits of *Carica papaya* were obtained from Station market, Kaduna State. The market is a popular fruit market located in Kaduna south local government area of Kaduna state. The state is located in north-western geopolitical zone. The Michigan Cell Foundation-7 (MCF-7) cell line was sourced from DNA Labs Kaduna. Gas Chromatography Mass Spectrometry(GC-MS) analysis of the plant extracts was carried out at National Agency for Food and Drug Administration and Control (NAFDAC) at Sabo, Kaduna state,

Preparation of *Papaya* Seeds Extract

The unripe pawpaw was washed with distilled water, blotted dry with paper towel, and cut into half with the aid of a sterile knife to access the seeds. The seeds were scrapped and washed properly with distilled water. The washed seeds were spread on plastic trays and left to air dry for 7days. The dried seeds were subsequently milled into fine powder with a blender, after which they were packed in a nylon bag to the laboratory for weighing and to carry out the extraction process. Weighing balance was used to weigh 50g of the unripe seeds powder.Cold percolation method of extraction was used. The plant extract was prepared by dissolving 50g of the powdered sample into 500ml of methanol and water each in a separate conical flask. These were all allowed to soak for two (2) days, after which the supernatant was collected and the residues washed twice by suspending them again in the respective solutions, mixing, and placing on a shaker overnight. The collected supernatant was pooled together and the residues were discarded. The aqueous and methanolic extracts were dried by filtering and concentrating to dryness by the use of a rotary evaporator machine overnight to ensure removal of traces of the solvents. The dried extract was stored at -20^{o}C.

Cell Culture

The MCF 7 breast cells were cultured in DMEM (Dulbecco Modified Eagle Medium). The medium is made up of D-Glucose, L-Glutamine and sodium pyruvate, the media was supplemented with 10% Foetal Bovine Serum (FBS) and 1% penicillin and streptomycin. The cells were incubated in a humidified incubator at 37^{o}C with 5% CO_2. Media was changed every 3 days and cells were sub cultured when culture becomes confluent.

Sterility Test of the Extracts

Purity of extract was determined by streak inoculation on freshly prepared blood agar plates which was then incubated at 37^{o}C for 24 hours. At the end of incubation period, plates were examined for growth.

Phytochemical Screening

The aqueous and methanolic extracts obtained were subjected to phytochemical analysis to screen for the presence of the active components such as tannins, alkaloids, flavonoid, phenol, glycosides, and Saponins.

Quantitative Analysis of Unripe *Carica papaya* Extracts Using Gas Chromatography Mass Spectrometry (GC-Ms) Technique

Gas Chromatography Mass Spectrometry (GC-MS) analysis of the plant extracts was carried out to ascertain the active secondary metabolites of the extracts using automated GC-MS machines (model GC-MS TQ8040 Germany). The methanolic unripe sample was injected at different times into the Gas chromatograph (GC), and then heated at 300^{o}C for 3 minutes; the material was then volatized and separated into components as sample flows through the column which was within a special oven which controls temperature from -20^{o}C to 320^{o}C.

The column surface was coated with a material which separated the various chemical compounds in the sample based on size and/or polarity, sample components that were more volatile and smaller in size travelled through

the column more quickly than others. The separated components flowed directly out of the column and into the MS through the ionization source, filtered and detected respectively, thereby counting the number of filtered ions. The information was then sent to a computer and a mass spectrum, the distribution of ions of different sizes was generated.

In-Vitro Anticancer Activity (CellTitre-Glo®)

The cytotoxicity of sample on MCF-7 was determined by cell titre-Glo® luminescent cell viability Assay. The monolayer cells were detached and single cell suspension was made using trypsinethylenediaminetetra-acetic acid (EDTA). A hemocytometer was used to count the viable cells and the cell suspension was diluted with a medium containing 5% FBS (foetal Bovine Serum) in order to obtain final density of 1x 105 cells/ml. Ninety Six (96) wells micro titre plate with the density of 10,000 cells/well were seeded with 100µ/l/well of cell suspension of the cell titre-Glo® 2.0 reagent and incubated for all attachment for 18hrs at 37^{o}C, 5% CO_2, 95% air and 100% relative humidity.

Aliquots of 100µl of different concentrations of sample A, B, C and D extracts (25, 50, 100, 200 and 400µg/ml respectively) dissolved in DMSO (1%) were added to the appropriate wells already containing the 100µl of media and then incubated for 48hrs at 37^{o}C, 5% CO_2, 95% air and 100% relative humidity. After which the plate and its contents was equilibrated to room temperature for approx. 30 minutes, after which it was then mixed for 2 minutes on an orbital shaker at 150 rpm to induce cell lysis and the plate was then incubated at room temperature for 15 minutes to stabilize. The viable cells were determined at 450nm using a Luminometer (MODULUS, Promega UK). The medium without sample was used as control and the assay was carried out in triplicate for all concentrations.

The effect of the samples on the proliferation of MCF-7 was expressed as the % cell viability and of cell growth inhibition using the following formulae;

$$\% \text{ cell viability} = \frac{Abs\ 450\ of\ treated\ \ Cell}{Abs\ 450\ of\ control\ \ cells} \text{ x } 100\%$$

$$\% \text{ cell growth inhibition} = \frac{100 - Abs\ (samples\ )}{Abs\ (control\ )} \text{x} \frac{100}{1}$$

Where:
Abs – Absorbance of Treated cells/ Control cells
Samples – Ripe Pawpaw Seeds Extract (Aqueous or Methanolic)
Control – Untreated Cell Lines

Statistical Analysis

The data was analysed using SPSS version 21.0 Student T-test was used to determine the significance by comparing the effect of both aqueous and methanolic extracts of ripe pawpaw (*C. Papaya*) seeds on MC-7 breast cancer cell line.

III. RESULTS

Phytochemical Analysis of Unripe Papaya Extract

The results of the quantitative phytochemical analysis of the unripe Papaya seeds are presented in Table 4.1. The concentration of Phenol was found to be higher in the methanolic extract (11.5) than in the aqueous extract (10.2). Flavonoid was slightly higher in the methanolic extract (3.9) than in the aqueous (3.4). However, the aqueous extract had a higher concentration of Saponins (34.2) compared to the methanolic extract (1.6). Alkaloids were slightly higher in the aqueous extract (1.8) than in the methanolic extract (1.0). Tannin was found to have slightly lower concentration in methanolic extract (2.1) than in the aqueous extract (2.7).

GC-MS Analysis of Unripe Methanolic Extracts

GC-MS analysis of unripe methanolic seed extracts showed a total of 9 compounds. The result is shown in table 4.2. Benzoic acid, Methyl ester had values of 40.78% (area), 23.38% (height) and 3.55% (ratio) in the unripe methanolic extracts. DodecylAcrylate had values of 1.17% (area), 1.14% (height) and 1.66% (ratio). Dibutyl Phthalate had values of 2.46% (area), 3.45% (height) and 1.45% (ratio). Octadecanoic acid, 2-Hydroxyl-1, 3-Propanediyl Ester had values of 1.16% (area), 2.74% (height) and 1.20% (ratio). Cis-9-Hexadecanal had values of 8.06% (area), 12.85% (height) and1.25% (ratio) in the unripe methanolic extracts. Bis (2-Ethyl Hexy) Phthalate had values of 1.17% (area), 2.20% (height) and 1.08% (ratio) in the unripe methanolic extracts.

Comparative Analysis of Percentage Cell Inhibition at Various Concentrations of Unripe Methanolic and Aqueous Extracts of *C. Papaya* Seeds extract

Cytotoxic activity is recorded as percentage inhibited cell growth as shown in figure 4.2. Analysis revealed that extract activity and cell growth inhibition followed a dose dependant pattern. The highest percentage (%) inhibition was recorded at 400 µg/ml concentration of extracts while the lowest percentage (%) inhibition was recorded at 25µg/ml concentration of extracts this declined with the reduction in dose of the extracts.

Effect of Aqueous and Methanolic Extracts on Breast Cancer Cell Line

Extracts activity as shown in figure 4.3 indicates that between the two extracts aqueous extracts were higher in cell inhibition than the methanolic extracts at each concentration level. There is a significant difference in the mean action of the two extracts at $p<0.05$.

Comparative Analysis of Aqueous and Methanolic Extracts of Unripe Seed on Breast Cancer Cells

The extracts of unripe seeds of *Carica papaya* inhibited the cancer cell in a dose related manner. The highest inhibition was recorded at 400µg/ml and the least was recorded at 25µg/ml. Figure 4.4 showed the inhibition percentages of both aqueous and methanolic extracts of the unripe seed. There was no significant difference ($P<0.05$) between the two extracts.

Table 4.1: Phytochemical Analysis of Unripe *C. papaya* Seed Extracts

CONSTITUENTS	UNRIPE EXTRACT	
	Methanolic (mg)	**Aqueous (mg)**
Phenols	11.5	10.2
Flavonoids	3.9	3.4
Saponins	1.6	34.2
Alkaloids	1.0	1.8
Tannins	2.1	2.7

Table 4.2: Phytocompounds in Unripe Methanolic Extracts of *C. papaya* Seeds

S/N	PHYTOCOMPOUNDS	UNRIPE METHANOLIC EXTRACTS		
		% AREA	**% HEIGHT**	**A/H RATIO**
1.	1-DODECANOL	2.79	3.39	1.67
2.	HEXADECANOIC ACID,METHYLESTER	2.92	4.15	1.43
3.	9-OCTADECANOIC ACID, METHYL ESTER	13.21	21.88	1.23
4.	BENZOIC ACID, METHYL ESTER	40.78	23.38	3.55
5.	DODECYL ACRYLATE	1.17	1.44	1.66
6.	DIBUTYL PHTHALATE	2.46	3.45	1.45
7.	OCTADECANOIC ACID, 2-HYDROXYL-1,3-PROPANEDIYL ESTER	1.16	2.74	1.20
8.	CIS-9-HEXADECANAL	8.06	12.85	1.25
9.	BIS(2-ETHYL HEXY)PHTHALATE	1.17	2.20	1.08

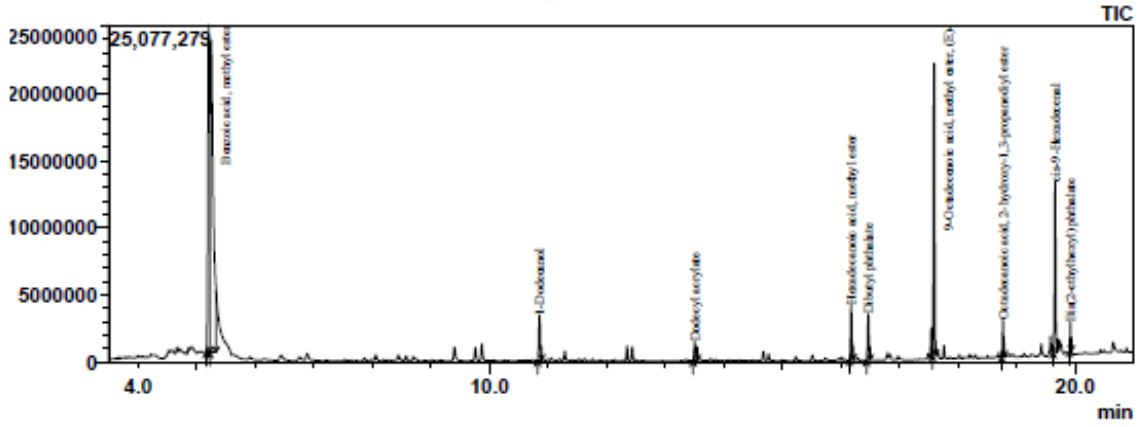
FIGURE 4.1: GC-MS ANALYSIS OF UNRIPE PAPAYA SEED

General GCMS Scan **13-S**

Sample Information

Analyzed by	: Admin
Analyzed	: 13-Sep-19 1:43:10 PM
Sample Type	: Unknown
Level #	: 1
Sample Name	: Unriped Sample
Sample ID	: $ID: $Option5EndIf[1]=1IS Amount:5$
Sample Amount	: $Sample Amount$
Dilution Factor	: $Dilution Factor$
Vial #	: $Vial #$
Injection Volume	: $Inj. VolumeIf$($Comment$!=)[Comment]
$Comment	

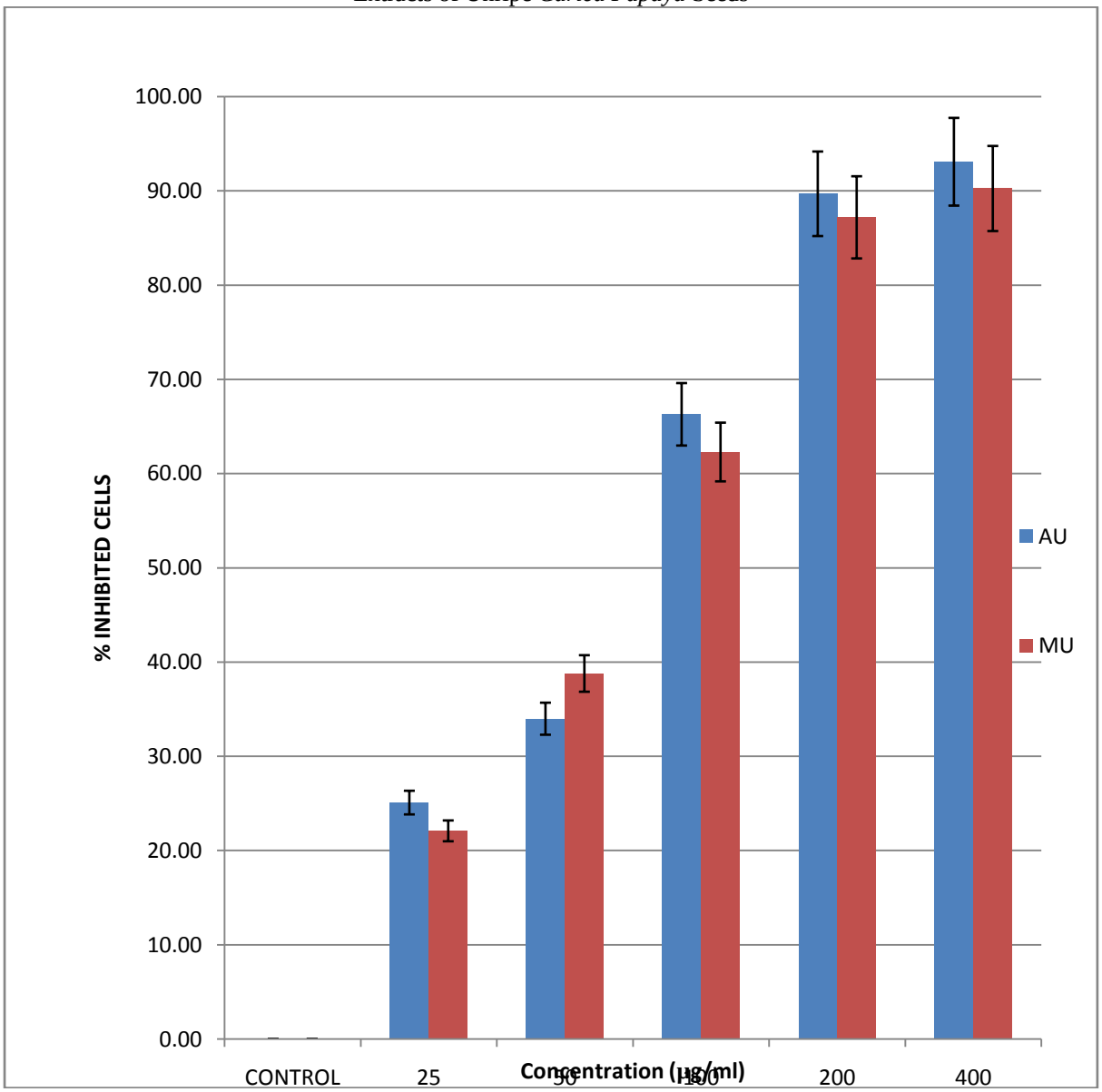
Chromatogram SOLVENT



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	5.210	5.165	5.230	52190118	25.82	24350064	24.51	2.14	MI	
2	5.251	5.230	5.335	82427487	40.78	23224182	23.38	3.55	MI	Benzoic acid, methyl ester
3	10.858	10.825	10.895	5643218	2.79	3369378	3.39	1.67	MI	1-Dodecanol
4	13.508	13.480	13.555	2369474	1.17	1430393	1.44	1.66	MI	Dodecyl acrylate
5	16.179	16.150	16.215	5911011	2.92	4125143	4.15	1.43	MI	Hexadecanoic acid, methyl ester
6	16.471	16.440	16.500	4970672	2.46	3423886	3.45	1.45	MI	Dibutyl phthalate
7	17.593	17.565	17.620	26703329	13.21	21730578	21.88	1.23	MI	9-Octadecenoic acid, methyl ester, (E
8	18.771	18.750	18.795	3256229	1.61	2722258	2.74	1.20	MI	Octadecanoic acid, 2-hydroxy-1,3-pn
9	19.661	19.625	19.695	16290635	8.06	12768812	12.85	1.28	MI	cis-9-Hexadecenal
10	19.922	19.905	19.940	2363446	1.17	2189508	2.20	1.08	MI	Bis(2-ethylhexyl) phthalate
				202125619	100.00	99334202	100.00			

Figure 4.2: Percentage Inhibition of MCF-7 Cells at Various Concentrations of Methanolic and Aqueous Extracts of Unripe *Carica Papaya* Seeds



KEY: AU- Aqueous Unripe,
MU - Methanolic Unripe

Figure 4.3: Inhibition of Aqueous and Methanolic Extracts on Cell Line

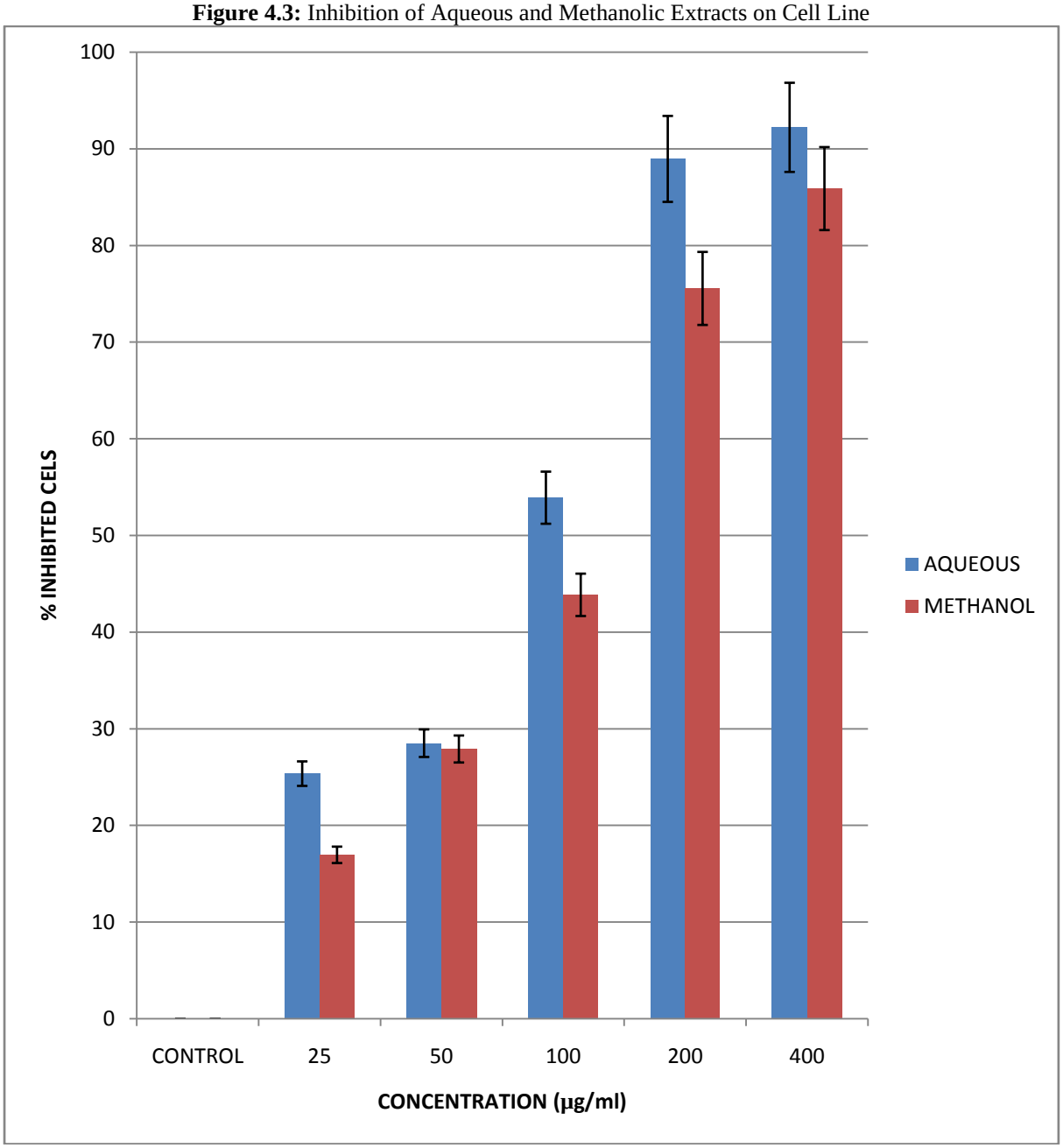
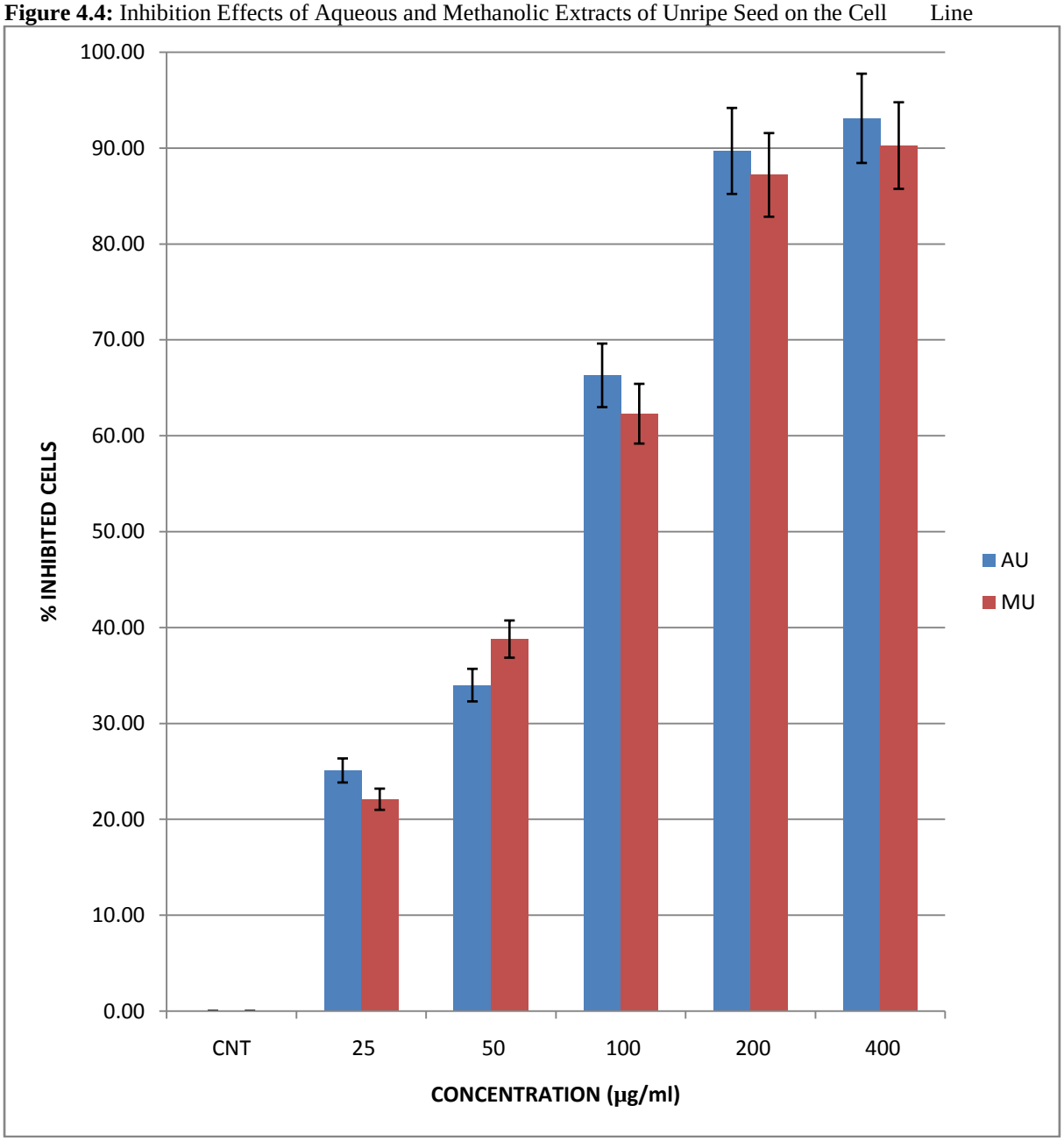


Figure 4.4: Inhibition Effects of Aqueous and Methanolic Extracts of Unripe Seed on the Cell Line



KEY: AU- Aqueous Unripe,
MU - Methanolic Unripe

IV. DISCUSSION

Breast cancer is a malignant tumor; collection of cancer cells arising from the cells of the breast. Traditional herbalists in Nigeria especially in the Northern region use a variety of herbal preparations, including *Caricapayaya* seeds to treat many cancer infection such as prostate cancer, cervical cancer among others, these in addition to other infections and diseases such as liver cirrhosis, digestion, renal failure. *Carica papaya* is well known for its nutritional and medicinal properties throughout the world. It is widely cultivated in the tropical and sub-tropical countries due to its multi-faceted properties. Its whole parts are utilised for their nutritional and medicinal properties.

The preliminary photochemical screening showed that aqueous and methanolic extracts of unripe seeds of *Carica papaya* contains active constituents such as Phenols, Saponins, Flavonoids, Tannins and Alkaloids. This is an indication that potential of unripe *C. papaya* seed extract as anti-proliferation of breast cancer cells is suspected due to the presence of these bioactive compounds which are in very high concentrations as observed in the study.This supports the report of (Huang *et al*. 2009), that three bioactive compounds have considerable anticancer effects: phenolics, saponins and flavonoids. The results are in line with those of Lambert *et al.*

(2005), who described an inhibitory effect of these compounds, particularly the effect of polyphenols on cancer cell proliferation.This is in accordance with the findings of Udoh and Udoh, (2005) who reported that Phenols, Saponins, Flavonoids, Tannins and Alkaloids were present in the extract. This current study is in contrast to the findings of Ikpeme*et al*. (2011) who reported that flavonoids were not present in the extract. This can be attributed to the different physiological conditions obtained in the unripe seeds and could be the reason for the extract inhibiting breast cancer cells. The plant's medicinal value depends on the presence of chemicals that showed positive pharmacological and physiological actions Yu *et al.* (1999).

GC-MS analysis of unripe seeds revealed differences in the composition of its phytocompounds. It was observed that there was a variation in lipids from unripe seeds which correlates with their activity against MCF-7 cell lines. This could possibly be responsible for the higher activity of unripe seed extracts when compared to the ripe seed extracts from other studies. This is in agreement with the work ofChukwuka*et al*. (2013), who reported that unripe pawpaw fruits had a higher amount of phytocompounds than ripe fruits.Theaqueousandmethanolic crude extract of unripe papaya seed shows significant activity at maximum concentration of 400µg/ml which exhibited high cytotoxicity across all concentrations and this is in accordance with the findings of Gul*et al.* (2013).

The aqueous and methanolic extracts of papayawere more effective at higher concentration when compared to controls. Results show a dose dependant inhibition of the MCf-7 cancer cells. The effect of *Carica papaya* seed extract on cancer growth inhibition in this study is also in concordance with studies performed by Morimoto *et al.* (2008), who demonstrated the extracts of different parts of papaya for the prevention, treatment, or improvement of many types of cancer. Data obtained from this current study indicate that aqueous extract of seeds from unripe *Carica papaya* was effective in inhibiting cell proliferation of breast cancer cells.

V. CONCLUSION

In conclusion, the results obtained from this study indicates that unripe papaya seeds extract specifically reduced cell viability of human breast cancer cells MCF-7. The preliminary photochemical screening showed that aqueous and methanolic extracts of unripe seeds of *Carica papaya* contains active constituents such as Phenol and Saponins which were present in excess and are known from other similar studies for their anti-cancer and anti-inflammatory activity. However, the aqueous extract of the unripe Papaya seeds were found to be more effective on the breast cancer cells (MCF-7) at a higher concentration (400µg/ml) with a higher cytotoxicity activity and lower cell viability on the cells than the methanolic crude extract of the seeds. It was also found that the aqueous and methanolic extracts of papaya were more effective at higher concentration when compared to controls. Results indicated a dose dependant inhibition of the MCf-7 cells. Thus, *Carica papaya* aerial parts could be helpful in cancer prevention and treatment. Therefore, papaya seed has anti-proliferative potentials and apoptotic induction on breast cancer cells (MCF-7).

VI. RECOMMENDATIONS

The findings of this study have produced many ideas for future research, and continuation of the exploration of bioactive compounds in *Carica papaya*.

1. Further studies is required to identify bioactive compound(s) in papaya seeds confirm that *Carica papaya* probably belongs to category of plants with a combination of complementary active compounds, this will allow for standardization of the whole extract, formulation and the conduct of clinical trials to provide sufficient scientific rationale for its use in cancer treatment.
2. Thus, further investigations are needed to ascertain the molecular mechanisms of action of the active components to fully understand the active ingredient and potential of *Carica papaya* seed as a chemo-preventive food.

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