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Original Article

Phytochemical contents, *in vitro* and *in vivo* antioxidant and anticancer activities of avocado (*Persea americana* Mill.) seed extract against MCF-7 and HMVII cancer cells

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Abstract

Background and aims: Avocados are unique in Nigeria for their taste and medicinal uses. This study was performed to determine the phytochemicals and evaluate the antioxidant and cytotoxic effects of avocado seed extracts (ASEs).

Methods: The phytochemicals were analyzed using the gas chromatography-mass spectrometry (GC-MS) apparatus. The total phenolic and flavonoid contents and *in vitro* antioxidant activity were evaluated using standard methods. In contrast, *in vivo*, antioxidants were used in a group of thirty healthy Wistar rats randomly grouped into five groups of six, with cytotoxicity activity by benchtop and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assays and apoptosis by assessing caspase-3-like activity.

Results: The GC-MS revealed polyphenols and fatty acids. Phenolic and flavonoid contents were 1178.67 mg gallic acid equivalent per gram (GAE/g) and 86.24 mg rutin equivalent per gram (RE/g), respectively. High radical scavenging toward 2,2-diphenyl-1-picrylhydrazyl (DPPH), ABTS, and ferric reducing antioxidant power (FRAP) reducing power with IC_{50} values of 25.25 ± 1.01 µg/mL, 38.22 ± 2.01 µg/mL, and 43.99 mM Fe (II) equivalent, respectively, *in vitro* antioxidation as well as dose-dependent *in vivo* antioxidant activities with reduced malondialdehyde (MDA), total superoxide dismutase (TSOD), and catalase (CAT) and increased glutathione peroxidase (GSH-Px) contents in vital organs were obtained. Cytotoxic effects on MCF-7 and HMVII cells were detected, with IC_{50} values of 16.51 ± 0.02 µg/mL and 31.71 ± 2.03 µg/mL, respectively, which was statistically significant ($P = 0.001$) when compared to doxorubicin standard drug and a higher selective index on breast cancer (BC).

Conclusion: The findings revealed that avocado seed possessed antioxidant and anticancer activities on human MCF-7 and HMVII cancer cell lines.

Keywords: Polyphenols, Fatty acids, Avocado seeds, Antioxidant, Anticancer

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Introduction

Medicinal plants are natural antioxidant and anticancer sources due to many secondary metabolites such as triterpenes, flavonoids, saponins, alkaloids, polyphenols, and the like. The ethnomedicinal uses of these plants were derived from historical documentation of the traditions and beliefs of the indigenous peoples over a long period (1). The antioxidant potentials of compounds from plants have demonstrated immense body protection from the scavenging effects of free radicals, thereby preventing diseases resulting from oxidative stress. Medically, antioxidants ensure the neutralization of free radicals by donating some electrons they possess, thus making them natural off-switches for scavenging radicals in the body (2). This mechanism assists in stopping specific redox reactions that can affect some molecules within the cells and the body. Regardless of their hazardous effects on the body, these free radicals are still part of human life. The body generates free radicals in response to environmental factors such as air pollution, chemical mutagens (e.g., nitrous oxide, sodium azides, acridine dyes, and the like), UV rays, and tobacco smoking. However, they are the usual by-products of cellular processes in the body (2-4). Medicinal plants comprise many metabolites collectively called phytochemicals, many of which possess antioxidant activities. For instance, ascorbic acid (vitamin C) can stop a free radical by acting as an electron donor to the free radical. In contrast, hesperidin, a phytochemical found in citrus fruits such as oranges, returns the ascorbic acid to its original form as an antioxidant. Other antioxidants, such as carotenoids (e.g., carotene in carrots and lycopene in tomatoes) and flavonoids (e.g., anthocyanins in berries, quercetin in apples, onions, and orange peels, flavanols in cocoa, and green tea catechins), are richly present in plants (4-6).

Currently, cancer has been described as the primary cause of death globally because of its poor prognosis and treatment challenges. After malaria, cancer comes second in causing death globally, which was responsible for 1 out of 6 deaths and about 9.6 million deaths in 2018, especially in Africa and mid-income countries (5,7). Though the significant causes of cancer are unknown, it is believed to

have been caused by agents such as smoking, ultraviolet radiation, certain chemicals (e.g., asbestos), certain viruses (e.g., the human papillomavirus), certain bacteria, and genetic factors (e.g., oncogenes), often developing from the change of normal cells into tumor cells, a process that is called tumorigenesis (8). Many plant products and bioactive compounds have been effectively used to treat and manage various cancer types, including avocado seed (*Persea americana* Mill.).

The avocado (*P. americana* Mill.) is a tree belonging to the Lauraceae family and is originally native to South and Central America. It has now been distributed in West African countries such as Nigeria, Ghana, Ivory Coast, Liberia, and India (9). Although the fruit is generally consumed for its nutritious contents, all parts of the plant are essential to human healthcare. The seed contains mainly polyphenols and fatty acids in addition to other secondary metabolites (e.g., flavonoids, alkaloids, vitamin C, catechins, and the like) that have potential pharmacological benefits to humans as antioxidant, antidiabetic, and anticancer agents (10). In addition, the avocado has several varieties due to biodiversity and chemical races within the genus and species of plants. For example, in Nigeria, the 'Mambilla' avocado is regarded as the most nutritious and largest fruit and the most sorted. The seed of avocado has been reported to possess anticancer activity against the malondialdehyde (MDA)-MB-231 human breast cancer (BC) cells (11) and other types of cancers (5).

The 'Mambilla' (where the seeds were collected) is a region in Nigeria located on the plateau in Taraba State. It borders the Republic of Cameroon and is 1600 m above sea level, making it the highest and coldest region in Nigeria. The daily temperature hardly exceeds 25 °C, and due to its cold temperature almost yearly, 'Mambilla' has attracted many visitors, such as tourists and miners (12).

This current study aims to determine the polyphenol and fatty acid contents of avocado (*P. americana)* seeds in Nigeria. Moreover, it seeks to evaluate the antioxidant and *in vitro* anticancer activities of the seed extract with a view to generating valuable information on its ethnopharmacological relevance and a comparison between this avocado seed and existing works of literature on avocado seeds from other regions or zones.

Materials and Methods

Chemicals and apparatus

All chemicals used in this study were of analytical grades. Methanol, rutin, gallic acid (GA), Folin-Ciocalteu (FC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, and 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from JoeChem Nigeria (Ltd. for Sigma-Aldrich; St. Louse Mo, USA). Aluminum chloride ($AlCl_3$), nitric oxide, sodium nitrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, trypan blue dye, apo alert caspase-3 kits, and ferric chloride were also obtained from the same company. The applied apparatuses included a UV–Vis spectrophotometer (Thermo Fisher, UK), Agilent 7890A gas chromatography-mass spectrometry (GC-MS; Agilent Technologies, UK), and a rotary evaporator (Thermo Fisher, UK).

Cancer cell lines

The MCF-7 human BC cell line, HMVII human vaginal melanoma cell, and Vero E6 normal cell from an African green monkey were obtained on demand from culture collections. The cancer cells were kept in the RPMI-1640 medium, while the Vero E6 normal cell was maintained in the Eagle's minimum essential medium. All the cells were enriched with 100 units/mL penicillin, streptomycin, and 10% fetal bovine serum and kept at 37 °C in a 5% CO_2 refrigerator.

Plant collection, identification, and preparation

Mature fresh avocado (*P. americana*) seeds were obtained from the Gembu community in the Mambilla plateau, Taraba State, Nigeria, in June 2023. The plant was identified by Dr. C.A. Ukwubile of the Department of Pharmacognosy, Faculty of Pharmacy, University of Maiduguri, Nigeria. A voucher specimen number of UMM.FPH/LAR/001 was deposited for the plant at the herbarium of the Department of Pharmacognosy. The seeds were sliced into pieces and were dried under shade for two weeks until constant weight was obtained. They were pulverized into fine powder using an electronic blender, weighed, and stored in a clean plastic bag for further use.

Extraction of plant material

The 800 g powdered seeds were extracted using 2.5 L of 90% methanol (v/v) by cold maceration for 72 hours. The resulting filtrate was filtered using Whatman No. 1 filter paper into a 1000 mL capacity flask. It was then concentrated in vacuo using a rotary evaporator at 64.7°C to obtain a brownish jelly extract weighing 68.8 g (% yield: 8.6). The extract was then stored in a sample bottle, labeled, and kept in a desiccator until needed.

Phytochemical analysis of seed extract

The preliminary phytochemical contents of the extract were determined using standard procedures to check for the presence of triterpenoids, alkaloids, tannins, flavonoids, saponins, anthraquinones, steroids, phytosterols, and polyphenols (13).

Gas chromatography-mass spectrometry analysis of the extract

The phytoconstituents of compounds present in the crude methanol extract of stembark were evaluated in the Agilent Technologies 7890A GC coupled with a mass spectrum detector (Agilent Technologies, USA). The carrier gas was helium with a column velocity flow of 1.0 mL/min. In addition, the ion-source temperature and the

interface temperature were 250 ˚C and 300 ˚C, respectively, and the operating pressure was 16.2 psi. Further, the out time and the injection temperature were 1.5 minutes and 300˚C in the split mode in a 1 µL injector, respectively. In comparison, the temperature of the column was initially 50 ˚C for 5 minutes and raised to 250 ˚C at the rate of 20 ˚C/min for 5 minutes (12). The total elution time was 25 minutes, and each compound was calculated in terms of relative abundance, peak areas, and retention times. The compounds were identified by comparing them with data from the National Institute of Standards and Technology library (14).

Isolation and purification of polyphenols and fatty acids

The crude avocado seed extract (ASE) was further subjected to silica gel column chromatography on a glass column with 35 × 90 mm dimensions using hexane, that is, ethyl acetate (7:3) as the solvent system by the gradient elution technique (12). Fifty fractions were collected and grouped into six groups (A, B, C, D, E, and F) based on their profiles on the *thin-layer chromatography* plate. Each fraction was further purified using short silica gel column chromatography. The purity of each was confirmed by obtaining a single spot on the *thin-layer chromatography* plate. Simultaneously, the boiling points of each compound were determined using the Mettler-Toledo MP80 digital boiling point apparatus (Mettler-Toledo, LLC, USA). The names of the isolated polyphenols and fatty acids were further confirmed using mass spectrometry by comparing them with data from the National Institute of Standards and Technology library (15).

Total phenolic contents

The total phenolic content (TPC) was determined using the FC method with slight modifications. Briefly, the FC stock solution was prepared by dissolving 10 g of FC in 10 mL of deionized water (i.e., 1:10) before the commencement of the experiment. Similarly, 7.5% (w/v) sodium carbonate was dissolved in 10 mL of deionized water. In comparison, the stock solution of the extract was prepared by dissolving 10 g of the extract in 10 mL of 98.1% (v/v) methanol (i.e., 1000 µg/mL). Then, the three stock solutions were all mixed and allowed to stand for 6 minutes. A GA (500 µg/mL) standard solution was prepared in the same way. The concentrations of 0 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, 125 µg/mL, and 150 µg/mL were used as diluting concentrations for plotting the GA calibration curve (16). The absorbance of each solution was taken at 765 nm using the UV-Vis spectrophotometer (Thermo Fisher, UK). The GA concentration in each extract was then calculated using the linear equation from the calibration curve using their absorbance. Next, the TPC was estimated and expressed as mg GA equivalent per gram (GAE/g) using Eq. (1):

$$TPC = C \times V/m \quad (1)$$

where TPC is the total phenolic content (in mg GA equipment; GAE/g), and C represents the GA concentration (but C = x/1000 mg/g). Moreover, V and m denote the volume of the extract per solvent (i.e., 1 mL) and the weight of the extract (in g), respectively.

Determination of flavonoid contents

The total flavonoid content (TFC) of the extract was evaluated using an $AlCl_3$ colorimetry assay with slight modifications. Briefly, 0.5 mL of the avocado methanol seed extract was added to a test tube containing 2 mL of 98.1% (v/v) methanol. Then, 3 mL of 10% sodium nitrite was added to the test tube and kept in the dark for 5 minutes. Next, 3 mL of 10% $AlCl_3$ was added to the mixture and left for 1 minute. Subsequently, 1 mL of the 1 M NaOH solution was added, made up to 5 mL volume with distilled water, and kept for 10 minutes. Finally, the absorbance of the solution was measured at 510 nm (3). Rutin was used as the standard, and the result obtained for TFC was expressed as mg rutin equivalent per gram (RE/g) of the sample. The concentrations of rutin were taken from 25 to 800 µg/mL. The TFC was derived from the rutin standard curve and calculated using Eq. (2). All the readings were taken in triplicate.

$$TFC = C \times V/m \quad (2)$$

where TFC and C denote total flavonoid contents (in mg rutin equipment; RE/g) and rutin concentration (but C = x/1000 mg/g), respectively. Additionally, V and m represent the volume of the extract per solvent (i.e., 1 mL) and the weight of the extract (in g), respectively.

Evaluation of in vitro antioxidant activity

Three methods, namely, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and ferric reducing antioxidant power (FRAP), were used to evaluate the *in vitro* antioxidant activity of the ASE. Stock solutions of the crude seed extract were separately prepared in methanol (10 mg/mL). These stock solutions were further diluted in distilled water at six different concentrations of 100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL, and 600 µg/mL. Ascorbic acid, Trolox, and iron (II) standard solutions were prepared similarly.

Evaluation of Folin-Ciocalteu, DPPH radical scavenging activity

The DPPH radical scavenging activity of the avocado seed methanol extract was determined with modifications (16). Briefly, the DPPH solution was prepared by dissolving 1 g of DPPH in 10 mL of 98.1% (v/v) methanol (Sigma-Aldrich St. Louis Mo) and mixed with the extract stock solution and then diluted to different concentrations (100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL, and 600 µg/mL). The absorbance of each concentration was observed at 517 nm. Ascorbic acid was prepared in a similar way and used as a control. The scavenging radical

activity was calculated using Eq. (3):

% DPPH radical scavenging = (AbsC – AbsE/AbsC) 100 (3)

where AbsC and AbsE denote the absorbance of the control and the absorbance of the extract, respectively.

Evaluation of ABTS diammonium salt radical scavenging activity

This assay was used to obtain ABTS radical cation by mixing 5 mM of the ABTS solution (Sigma-Aldrich, St. Louis, Mo) and 1 mM of the potassium persulfate solution in 10 mL distilled water. The mixture was incubated in a dark cupboard for 24 hours, and the solution was diluted with methanol to obtain the working solution of ABTS, having an initial absorbance of 0.70 ± 0.02 at 734 nm. Then, 100 mL of the crude ASE and the Trolox standard (100–600 µg/mL) were mixed with 3 mL of the ABTS stock solution. The absorbance was measured six minutes later at 745 nm against the blank (10 mL methanol). All readings were performed in triplicate. The percentage of ABTS scavenging was then determined using Eq. (4) as follows (2):

% ABTS radical scavenging = (AbsC – AbsE/AbsC) 100 (4)

where AbsC represents the absorbance of the control, and AbsE is the absorbance of the extract.

Evaluation of ferric-reducing antioxidant power

The previously described method was used with some modifications to evaluate FRAP (17). The FRAP reagent was prepared by mixing 200 mM sodium acetate buffer, 20 mM 2,4,6-*tripyridyl-s-triazine* solution (dissolved in 50 mM *hydrogen chloride* solution), and the 10 mM ferric chloride solution. The reagents were kept for 40 minutes in a dark cupboard, and 1 mL of the plant extract, positive control ascorbic acid positive control (50 µg/mL, 100 µg/mL, 200 µg/mL, and 400 mg/mL), and *ferrous sulfate* (50 mM, 100 mM, 200 mM, and 400 mM) were separately mixed with 3 mL of the FRAP reagent. The blank (control) was the 0.1 mL dimethyl sulfoxide solution. The absorbance for the reaction mixture for each sample was measured at 593 nm. The results were compared with ascorbic acid (control), while *ferrous sulfate* was used for the calibration curve. The FRAP activity was calculated as ferrous equivalent and expressed in mM (18).

In vivo antioxidant activity of avocado seed extract

Experimental animals and grouping

Thirty Wistar rats of opposite genders weighing between 100 g and 150 g were purchased from PJ Rat Farms Ltd., Jos, Nigeria. The animals were allowed to acclimatize in the laboratory for seven days, with free access to food and water and under average body temperature (37 °C). They were randomly assigned to five groups of six. Group I was the standard control group, which received 5 mL of distilled water. Group II was the negative control group, which received 0.3 mL of the D-galactose solution (Pfanstiehl, Inc., USA). Group III was the positive control group, which received a 200 mg/kg oral dose of vitamin C daily. In contrast, groups IV and V received oral dosages of 200 mg/kg and 400 mg/kg of ASE, respectively, for one week (19). Before treatment, rats in groups II to V were administered 0.3 mL of the D-galactose solution to induce oxidative damage in the animals. *In vivo,* antioxidant analysis was evaluated on parameters such as lipid peroxidation (LPO), catalase (CAT), total superoxide dismutase (TSOD), glutathione peroxidase activity (GSH-Px), and MDA content (19). The experiments involving the animals used in this study were performed following the guidelines of international laws and regulations relating to animal use. The Animal Care and Use Committee of PJ Farms Ltd., Jos, Nigeria, approved the ethical approval (with approval number PJF/001/RAT/2023).

Determination of Lipid peroxidation content (malondialdehyde), catalase, total superoxide dismutase, and glutathione peroxidase activity

The LPO content of the extracts was evaluated using the method previously described in previous research (20), while CAT, TSOD, and GSH-Px were determined using methods provided in another research (19).

Evaluation of anticancer activity of avocado seed extract

Benchtop assay for preliminary cytotoxic and growth inhibitory effects

To evaluate the preliminary cytotoxic effect of the extract, ten tadpoles (*Raniceps raninus*) were put in 50 mL beakers containing 15 mL of water from the tadpoles' source and 39 mL of distilled water, which were made up to 50 mL with 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL, and 160 µg/mL. The control beaker contained tadpoles with only 50 mL of distilled water. The mortality of tadpoles in treated beakers was then observed at 5 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes, and 30 minutes. Similarly, the growth inhibitory effect was evaluated using the guinea corn (*Sorghum bicolor*) radicles. This method put twenty viable guinea corn in six Petri dishes underlaid with cotton wool and Whatman No. 1 filter paper. Then, the plates were treated with 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL, and 160 µg/mL of the extract. The control Petri dish contained seeds treated with 10 mL of distilled water. All the Petri dishes were kept in the dark cupboard, and the lengths of emerging radicles were measured using a transparent ruler at 24 hours, 48 hours, 72 hours, and 96 hours. The experiments were performed in triplicate (21).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The cytotoxic effect of ASE was determined by previously described methods (22). In this method, two cancer cell lines (human breast MCF-7 and human vaginal melanoma HMVII cell lines) were seeded in a 96-well plate at a cell

density of 10^5 cells per well using a multichannel pipette and incubated for 24 hours in 5% CO_2 (7). Afterward, various concentrations (040 µg/mL) of the extracts and doxorubicin standard anticancer drug (positive control) were added and incubated for 48 hours at 37 ˚C. Subsequently, 10 µL of the MTT reagent was added to the wells, and the plates were incubated for another four hours to form formazan crystals. To dissolve the formazan crystals formed, 100 µL of dimethyl sulfoxide solution was added to the wells and incubated for two hours. The absorbance of the plates was then read using an enzyme-linked immunosorbent assay scanning multiwell UV-Vis spectrophotometer (Thermo Fisher, UK) at 562 nm (22). Percentage cell cytotoxicity was calculated using Eqs. (5) and (6):

$$\% \text{ cytotoxic activity} = (AbsC\text{-}AbsT/AbsC)\ 100 \quad (5)$$

$$\text{Selective index (SI)} = CC_{50} \text{ of Vero E6}/IC_{50} \text{ of cancer cells} \quad (6)$$

where AbsC and AbsT imply the absorbance of the well without and with treatment, respectively, and CC_{50} denotes the concentration at which the extract showed cytotoxic potentials on half of the population of Vero E6 normal cells. In addition, IC_{50} represents the concentration at which the extract inhibited the growth or proliferation of half of the cancer cells.

Trypan blue exclusion assay of cell viability

In this procedure, the cancer cell lines at a cell density of 105 cells per well were each seeded in a 24-well plate (Costa, US) for 24 hours. Cell suspensions were then prepared at 1000 µL and filled in the *INCYTO Neubauer chamber. No disposable* hemocytometer was stained with 20 µL of trypan dye (Sigma-Aldrich St. Louis, Mo). The cells were then viewed under the microscope (40x) by counting the viable and dead cells using the middle squares of the hemocytometer (23).

Evaluation of caspase-3-like activity

To determine the involvement of the caspase-3 protease enzyme in inducing apoptosis, the cancer cells were each seeded at a density of 1 x 10^8 cells on coverslips and incubated for 24 hours. Then, various concentrations (0 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL) of ASE were added to each well and further incubated for 4 hours (23).

With utmost precision, caspase-3 activity was estimated by fluorescence staining (annexin V) using the ApoAlert Caspase-3 test kits (Biocompare, US) prepared following the instructions of the manufacturer. Doxorubicin was used as the control, and readings were taken in triplicates (24).

Statistical analysis

The obtained data were expressed as means ± standard deviations (n = 3). A P value of < 0.05 was considered statistically significant versus the control using one-way analysis of variance, followed by Dunnett's post hoc test. The analysis was performed using the IBM SPSS, version 23.

Results

Phytochemical contents

The results of the phytochemical screening of the avocado seed methanol extract revealed the presence of alkaloids, flavonoids, tannins, triterpenes/steroids, fats/oils, and phenols while not representing anthracenes and saponins (Table 1).

Gas chromatography mass spectrometry analysis

The GC-MS analysis of ASE demonstrated the presence of nineteen compounds belonging to different classes, including six saturated and unsaturated fatty acids, seven fatty acid derivatives, and six polyphenols with their respective retention times, mass-to-charge ratio (m/z), peak areas, and chemical formula (Table 2).

Total phenolic, total flavonoid contents, and in vitro antioxidant activities

The TPC of 'Mambilla' ASE was determined to be 1178.67 mg GAE/g obtained from the GA calibration curve with the linear equation $y = 0.0087x + 0.064$; $R^2 = 0.9811$, while the TFC was 86.24 mg RE/g obtained from the rutin calibration curve with the linear equation $y = 0.0003x + 0.2476$; $R^2 = 0.2359$ (Figures 1a and 1b). The avocado seed methanol extract also showed IC_{50} values of 25.25 µg/mL and 38.82 µg/mL for DPPH and ABTS radical scavenging activities, respectively, and a ferric reducing power (FRAP) percentage scavenging value of 43.99 mM Fe (II) equipment (Figures 2a, 2b, and 2c). The obtained activities were dose-dependent and comparable to the controls at 100 µg/mL and 600 µg/mL.

In vivo antioxidant activities of avocado seed extract

The MDA content, CAT, TSOD, GSH-PX, and total antioxidant activities in the serum, brain, liver, kidney, and heart of rats were estimated to assess the *in vivo* antioxidant activities of ASEs. The results showed that the serum MDA content and other tissues decreased in a dose-dependent manner (Figures 3a, 3b, 3c, 3d, and 3e) in

Table 1. Phytochemical contents of crude avocado seed methanol extract

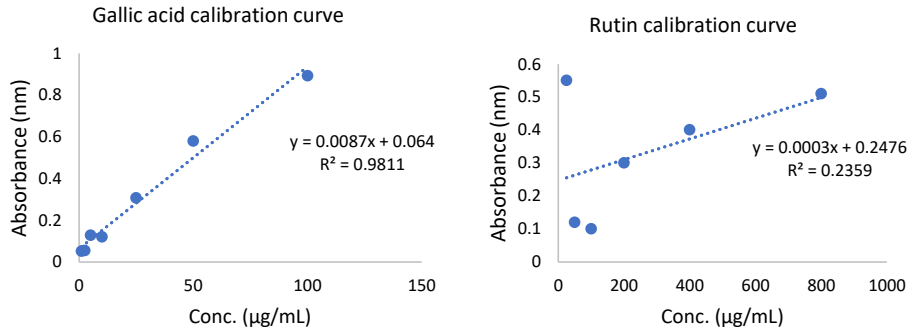
Constituents	Test	Inference
Alkaloids	Dragendorff-Wagner	+ +
Flavonoids	Shinoda's NaOH	+ +
Tannins	$FeCl_3$ Goldbeater's	+ +
Triterpenes/steroids	Liebermann's	+
Anthracenes	Bontrager	-
Fats/oils	Spot Sudan III	+ +
Phenols	$FeCl_3$	+
Saponins	Frothing	-

Note. NaOH: Sodium hydroxide; $FeCl_3$: Ferric chloride. + and – denote present and absence, respectively.

Table 2. GC-MS bioactive compounds of crude avocado seed methanol extract

Compounds	RT (min)	PA (%)	Formula	m/z (g/mol)	Class
Stearic acid	15.46	1.12	$C_{18}H_{36}O_2$	284.48	Fatty acid
Oleic acid	19.12	18.04	$C_{18}H_{34}O_2$	282.46	Fatty acid
Linoleic acid	19.15	12.11	$C_{18}H_{32}O_2$	280.45	Fatty acid
Catechin	19.84	14.23	$C_{15}H_{14}O_6$	290.27	Polyphenol
Avocatin	20.51	28.01	$C_{34}H_{66}O_6$	570.90	Fatty acid +
Palmitic acid	15.31	5.81	$C_{16}H_{32}O_2$	256.43	Fatty acid
Myristic acid	18.24	3.88	$C_{14}H_{28}O_2$	228.37	Fatty acid
Ferulic acid	15.88	6.78	$C_{10}H_{10}O_4$	194.18	Polyphenol
Coumaric acid	15.00	9.01	$C_9H_8O_3$	164.16	Polyphenol
Caffeic acid	15.13	12.02	$C_9H_8O_4$	180.16	Polyphenol
Rutin	22.01	26.05	$C_{27}H_{30}O_{16}$	610.52	Polyphenol
Chlorogenic acid	21.82	2.41	$C_{16}H_{18}O_9$	354.31	Polyphenol
OH-benzoic acid	14.44	1.88	$C_7H_6O_3$	138.12	Polyphenol
Persin	25.04	2.12	$C_{23}H_{40}O_4$	380.57	Fatty acid +
Pahuatin	20.44	0.26	*$C_{19}H_{34}O_4$*	*326.47*	Fatty acid +
Tetracosanoic acid	19.10	1.31	$C_{24}H_{48}O_2$	368.60	Fatty acid
2-OH-palmitic acid	21.18	14.26	$C_{16}H_{32}O_3$	272.42	Fatty acid +
Vanillic acid	15.02	0.51	$C_8H_8O_4$	168.15	Fatty acid +
Kaempferol	18.89	0.11	$C_{15}H_{10}O_6$	286.23	Fatty acid +
Procyanidin B	18.22	1.88	$C_{30}H_{26}O_{13}$	594.50	Fatty acid +

Note. GC-MS: Gas chromatography mass spectrometry; RT: Retention time; PA: Peak area; m/z: Molecular ion; OH: Hydroxy. + indicates fatty acid derivatives.



(a) (b)

Figure 1. Standard curves for the determination of total phenolic **(a)** and flavonoid **(b)** contents of the avocado seed extract

the extract-treated groups when compared to the normal control group.

Anticancer activities

Benchtop growth inhibitory assay

The result confirmed that the extract significantly ($P = 0.001$) increased the mortality rate of tadpoles and inhibited the growth of guinea corn (*Sorghum bicolor*) radicles in a concentration-dependent manner. At the highest (160 µg/mL) concentration, 100% mortality was detected after 30 minutes (Figure 4b). The lowest growth was found at a concentration of 160 µg/mL. At the same time, the control (untreated) had the highest growth of radicles at 96 hours; the radicles had average lengths of 16.04 ± 2.01 mm in a control Petri dish, while the Petri dish containing seeds treated with 160 µg/mL of the seed extract had the lowest average length of radicles of 2.24 ± 0.01 mm, representing a 76.14% reduction (Figure 4a).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay

The anticancer effects of ASE were evaluated on MCF-7 and HMVII at 0 µg/mL to 40 µg/mL concentrations. The result revealed a dose-dependent activity that was much more noticeable on the human MCF-7 BC cell lines than on the vaginal melanoma HMVII cell lines after 48 hours of exposure. The ASE showed IC_{50} values of 16.51 ± 1.02

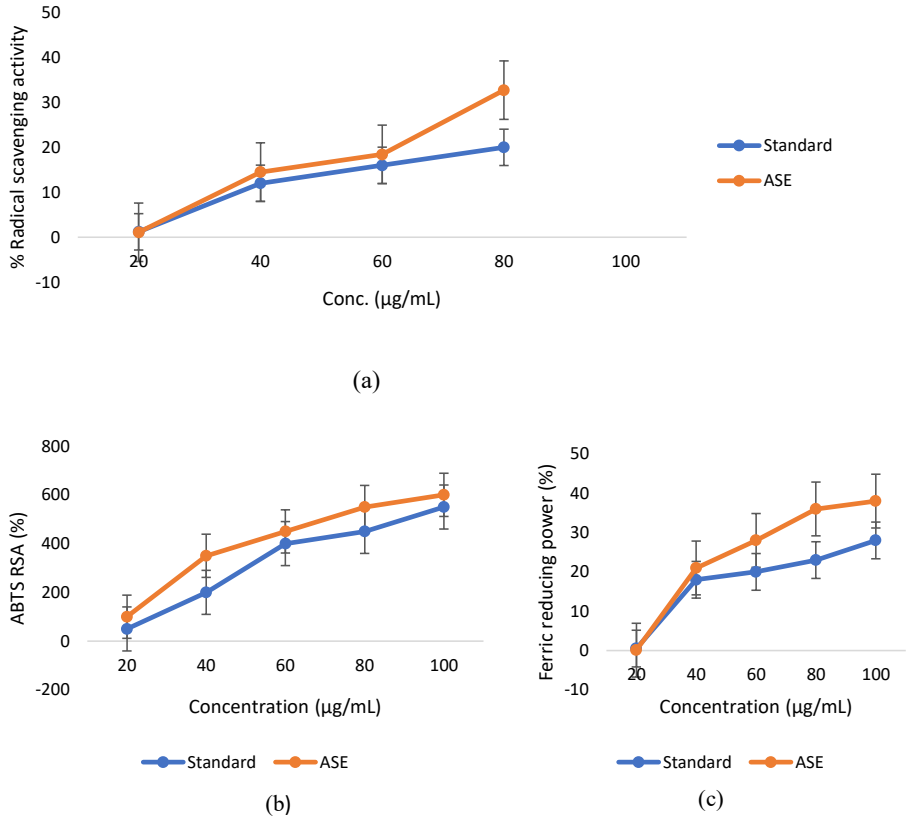


Figure 2. *In vitro*, antioxidant activity of the avocado seed extract and control (standard) using DPPH **(a)**, ABTS **(b)**, and FRAP assays. *Note*. ANOVA: Analysis of variance; DPPH: Folin-Ciocalteu, 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; FRAP: Ferric reducing antioxidant power. The results are expressed as mean ± standard deviations (n = 3). A *P*-value < 0.05 was statistically significant versus the control using one-way ANOVA, followed by Dunnett's post hoc test (*P* = 0.001)

(SI = 26.56) against MCF-7 and 31.71 ± 2.03 (SI = 7.3) against HMVII cell lines when compared (*P* = 0.001) to doxorubicin standard drug (Tables 3 and 4). Similarly, there were significant (*P* < 0.05) reductions in MCF-7 cancer cell percentage viability compared to HMVII cells by ASE, isolated compounds, and doxorubicin in a concentration-dependent manner, as revealed by the trypan blue exclusion assay (Figures 5 and 6).

Caspase-3-like activity

The result demonstrated a concentration-dependent (0–40 µg/mL) increase in caspase-3-like activities between the extract and doxorubicin against MCF-7 cancer cell lines (Figure 5). This increase also resulted in early and late apoptosis and necrotic cells observed from the flow cytometry fluorescence images. The extracts potentially reduced the percentage of live cells in both MCF-7 and HMVII cell lines when compared (*P* = 0.001) to untreated cells at the same concentrations, with rutin having the most elevated levels of caspase-3-like activity (Figures 7-9a and 9b).

Discussion

For decades, the use of medicinal plants has been centered on the treatment of diseases rather than prevention in traditional medicine. However, research on pharmacognosy and medicinal plant chemistry has shown that medicinal plants contain secondary metabolites that can potentially prevent diseases. In most developing and underdeveloped countries, the use of medicinal plants has gained much popularity through the practice of traditional medicine. Traditional medicine has been defined as the total of all skills and practices, whether explicable or not, used in the diagnosis, prevention, and eradication of physical, mental, or social imbalance, which exclusively relies on practical experience and observation handed down from generation to generation, whether verbally or in writing (1).

The current study's preliminary phytochemical analysis revealed that the avocado seed methanol extract contains alkaloids, flavonoids, phenols, tannins, triterpenoids/steroids, and fats/oils (Table 1). These phytoconstituents play crucial roles in achieving specific therapeutic effects in the body. For instance, flavonoids have been reported to possess an antioxidant effect, thereby protecting the cells in the body from developing some diseases, especially cancers resulting from oxidative damage by reactive oxygen species (ROS) (25). Flavonoids have also been shown to inhibit the activity of certain enzymes and facilitate the production of certain hormones and neurotransmitters and free radical scavenging activity. The phytochemicals found in this study were also obtained by previous research (9), except saponins and anthracenes, which were absent in the current study.

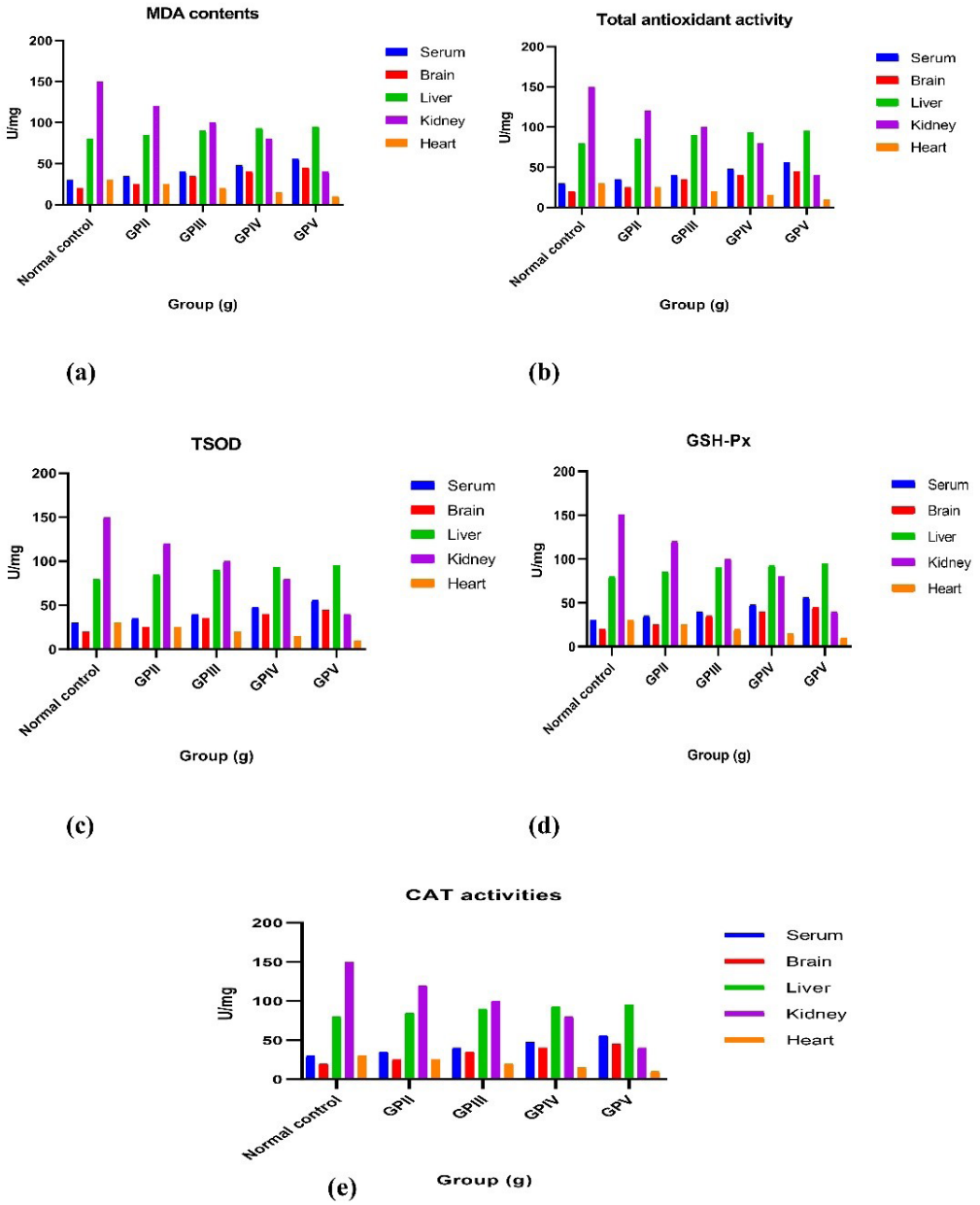


Figure 3. *In vivo* antioxidant effects of crude ASE on malondialdehyde contents **(a)**, total antioxidant activity **(b)**, total superoxide dismutase activities **(c)**, glutathione peroxidase activities **(d)**, and catalase activities **(e).** *Note*. ANOVA: Analysis of variance; SD: Standard deviation. The results are expressed as means ± SD (n = 6). *P*-values < 0.05 are statistically significant (one-way ANOVA, followed by Dunnett's post hoc test) compared with the negative control group ($P = 0.001$)

Similarly, the GC-MS demonstrated the presence of many fatty acids, fatty acid derivatives, and polyphenols such as stearic acid, oleic acid, linoleic acid, avocatin, rutin, catechin, and the like (Table 2).

The GC-MS apparatus has emerged as the most important analytical technique in natural product research because of its numerous advantages, such as the small amount of sample needed, easy operation, and ability to analyze samples based on mass-to-charge ratio, base ion peak, fragmentation patterns, and molecular ion number (9,26-28). Some of these compounds revealed by GC-MS have been confirmed to possess antioxidant and anticancer activities. For example, rutin, oleic acid, linoleic acid, avocatin, and catechin have shown potential cytotoxic effects on various types of cancers, such as colon, lung, breast, and leukemia, both in *in vitro* and *in vivo* experiments (29). These compounds undoubtedly played similar roles in the current study. For instance, phenolic compounds consist of large groups of plant compounds having an aromatic ring containing one or more than one hydroxyl group (6). In this study, the TPC was evaluated to be 1178.67 mg GAE/g. In contrast, the TFC was 86.24 mg RE/g (Figure 1). The high TPC and TFC obtained for 'Mambilla' ASE may be due to cold climatic conditions throughout the year in the 'Mambilla' plateau, Nigeria. This is because polyphenols are mainly affected by changes in temperature, resulting in various chemical properties, such as redox properties and the presence of hydroxyl groups, which help in structural modifications and chemical races in many plant families (30). Furthermore, medicinal plant polyphenols are essential for human nutrition because of their antioxidant

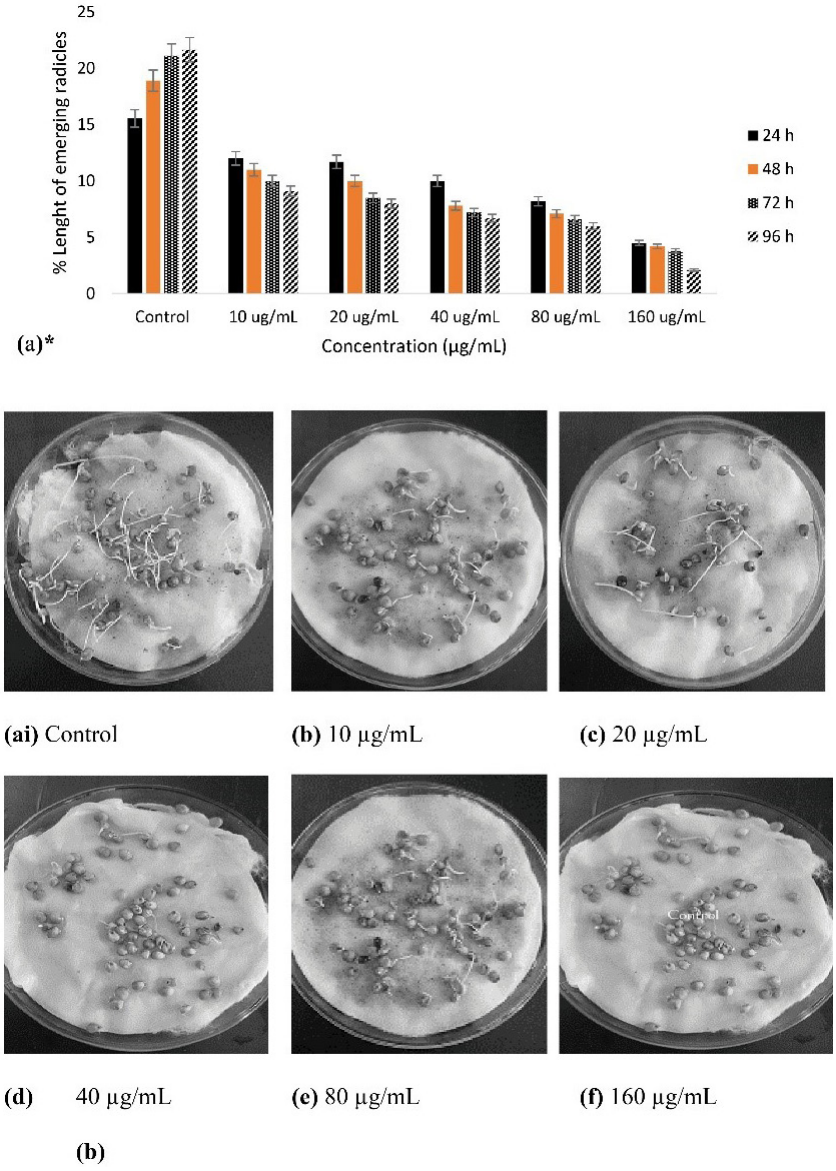


Figure 4. Effects of various concentrations of the avocado seed extract on tadpole mortality (a)* and the growth of emerging *Sorghum bicolor* radicles (b) after 96-hour incubation. *Note*. Figures a-f illustrate Petri dishes used for growing the seeds of *Sorghum bicolor*

Table 3. Effects of avocado seed crude extracts on human breast cancer cell lines (MCF-7)

Sample	MCF-7 IC_{50} (µg/mL)	Vero E6 CC_{50} (µg/mL)	SI
Normal control	368.15 ± 5.04	504.04 ± 2.08	-
Crude ASE	16.51 ± 1.02*	438.54 ± 5.14	26.56
Doxo. (2 mg)	6.62 ± 0.01*	112.02 ± 2.44	16.92
Oleic acid	26.12 ± 2.01*	226.14 ± 2.04	8.66
Linoleic acid	8.02 ± 0.01*	302.11 ± 4.01	37.67
Palmitic acid	10.01 ± 1.06*	108.06 ± 2.12	10.80
Ferulic acid	34.25 ± 2.11*	120.23 ± 1.24	3.51
Rutin	4.14 ± 0.01*	240.04 ± 2.22	57.98
Coumaric acid	22.08 ± 2.04*	234.30 ± 2.08	10.61

Note. The results are expressed as means ± standard deviations (n = 3). ASE: Crude avocado seed extract; Doxo.: Doxorubicin; SI: Selective index; IC_{50}: Half-maximal inhibitory concentration; CC_{50}: 50% cytotoxic concentration; ANOVA: Analysis of variance. *Statistically significant ($P = 0.001$, one-way ANOVA, followed by Dunnett's post hoc test) compared to control. Distilled water (10 mL) was used as the normal control. The concentration of 40 µg/mL was utilized in this study.

Table 4. Effects of avocado seed crude extracts on human vaginal melanoma cancer cell lines (HMVII)

Sample	HMVII IC_{50} (µg/mL)	Vero E6 CC_{50} (µg/mL)	SI
Normal control	382.44 ± 4.02	608.26 ± 4.04	-
40 µg/L ASE	31.71 ± 2.03*	248.12 ± 2.11	7.83
Doxo. (2 mg)	10.12 ± 1.02*	108.24 ± 1.08	10.70
Oleic acid	33.01 ± 2.04*	201.23 ± 4.04	6.10
Linoleic acid	28.04 ± 0.01*	112.42 ± 2.01	4.00
Palmitic acid	32.08 ± 2.02*	128.08 ± 1.14	3.99
Ferulic acid	42.15 ± 2.24*	130.44 ± 2.34	3.09
Rutin	14.06 ± 1.01*	160.04 ± 1.02	11.38
Coumaric acid	42.18 ± 4.01*	110.24 ± 2.02	2.61

Note. The results are provided as means ± standard deviations (n = 3). ASE: Crude avocado seed extract; Doxo.: Doxorubicin; SI: Selective index; ANOVA: Analysis of variance; IC_{50}: Half-maximal inhibitory concentration; CC_{50}: 50% cytotoxic concentration. *Statistically significant ($P = 0.001$, one-way ANOVA, followed by Dunnett's post hoc test) compared to the control. Distilled water (10 mL) was utilized as the normal control. The concentration of 40 µg/mL was considered in this study.

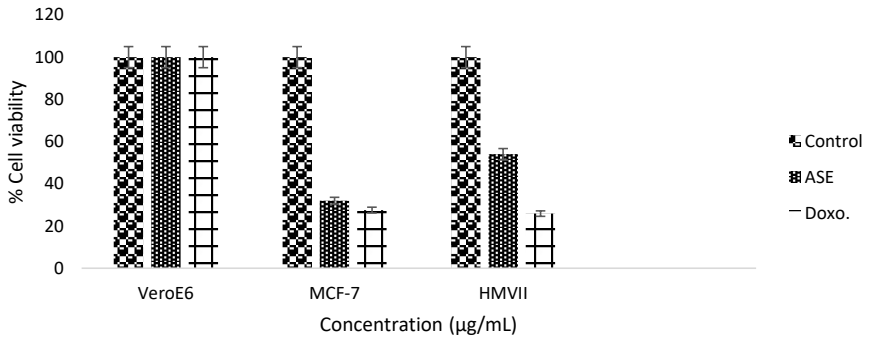


Figure 5. Effects of the avocado seed extract on percentage cell viability of cancer cells by trypan blue exclusion assay method. *Note*. ANOVA: Analysis of variance. The results are expressed as means ± standard deviations (n = 3). $P < 0.05$ was statistically significant versus the control using one-way ANOVA, followed by the Dunnett's post hoc test ($P = 0.001$)

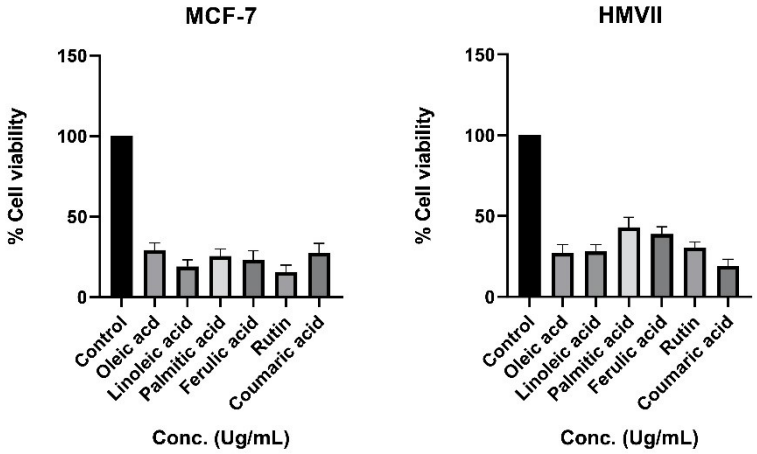


Figure 6. Effects of isolated polyphenols and fatty acids from avocado seed extract on percentage cell viability of cancer cells at various concentrations ((0-40 µg/mL) by trypan blue exclusion assay method. *Note*. The results are presented as means ± standard deviations (n = 3). ANOVA: Analysis of variance. $P < 0.05$ was considered statistically significant versus the control using one-way ANOVA, followed by the Dunnett's post hoc test ($P = 0.001$).

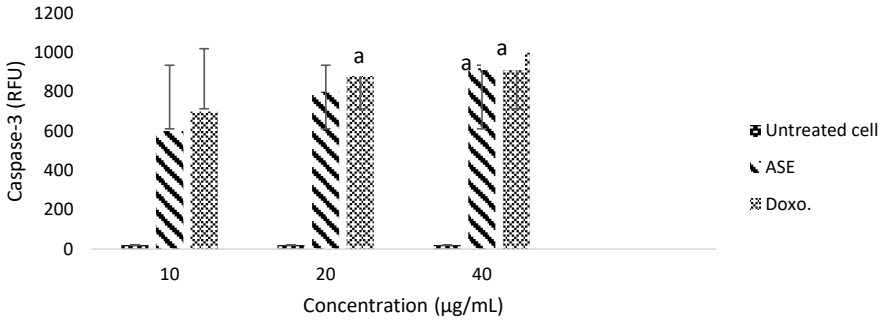


Figure 7. Effects of avocado seed extract on caspase-3-like activity of treated and untreated MCF-7 breast cancer cell lines. *Note*. ANOVA: Analysis of variance. The results are expressed as means ± standard deviations (n = 3). $^{a}P < 0.05$ was statistically significant versus the control using one-way ANOVA, followed by the Dunnett's post hoc test ($P = 0.001$)

potential and ability to reduce tissue damage caused by oxidative stress, which is linked to many human diseases.

In addition, the high TPC and TFC are responsible for the antioxidant and anticancer activities of the 'Mambilla' ASE. The findings revealed concentration-dependent activities of DPPH, ABTS, and FRAP radical scavenging. For instance, the DPPH and ABTS radical scavenging assays had IC_{50} values of 25.25 µg/mL and 38.82 µg/mL, respectively. FRAP radical scavenging had an IC_{50} value of 43.99 mM Fe (II) E (Figures 2a-2c). These superoxides are reactive species capable of causing cellular and DNA damage in the body, resulting in various diseases (27). The ability of the extract to inhibit radical scavenging activities of these superoxide species, based on the values obtained in the IC_{50}, further demonstrated its antioxidant potential.

Furthermore, the *in vivo* antioxidant assays confirmed that the crude ASE decreased MDA contents in most organs and tissues, except for the kidney (Figure 3a). The results of the current study showed a dose-dependent reduction in MDA contents within the experimental animals. It has been reported that MDA is one of the end-products of unsaturated fatty acid peroxidation. An

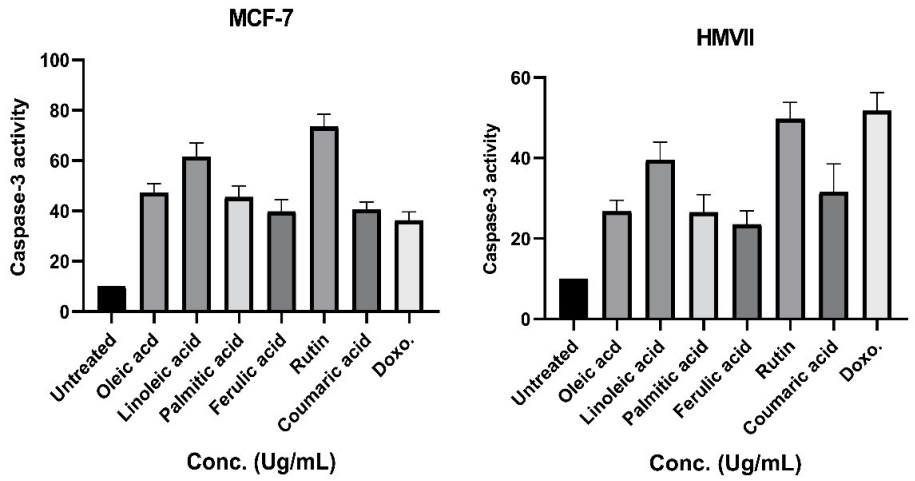


Figure 8. Effects of isolated polyphenols and fatty acids isolated from avocado seed extract on caspase-3-like activities at various concentrations (0-40 µg/mL). *Note*. ANOVA: Analysis of variance. The results are expressed as means ± standard deviations (n = 3). *P*-value < 0.05 was considered statistically significant versus the control using one-way ANOVA, followed by the Dunnett's post hoc test (*P* = 0.001)

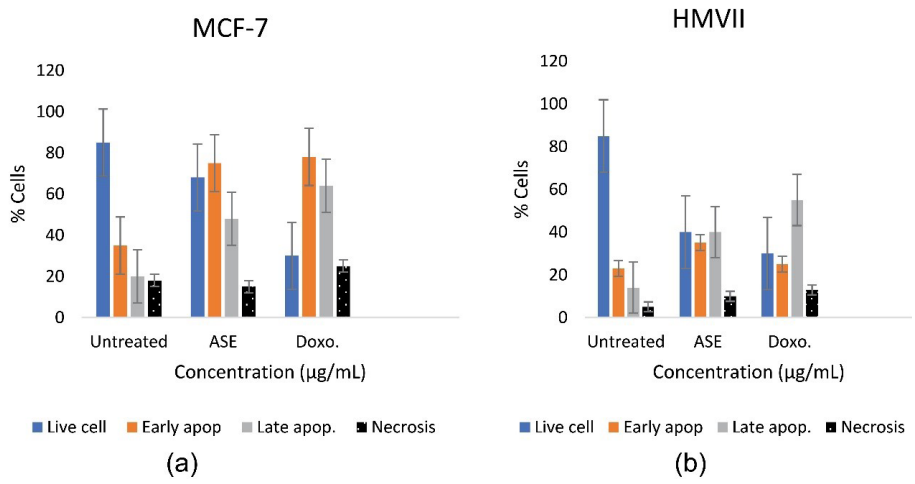


Figure 9. Effects of avocado seed extract on apoptosis induction in treated and untreated cell lines. *Note*. The results are expressed as means ± standard deviations (n = 3). A *P*-value of less than 0.05 was statistically significant versus the control using one-way ANOVA, followed by the Dunnett's post hoc test (*P* = 0.001). ANOVA: Analysis of variance; Apop: Apoptosis; Doxo.: Doxorubicin

elevated rise in free radicals results in the production of MDA above the normal range (31). The MDA level is usually used as a marker for oxidative stress and the antioxidant status in cancer patients. Therefore, reduced values of MDA represented the antioxidant capacity of the extract.

Similarly, the numerical value obtained for total antioxidation (Figure 3b) is the body's overall antioxidant effects. It could also denote the body's ability to prevent antioxidant damage (11). Moreover, TSOD, CAT, and GSH-Px are antioxidant endogenous enzymes vital in antioxidant prevention (20). These enzymes are the first to defend the body against ROS by converting superoxide anions into hydrogen peroxide (17). The roles of these enzymes were not different in the current research (Figures 3c-3e). Based on our findings, the levels of these enzymes decreased dose-dependently in ASE-treated groups compared to control ($P < 0.05$). This implies that the body's defense against ROS was achieved using both non-enzymatic and enzymatic methods, according to the *in vitro* and *in vivo* experiments. The elevated values of GSH-Px observed in the study justify the reduced levels of the obtained LPO. Many polyphenols and fatty acids have been reported to have shown various levels of antioxidant activities in animals (32), which conforms to the results of this study.

In evaluating the anticancer activity of the 'Mambilla' ASE, the preliminary cytotoxic and antiproliferative effects of the extract were determined by the benchtop assay (21). Benchtop assays have been used to assess the potential of medicinal plant extracts to exert cytotoxic effects on some organisms, such as nauplii of *Artemisia salina*, larvae of mosquitoes, and tadpoles (21). These assays have reproducibility, are easy to perform, and are used to evaluate plant extracts for antitumor activity. In the current study, the extract showed significant concentration-dependent activities in both experiments ($P < 0.05$). This is because 100% mortality was detected in the beaker containing tadpoles (*R. raninus*) treated with the highest extract concentration (160 µg/mL) after

30 minutes compared to tadpoles in the control beaker. Similarly, the extract caused a potential decrease in the length of emerging guinea corn radicles at a concentration of 160 µg/mL with an average length of 2.24 ± 0.01 mm after 96 hours of incubation (Figure 4). The extract may have exhibited cytotoxicity and antiproliferative activities by interfering with some growth-regulating processes such as mitosis and creating biochemical imbalances within the cells of tadpoles and guinea corn (21).

Moreover, the cytotoxicity study of the extract on selected cancer cell lines further corroborates the findings from the benchtop assays. For instance, the 'Mambilla' ASE represented an IC_{50} value of 16.51 ± 1.02 µg/mL and an SI value of 26.56 against the MCF-7 cell lines, as well as an IC_{50} value of 31.71 ± 2.03 µg/mL and an SI value of 7.83 against the woman vaginal melanoma cancer cell line HMVII (Tables 3 and 4). The obtained results are statistically significant ($P < 0.05$) when compared to the standard drug doxorubicin with IC_{50} values of 6.62 ± 0.01 µg/mL (SI = 16.92) and 10.12 ± 1.12 µg/mL against the MCF-7 and HMVII cells, respectively. The findings confirmed that the extract was cytotoxic on both cell lines with an IC_{50} less than 50 but more potent on the MCF-7 BC cell lines than the vaginal melanoma cell line HMVII. The extract could also discriminate the MCF-7 cancerous cell from the regular Vero E6 cell lines than it did against HMVII cell lines. The variation in SI values was because different cancer cells possessed unique markers recognized by chemotherapeutic agents differently (8). This therapeutic characteristic is crucial for any anticancer agent's effectiveness in cancer treatment (33). Similarly, a reduction in cancer cell viability was observed when the cell lines were exposed to various extract concentrations in a concentration-dependent manner (Figures 5 and 6).

It is known that cancer cells have higher levels of oxidative radicals or ROS due to their higher rate of metabolism and cancerous activities different from normal cells (34). Thus, cancer cells lack adequate antioxidant processes needed to inhibit the radical scavenging activity of these reactive species. Therefore, the observed anticancer effects of the ASE may be due to the high number of polyphenols and fatty acids that distort the redox balance required for the viability of the MCF-7 and HMVII cell lines. In the current study, the crude ASE and isolated polyphenol and fatty acids significantly increased the caspase-3-like activity in a concentration-dependent manner (Figures 7 and 8). Caspase-3 is necessary for some processes connected with the disassembling of cells and the formation of apoptotic bodies initiating cell apoptosis (35). It may also play its role before or at the stage when loss of cell viability is achieved (36). Based on the results of the study, elevated levels of caspase-3-like activity induced by the extract implied that the extract induced both early and late apoptosis in both MCF-7 and HMVII cancer cell lines (Figure 9). The ability of the extract to achieve this was because of the presence of specific bioactive compounds revealed in the plant part. For example, certain fatty acids have been reported to induce apoptosis using the caspase-3 enzyme pathway in cancer cells (24). The activity of the ASE in the current study was similar due to the presence of various classes of compounds such as fatty acids, fatty acid-derived compounds, and polyphenols.

Conclusion

Our findings revealed that the avocado seed (*P. americana*) methanol extract contains a high number of polyphenols and fatty acids responsible for its *in vitro* and *in vivo* antioxidant and anticancer activities against the MCF-7 (human BC cell lines) and HMVII (woman vaginal melanoma cells) with a high SI. The findings further justified the preference for the 'Mambilla' avocado pear over any other avocado in Nigeria and the traditional use of the seeds for cancer treatment and management. It is suggested that further studies be conducted to isolate the main bioactive compound responsible for the observed activities related to drug discovery.

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Authors' Contribution

Conceptualization: Cletus Anes Ukwubile.
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Formal analysis: Cletus Anes Ukwubile, Aishat Omolabake Makinde.
Investigation: Cletus Anes Ukwubile, Aishat Omolabake Makinde.
Methodology: Cletus Anes Ukwubile, Aishat Omolabake Makinde.
Project administration: Cletus Anes Ukwubile.
Writing-original draft: Cletus Anes Ukwubile.
Writing–review & editing: Cletus Anes Ukwubile, Aishat Omolabake Makinde.

Competing Interests

The authors declare that they have no conflict of interests.

Ethical Approval

This study was approved by the Research and Ethics Committee of the PJ Rat Farms Jos Nigeria (with approval number PJF/001/RAT/2023).

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