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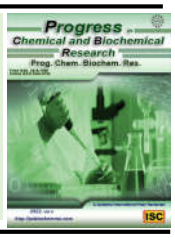

Original Research Article

Phytochemical evaluation, *in vitro-in vivo* antioxidant and cytotoxicity activities of various layers of watermelon fruit *Citrullus lanatus* (Cucurbitaceae) Matsum. & Nakai

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ABSTRACT

Citrullus lanatus (watermelon) is a popular plant whose fruits are use in all parts of Nigeria as remedy for various diseases. In this present study, the phytochemical contents, antioxidant and cytotoxicity activities of extracts from the various layers (peel, rind, pulp and seeds), of watermelon fruit were evaluated. The phytochemical contents were evaluated qualitatively while NMR and GC-MS used to identify phytoconstituents present. Antioxidant activities were examined by DPPH, H2O2 radical scavenging, ORAC, FRAP, and ABTS assays. In vitro cytotoxic activity was examined by MTT assay while apoptosis was detected by flow cytometry as well as fluorescence microscopy. Phytochemical evaluation showed the presence of various metabolites in the pulp and seed extracts. GC-MS and NMR elucidations of compound revealed the presence of methyl stearate ester. Antioxidant evaluations by various radical scavenging showed that the pulp (PP) had IC50 value of 8-22 µg/mL, indicating potential antioxidant effect. In vivo evaluation of superoxide dismutase, catalase activities and total proteins in serum of rats further confirmed the in vitro studies. Antiproliferative study showed that the pulp and the seeds significantly displayed concentration-dependent effects. These results were further replicated in the cytotoxicity effect against MCF-7 and HMVII cells with much reduced IC50 on MCF-7, as well as early and late apoptosis after 72 h caused by exposure of pulp extract in vitro. Our study showed that the pulp and seed layers of watermelon contain phytochemicals which were responsible for its antioxidant and cytotoxicity effects

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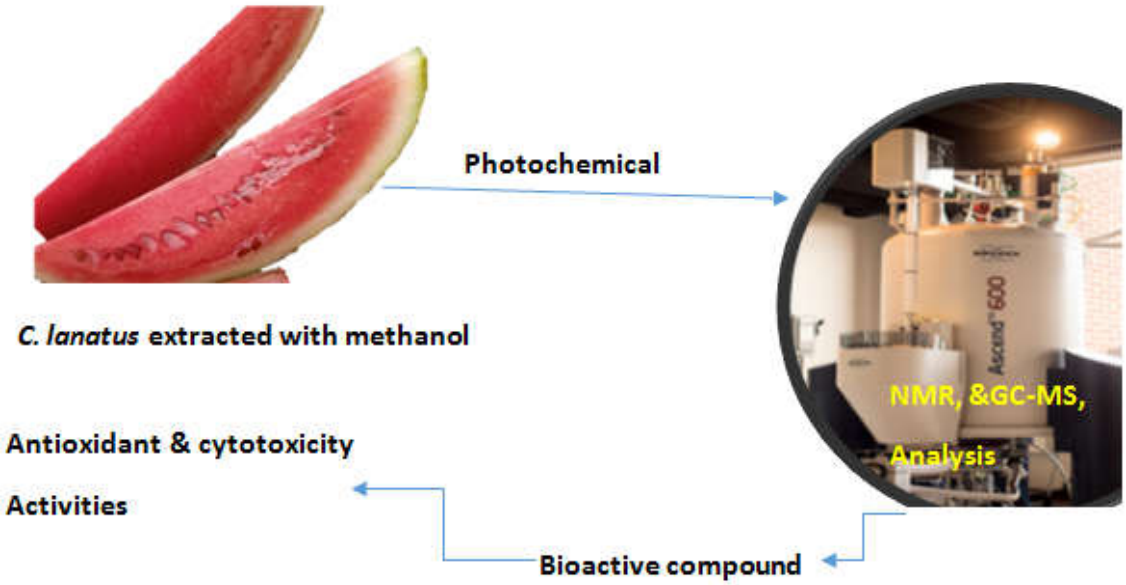
GRAPHICAL ABSTRACT



1. Introduction

Researches on the investigation of compounds of plant origin are expanding worldwide, especially in the developing countries like Nigeria, in which the use of herbal medicine is widely popular for their primary health needs. It is known that medicinal plants have been utilized in all over the universe since the ancient times for the treatment of various diseases, including asthma, abdominal disorders, skin diseases, respiratory, and urinary complications, as well as liver and cardiovascular disease [1]. Thus, the importance of the search for natural products with antioxidant effect is emphasized, as they are able to prevent, stabilize, or disable free radicals before they attack biological targets in cells like DNA, proteins, and lipids [2].

Antioxidants are substances which protect the body cells against free radicals, which may play a role in heart diseases, cancer, and the other diseases [3]. Free radicals are formed from molecules through the breakage of food enzymes in the body or breakage of a chemical bond. Antioxidants, such as vitamin C and E as well as carotenoids may help protect cells from damage by free radicals. The other naturally occurring antioxidants include flavonoids, tannins, phenols, and lignans. These include fruits, vegetables, whole grains, nuts, seeds, herbs, and spices [4]. Dietary antioxidants can act as free radical scavengers, radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors [5]. Therefore, there is an increasing interest in extending the range of antioxidants which can be used as food ingredients to prevent food oxidation.

Citrullus lanatus (Thunb.) is one of the popular fruits grown in the tropics. It is grown in the favorable climates from tropical to temperate regions worldwide. The juicy part is usually deep red or pink in color, and the seed is black, although there are some seedless varieties. It is consumed as juice or as an ingredient in mixed beverages. The large fruit is a kind of modified berry called a *pepo* with a thick rind (peel) and fleshy center (pulp). The fruit rind is mid- to dark [6], green and usually mottled or striped, and the flesh, containing numerous pips spread throughout the inside, can be red, pink, orange, yellow, or green [7]. Due to the massive water and significant electrolyte content of the fruit, it is largely consumed during hot weather conditions such as summer and heat waves to quench thirst and provide relief for dehydration.

The seeds taste like nuts and can be roasted and eaten raw as a snack, or grounded into flour. The rind, which is the hard exterior of the fruit is used in some countries as a vegetable for making stews, and pickles [8] .

The proposed study was carried out in order to determine the phytochemical contents, *in vitro-in vivo* antioxidant and cytotoxicity activity of various layers of watermelon fruits (Fig. 1) which were collected from Maiduguri, Nigeria.

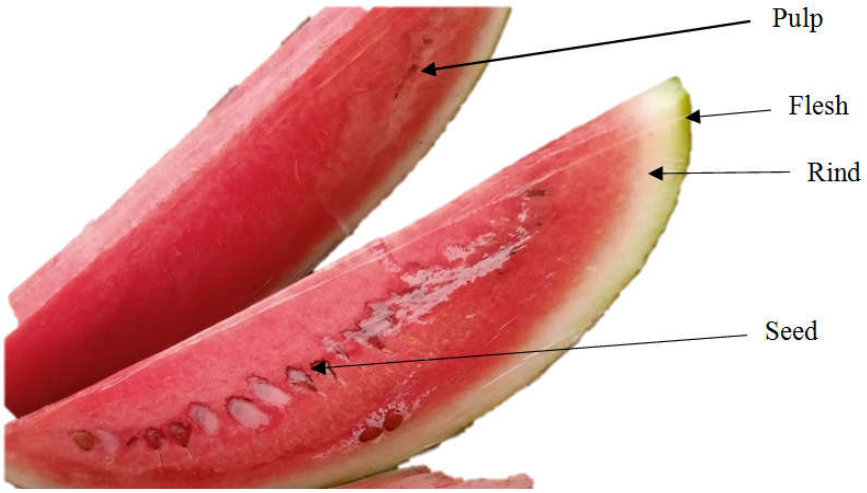


Fig.1. Transverse section through watermelon fruit showing various layers.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and equipment

80 % (v/v) methanol, Folin-Ciocalteu, Rutin, H_2SO_4, annexin v, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid were purchased from Sigma Aldrich (St Louis, USA), UV-vis spectrophotometer (Jenway™ model 7315, Fisher Scientific, UK), Carry 3500 UV-vis spectrophotometer (Agilent Technologies, USA), flow cytometry, Bio-assay reader (Perkin Elmer HTS 7000, USA), rotary evaporator (BUCHI, Germany). The other chemicals were of analytical grades.

2.1.2. Laboratory animals

Fifty (50) Wistar rats of both sexes weighing 150-180 g were purchased from the animal house of the Department of Pharmacology, University of Jos, Nigeria. Ethical consideration guiding the use of animals in research by the Ethical Committee of the University of Jos was strictly followed such careful handling of the animals among others. The animals were allowed to acclimatize in the laboratory for one week with access to water and feeds *ad libitum*.

2.2. Methods

2.2.1. Collection and identification of plant materials

Citrullus lanatus fruits were collected fresh in the early morning hours in June, 2021, from Jakana, Borno State, Nigeria. The selected fruits were greenish in color and heavy in weight. The plant was authenticated by Mr. C. A. Ukwubile (a Pharmacognocist) of the Department of Pharmacognosy, Faculty of Pharmacy, University of Maiduguri, Nigeria, in which a voucher number of *UMM/FPH/CUB/002* was deposited for the plant in the herbarium.

2.2.2. Extraction of plant materials

The fruits were peeled and both the exocarp and then, the seeds were shade-dried at room temperature for a period of two weeks prior to grinding into fine powder. The powdered sample was stored in a freezer prior to the extraction.

500 g of each extract was weighed and added to 1000 mL of methanol and kept for 24 h at room temperature using cold maceration technique. The resulting mixtures were filtered with Whatman No.1 filter papers. The exocarp filtrate was concentrated using water bath at a temperature of 64.7 ^{0}C. The solvents were then taken out of the seed filtrate by rotary evaporation at 100 r/min (220 v; 50/60 Hz).

2.2.3. Phytochemical evaluation of extracts

The presence of major metabolites in the three layers of the fruit such as alkaloids, glycosides, carbohydrates, fats and oils, etc. was determined following the standard procedures previously described by [9-11]. At least two different tests were carried out to determine the presence or absence of the metabolites in the extracts. GC-MS and NMR were used to determine the type of the presented compounds [12].

2.2.4. Antioxidant activity by DPPH radical scavenging activity of extracts

The percentage antioxidant activity (% RSA) of each extract was obtained using the DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical absorbance assay, according to the procedure described by [13-14]. Exactly 24 mg DPPH was dissolved with 100 mL methanol to attain an absorbance of about 0.98 ± 0.02 at 517 nm using UV-vis spectrophotometer (Jenway™ model 7315, Fisher Scientific, UK), and was stored at 20 ^{0}C prior to analysis. The reaction mixture contained 100 µL of extract and 3 mL of DPPH in methanol solution at different concentrations of 100, 200, 300, 400, and 500 µg/mL. The positive control was ascorbic acid at the same sample concentration. The percent DPPH scavenging effect was calculated using the following equation:

$$\% \ RSA = [(Absorbance_{control} - Absorbance_{test\ sample})/Absorbance_{control}] \times 100 \quad (ii)$$

2.2.5. Hydrogen peroxide (H_2O_2) radical scavenging activity of extracts

H_2O_2 radical scavenging activity was determined using the procedure previously described by [15]. Briefly, various concentrations 100, 200, 300, 400, and 500 µg/mL of the extracts were separately mixed with 0.1 mL of 2 mM H_2O_2 solution in 50 mM PBS solution (pH 7.4), and incubated at 45 ^{o}C for 10 min. The solution was measured at 230 nm absorbance. Ascorbic acid was used as positive control. The % of inhibition was then evaluated by comparing the control absorbance to that of the test samples using the following formula. The raw data obtained for anti-oxidation were compared with that of the reference ascorbic acid.

$$\% \ H_2O_2 \ RSA = (Absorbance_{control} - Absorbance_{test\ sample})/Absorbance_{control}] \times 100 \quad (iii)$$

In which, RSA = radical scavenging activity.

2.2.6. Oxygen radical absorbance capacity (ORAC) assay

An automated plate reader (KC4, Bio Tek, USA) containing 96-wells [16-17]. as well as Phosphate buffer (pH 7.4; 37 ^{o}C) were used. In order to generate peroxyl radical, freshly prepared 2, 2'-azobis (2-amidino-propane) dihydrochloride was utilized for each experiment. The excitation value of 485 nm and emission value of 520 nm were used as the fluorescence conditions. The results were expressed as mM TE/g fresh mass.

2.2.7. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the procedure described by [18], with slight modifications.

2.2.8. ABTS (2,2'-azinobis-3 ethylbenzothiazoline-6sulfonate) assay

The antioxidant capacity assay was evaluated by the method previously described [19-20]. Briefly, ABTS was prepared reacting 7 mM ABTS & 2.45 mM potassium persulfate, and incubated for 16 h at 23 °C. The solution was thereafter diluted with 90 % ethanol and measured at 734 nm absorbance using UV-vis spectrophotometer.

2.2.8. Determination of total phenolic contents

The total phenolic contents was evaluated using the Folin- Ciocalteu reagent method previously described by [21]. Briefly, 1 mL of extracts was each diluted with 1 mL of Folin- Ciocalteu reagent in a ratio of 1:1. After 5 min, 10 mL of 70 % Na_2CO_3 solution was added to the mixture and the volume was made up to 13 mL with deionized water, the mixture was thoroughly shaken. The mixture was measured with UV-vis spectrophotometer at 750 nm at room temperature. The total phenolic content was then determined from the linear calibration curve using gallic acid as a reference. It was expressed as mg of gallic acid equivalent per gram of dried extracts obtained from a standard curve of gallic acid.

2.2.9. Determination of total flavonoid contents

In determining the flavonoid contents, aluminum chloride ($AlCl_3$) colorimetric assay was used with slight modifications. Briefly, 5 mL extract was mixed with 10 mL of 30% methanol, 2 mL $NaNO_2$ and 2 mL of $MgCl_2.H_2O$. After 5 min, 1 mL of NaOH was added to the mixture. The mixture absorbance was recorded at 506 nm against blank. Rutin solution prepared at 0-100 µg/mL concentrations was used as a standard for the calibration curve. The total flavonoid content was then expressed in µg/mL rutin equivalent dry weight [14].

2.2.10. Determination of the in vivo antioxidant activity of the extract

Thirty Swiss albino Wistar rats of both sexes were randomly grouped into five groups of six animals. Group I was the negative control group which received 0.5 mL of distilled water orally (p.o.), Group II was the positive control group administered with 0.2 mL dimethyl sulfoxide (Rimso-50®, Guangdong Guanghua Sci-Tech Co. Ltd, China) (p.o.), Group III was administered 100 mg/kg of extracts, Group IV was administered 200 m/kg of extracts, while Group V was administered 400 mg/kg of extracts (p.o.). The animals were dosed three times a week for 14 days. They were observed daily for signs of toxicity and mortality in the period of the study. On the last day of the study, the animals were chemically sacrificed and blood was immediately collected from the animals by direct cardiac puncturing. Fresh blood collected were stored in EDTA bottles and kept in a refrigerator at -20 ^oC for further usage [14, 22-23].

2.2.10.1. Preparation of serum

Serum was prepared following the procedure previously described by [14,24]. In this procedure, fresh blood collected from the animals by cardiac puncturing into EDTA bottles were allowed to clot for 30 min and then, they were centrifuged at 2000 rpm for 15 min in order to harvest the serum from the upper layer of the bottles.

2.2.10.2. **Determination of lipid peroxidation (LPO)**

Thiobarbituric acid reactive substance (TBARS) and malondialdehyde (MDA) production levels were measured following modified procedures previously described by [14,25]. Briefly, 50 µL serum was de-proteinized by adding 1 mL of 14 % trichloroacetic acid plus 1 mL 0.6 % thiobarbituric acid. The mixtures were heated in a Precision 280 Digital Water Bath ±0.2°C at 37 °C (Marshall Scientific, USA) for 30 min and cooled on ice block for 5 min. It was then centrifuged at 2000 rpm for 10 min and the TBARS absorbance was measured at 535 nm using a Carry 3500 UV-vis spectrophotometer (Agilent Technologies, USA). The TBARS concentration was calculated using molar extinction coefficient of malondialdehyde (1.56×10^5 mol/L/cm) from the formula below:

$$A = \Sigma CL \qquad \text{(iv)}$$

In which, A = absorbance, Σ = molar coefficient, C = concentration, and L = path length.

The obtained results were expressed in nmol/mg of protein [14].

2.2.10.3. Determination of superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined following the procedure described by [26]. Briefly, xanthine-xanthine oxidase system was used to produce a superoxide flux, while nitro-blue tetrazolium (NBT) was used as superoxide production indicator. The degree of inhibition of enzyme unit reaction giving 50% inhibition of NBT reduction was taken as a measure of SOD activity (Onoja et al., 2014). The results obtained were expressed as U/mL.

2.2.10.4. Determination of catalase activity (CA) in serum

Catalase activity (CA) in serum was determined following the procedures which was previously described by [14, 27] with slight modification. In this case, 10 µL serum was added to test tube containing 2.50 mL 50 mM potassium phosphate buffer at neutral pH. Furthermore, 0.1 mL fresh 30 mM H_2O_2 solution was added to the mixture. H_2O_2 decomposition rate was measured at 240 nm absorbance for 5 min using a UV-vis (Carry 3500; Agilent Technologies, USA) spectrophotometer. To calculate the CA, 0.041 $mM^{-1} cm^{-1}$ molar extinction coefficient was used.

2.2.10.5. Evaluation of total protein in serum

The amount of protein in the serum was evaluated using total protein kit (TP0300, Micro Lowry, Peterson's modification; Sigma-Aldrich, USA) which uses colorimetric, endpoint method [14].

2.3. Cytotoxicity by benchtop assay

Preliminary anti-proliferative and cytotoxicity activities of the watermelon extracts were evaluated using the methods previously described by [28].

2.4. MTT cell viability assay of extracts

MCF-7 breast cancer cells were grown in 96-well plates (Costar, USA) at seeding rate of 1 x 10^5 cell /well in 100 µL of RPMI 1640 and were allowed to grow in a 5 % CO_2 incubator for 24 h. The medium is then removed and replaced with freshly prepared medium which contain various concentrations of watermelon extracts (1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL) for 48 h. The medium was then washed with 10 % PBS, and 0.5 mg/mL MTT solution was added to each well and incubated for 4 h in the dark at 37 °C for 4 h. The MTT solution was then replaced with 200 µL dimethyl sulfoxide (DMSO) and further incubated for 1 h and the medium was removed. The amount of MTT formazan was directly proportional to the amount of living cells which was determined by determining the optical density at 570 nm using a bio-assay reader (Perkin Elmer HTS 7000, USA). The concentration of the extract that exhibited 50 % lethality on the cancer cells (CC_{50}) was calculated as well as the selective index [29-32].

2.5. Apoptosis detection by flow cytometry and cellular morphology

Cancer cells were cultured in a 24-well culturing plates and treated with 200 µg/mL extract for 48 h. Cells were stained with propidium iodide (PI) and annexin V. Then, cell morphology was observed under 400x magnification using fluorescence microscopy [29,31].

2.6. Statistical analysis

Significant differences between the positive controls and test groups were determined using Graphpad prism 9.1. The value of $p < 0.05$ was considered as being statistically significant (one-way ANOVA).

3. Results

3.1. Phytochemical determination

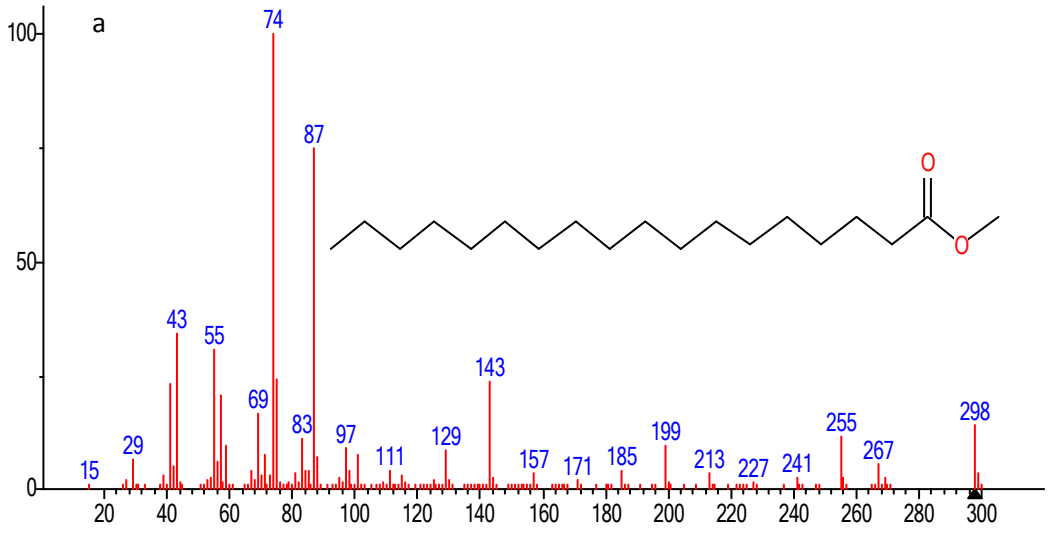
The phytochemical contents of the *Citrullus lanatus* extracts were evaluated using GC-MS and NMR, which revealed the presence of some compounds of which methyl stearate had the highest % abundance according to the GC-MS. Flavonoid component was detected in lager amount in the pulp (PP) and the seed (SD)

methanol extract as seen in Table 1 and Fig. 2. *Thin layer chromatography (TLC) spots revealed other major metabolites in the layers such as carbohydrates, saponins, tannins, terpenes/sterols, and proteins.*

Table 1. Phytochemical analysis of various layers of watermelon fruit methanol extract

Phytoconstituent	Test	Observation			
		Peel	Rind	Pulp	Seed
Alkaloids	Meyer's	-	-	-	-
	Dragendorff's	-	-	-	-
	Wagner's	-	-	-	-
	Tannic acid	-	-	-	-
Saponins	Frothing	-	++	++	-
	Hemolysis	-	++	++	-
Carbohydrates	Molisch's	+	++	++	+
	Fehling's	+	+	++	+
Fixed oils/fats	Spot	-	-	-	+
Tannins	$FeCl_3$	-	-	++	+
Proteins	Millon's	-	-	++	-
	Ninhydrin's	-	-	++	-
Flavonoids	NaOH	+	+	++	+
	Shinoda's	+	++	++	+
	Lead acetate	+	+	++	+
Anthraquinones	Borntrager's	+	-	-	-
Cardiac glycosides	Keller Kiliani's	-	-	++	++
	Kede's	-	-	++	+
Triterpenes	Liebermann's	-	-	-	-

Note: - (not detected), + (detected in moderate amount), ++ (detected in larger amount).



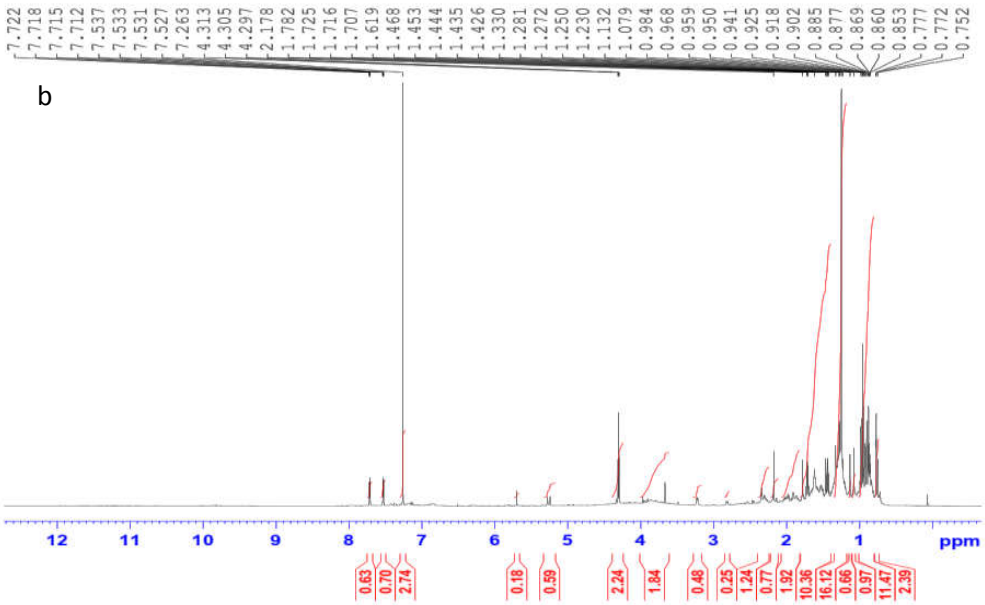


Fig. 2. Characterization of isolated compound from *C. lanatus* pulp extract: a; mass spectra of compound. It is white crystals, m/z: 298.5 mol/g, boiling point:_443 °C; methyl stearate ester (molecular formula: $C_{19}H_{38}O_2$), b; ^{1}H-NMR spectrum.

3.2. In vitro antioxidant activity, total phenolic and total flavonoid contents

The C. lanatus methanol extracts of various layers indicated potent antioxidant activity against DPPH, H_2O_2, ORAC, FRAP, and ABTS. From the study, the most antioxidant activities were obtained in the pulp layer (PP) with IC_{50} value of 8.12 *mg/mL to 22.02 mg/mL followed by the seed (SD) with IC_{50}* value of 14.20 *mg/mL to 32.14 mg/mL scavenging radicals. These values were statistically different compared to their standards ($p < 0.05$; one-way ANOVA). The peel (PL) and the rind (RD) do not illustrate a strong scavenging radical activity (Table 2; Fig. 3). Total phenolic content* of the PP *extract was 123.20 mg of GA/100 g extract, while the SD had 98.45 mg of GA/100 g extract. Similarly, flavonoid content of the PP was 23.30 mg of rutin/100 g extract, while the SD had 18.50 mg of rutin/100 g (Fig. 3).*

Table 2. *In vitro* antioxidant activity of various layers of the extract from *C. lanatus* fruits

Extracts (µg/mL)	Antioxidant activity (% inhibition)				
	DPPH	H_2O_2	ORAC	FRAP	ABTS
PL	28.71±0.65^b	22.10±0.31^b	23.65±0.44^b	42.10±0.23^b	56.14±0.53^b
RD	32.10±0.10^b	45.10±0.42^a	20.33±0.14^b	37.10±0.28^b	22.18±0.31^a
PP	78.56±1.01^b	75.12±1.12^b	88.56±0.06^a	68.04±0.22^b	72.04±0.31^b
SD	62.21±0.54^a	70.23±1.45^b	85.67±1.34^a	52.37±0.64^a	73.65±1.01^b
Standard	82.22±1.34^b	88.00±1.02^b	78.14±0.12^a	58.06±0.24^b	84.08±1.12^a

Values are expressed as mean±SD; n = 3. Values which contain the same superscript alphabets in the same column are significantly different ($p<0.05$; one-way ANOVA followed by Duncan's multiple range test). PL (peel), RD (rind), PP (pulp), and SD (seed).

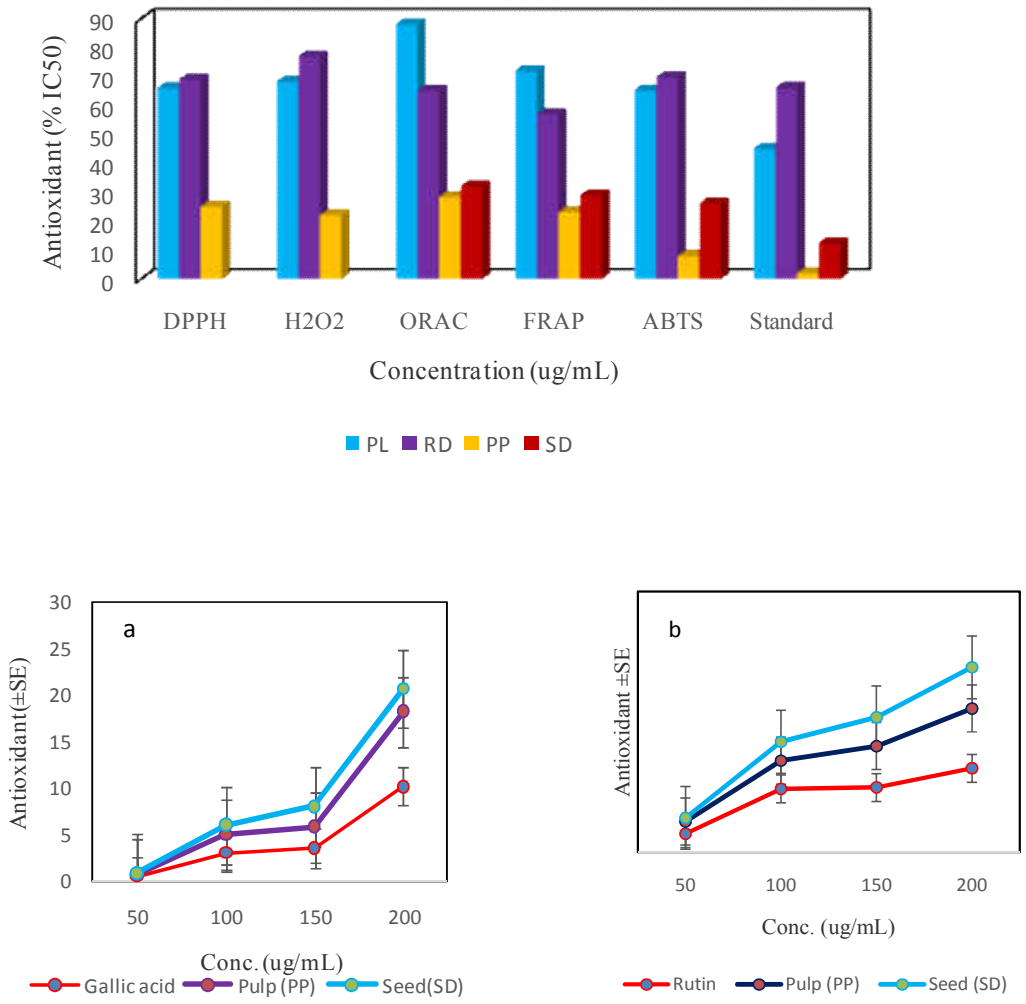


Fig.3. (a) IC_{50} potentials of various layers from *C. lanatus* fruit. Results are mean±SD. Extract concentrations of 50-250 µg/mL were used, (b) total phenolic and flavonoid contents of *C. lanatus* pulp (PP) and seed (SD) layers.

3.3. In vivo antioxidant effects of C. lanatus extracts in Wistar rats

The results of the in vivo antioxidant study of C. lanatus extracts on Wistar rats are displayed in Fig. 4. From the result the pulp (PP) and the seed (SD) extracts produced a dose dependent increase in serum catalase and superoxide dismutase (SOD) activities at dose of 100 to 400 mg/kg b.w. extracts, while there was also dose-dependent decrease in serum malondialdehyde *(MDA) levels. These values were significantly different at the highest dose 400 mg/kg b.w. when compared to the control group ($p < 0.05$).*

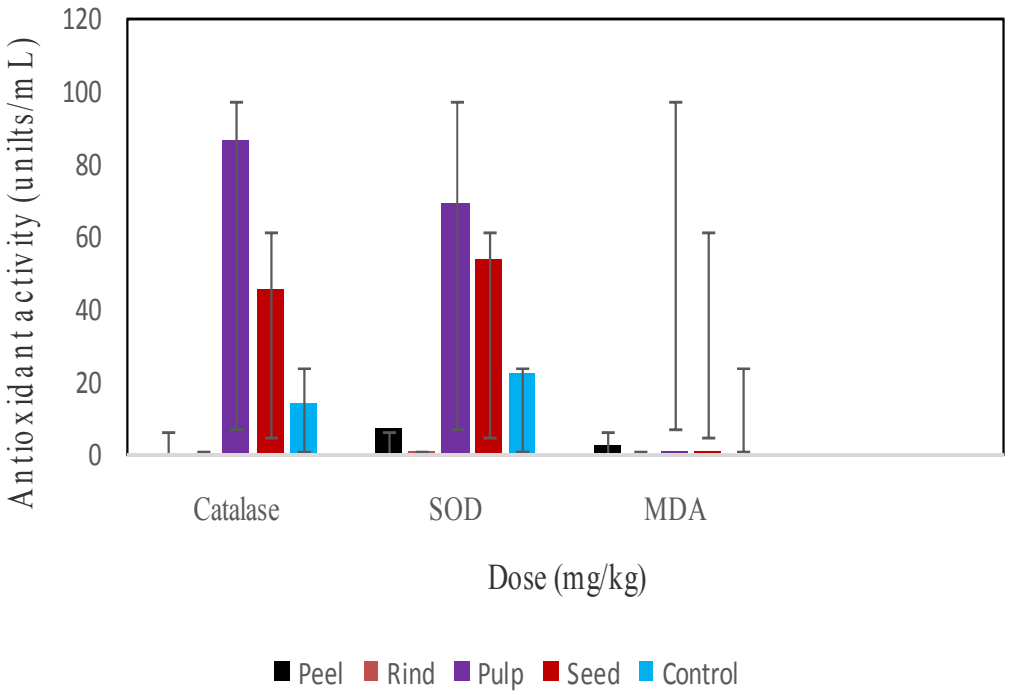


Fig.4. *In vivo antioxidant activity of various layers of C. lanatus fruit in Wistar rats. Extracts were administered at dosses of 100, 200, and 400 mg/kg b.w. (p.o.). Results were expressed as mean±SD; n = 5 per group. There was significant different between the treated and the control group at 400 mg/kg b.w (p < 0.05; one-way ANOVA).*

3.4. Anti-proliferative and growth inhibitory effects of C. lanatus extracts

The data obtained from the study indicated all layers of watermelon fruit, the pulp (PP) and the seeds (SD) exhibited concentration-dependent activity. From the results, length of guinea corn (*Sorghum bicolor*) radicles decreased progressively with increased concentrations of extracts from 5µg/mL to 40 µg/mL (Fig. 5). Likewise, more mortalities were witnessed among the tadpoles (*Raniceps ranianus*) with increased concentrations of the extracts.

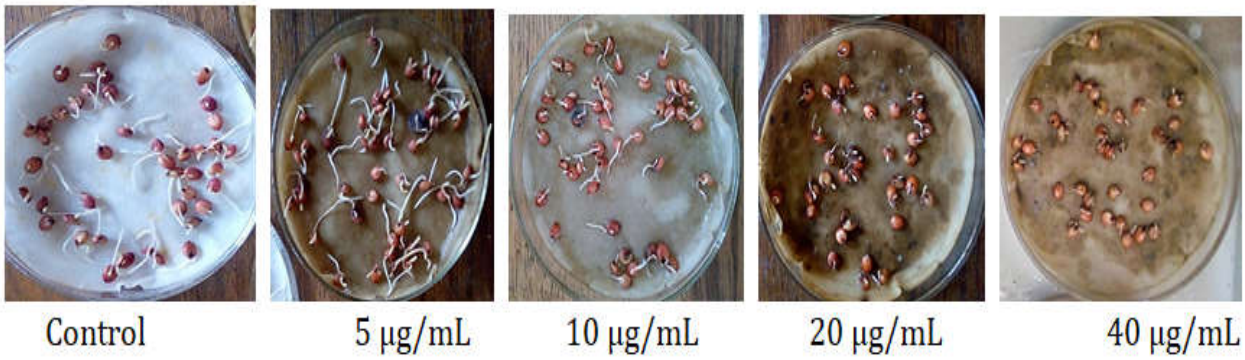


Fig. 5. Growth inhibitory effects of watermelon pulp on *Sorghum bicolor* radicles.

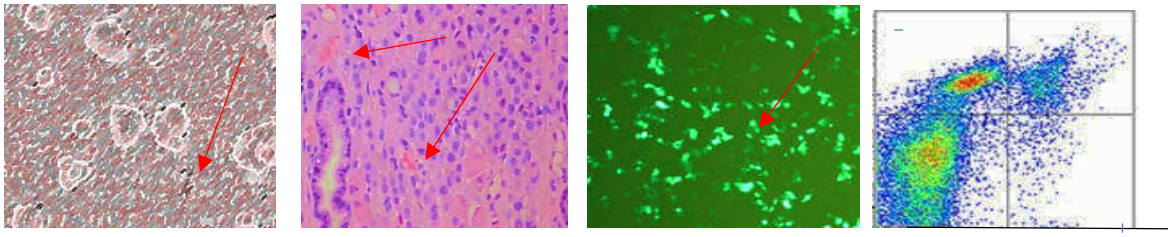
3.5. In vitro cytotoxic effects of C. lanatus extracts

From the cytotoxicity study carried out on MCF-7 and HMVII cancer cells using various layers of *C. lanatus* fruit, the results demonstrated a concentration-dependent effects as well as the induction of early and late apoptosis in cancer cells especially in breast cancer cells MCF-7 when compared to vaginal melanoma cancer cells HMVII as revealed by flow cytometry. The pulp and the seeds illustrated the most potent cytotoxic effects than the other layers with IC_{50} values 6.01± 0.01 µg/mL and 18.12± 0.20 µg/mL, respectively (Table 3; Fig. 6). These results were not significantly different from doxorubicin-treated cells ($p < 0.05$; one-way ANOVA).

Table 3. Cytotoxic effects of extracts from various layers of *C. lanatus* fruit

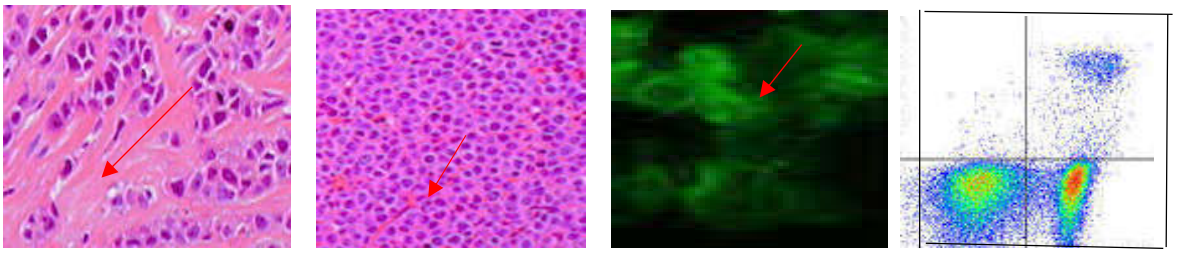
	IC50 (µg/mL)		IC50 (µg/mL)	
Extract (µg/mL)	MCF-7	SI[a]	HMVII	SI[b]
Peel (PL)	228.12 ± 6.32	ND	282.44 ± 4.56	ND
Rind (RD)	129.10 ± 6.22	ND	202.03 ± 2.34	ND
Pulp (PP)	6.01 ± 0.01*	66.12	22.14 ± 0.01*	48.50
Seed (SD)	18.12 ± 0.20*	45.66	8.12 ± 0.01*	58.04
Doxorubicin	0.20 ± 0.01*	16.88	0.60 ± 0.02*	14.28

Results are mean ± SD (n = 3). *P < 0.05 (one-way; ANOVA) are statistically significant when compared to the control drug, ND (not determined), SI[a] and SI[b] are selective index against MCF-7 and HMVII cells, respectively at concentrations from 10-200 µg/mL.



Control (doxorubicin) 200 µg/L (PP) Apoptosis (48 h) Annexin V 10^5

a. MCF-7 breast cancer cells



Control (doxorubicin) 200 µg/L (PP) Apoptosis (48 h) Annexin V 10^5

b. HMVII vaginal melanoma cells

Fig.6. Cytotoxic effects of *C. lanatus* pulp (PP). Cancer cells treated with pulp extract from *C. lanatus* fruit depicted early and late apoptosis in cells stained with propidium iodide/Annexin V and viewed by fluorescence microscopy and flow cytometry. The arrows indicated apoptotic cells.

4. Discussion

Breast and vaginal cancer incidence in Nigeria and Africa have been on increasing rate in recent decades. This is due to varying lifestyle of people in addition to poor health facilities to curb these cases. Several others factors were also reported to have contributed to these problems such as lack of exercise, consumption of alcohols, exposure to certain oncogenes or chemicals, and free radical ions, among others [33-34]. It worth to note that regular medical check-ups have assisted greatly in detecting breast and vaginal cancers early. Despite this, more than 10, 000 people have died in 2020 alone in Nigeria [35], including those who have receiving chemotherapeutic drugs. Therefore, it is important to seek an alternative natural anti-cancer agent which is not harmful to the normal body cells, that is capable of reducing the negative effects of free radical scavenging elements which are usually implicated in cancer cases [36]. For this, our study evaluated the phytochemical contents, antioxidant and cytotoxicity activities of the peel (PL), rind (RD), pulp (PP), and seeds (SD) methanol extracts of *Citrullus lanatus* (watermelon) fruit against breast MCF-7 and vaginal melanoma HMVII cancer cells, while Vero E6 was used as the normal cell.

From the conducted study, phytochemical analysis of the four layers of watermelon fruit showed that there are many phytoconstituents in the pulp (PP) followed by the seed (SD) (Table 1). The presence of these metabolites was responsible for the observed antioxidant and anticancer activities of *C. lanatus* fruit. For instance, metabolites such as saponins, tannins, proteins, cardiac glycosides, and flavonoids have been reported to play crucial roles in healing during disease condition in humans. The presence of flavonoids in the pulp more than any other layer of the fruit should be the major reason it indicated most potent biological activities in the current study.

Flavonoid compound quercetin has been reported to have possessed many pharmacological activities in both *in vitro* and *in vivo* studies. This is because it is readily present in fruits and vegetables, and it has been shown to possess a neuron-protective effect by reducing oxidative stress inhibiting cell proliferation as well as inflammation [37]. The roles played by flavonoid in this current study were not different from the aforementioned. It is also evident from the study that isolation of methyl stearate ester as identified by the GC-MS and NMR. From the study, the methyl stearate ester (a fatty acid) isolated depicted eight signals (1H-NMR) which are mainly singlets and triplets (Fig. 2; a & b), has been reported to possess antifungal and antioxidant activities [38-39]. The compound methyl stearate ester must have contributed to the antioxidant activity witnessed in the current investigation, because most ROS are highly susceptible to polysaturated fatty acids [40]. It also suggests that the pulp contains significant amount of fatty acid ester which have been widely reported to be beneficial to humans aside other metabolites revealed from our study.

From our study, the antioxidant activity of *C. lanatus* extracts were evaluated using DPPH, H_2O_2, ORAC, FRAP, and ABTS (Table 2). The proposed scrutiny indicated that the pulp reduced the scavenging radical activities by DPPH and other assays examined in concentration dependent-fashion *in vitro*. For instance, H_2O_2 is a bacteriostatic agent that is small and relatively stable molecule, that diffuses readily and thus, gas the potential to act as a second messenger and modulate gene expression [41]. Thus, exposure of an agent to H_2O_2 is a widely use method to cause oxidative damage in cellular models [42]. From the study, most of the assays showed the IC_{50} values of less than 50

μg/mL with the pulp and seeds of watermelon fruit (Fig. 3). However, the pulp was more potent than the seeds at reducing the scavenging radical activities of reactive oxygen species (ROS) at the evaluated concentrations. Flavonoids help regulate cellular metabolisms and fight free radicals which cause oxidative stress on the body.

It also have anti-inflammatory effects and strengthens the blood vessel [43]. The anti-radical activity of the total phenolic contents and flavonoids obtained from the current study are principally based on the structural relationships between the different layers of *C. lanatus* fruit in their chemical structures of compound isolated from the pulp. This was in agreement as reported by [44] Similarly, the total antioxidant capacity of *C. lanatus* fruit is a combination of different activities of the ROS which are concentration-dependent as being comparable to the positive controls (Fig. 3).

Furthermore, the results obtained from the *in vitro* studies were affirmed in the *in vivo* rat models. From the *in vivo* study, antioxidant evaluation of the various layers of *C. lanatus* fruit illustrated that the pulp and the seed extracts possessed potent antioxidant effects with increased activities of serum superoxide dismutase (SOD) and catalase (CA), while there were decreases in the level of MDA (Fig. 4). It was reported that catalases are enzymes that catalyze the decomposition of hydrogen peroxide, which is a toxic bye product of various metabolic processes [45-46], while SOD is responsible for accelerating the activity of dismutation of superoxide to H_2O_2 and oxygen, thus, alleviating the possibility of superoxide anion reacting with nitric oxide to form reactive species [14, 45]. These increased levels in serum activities of the catalase and SOD obtained from the current study showed that the *C. lanatus* fruit extracts have an *in vivo* antioxidant activity which is capable of affecting the scavenging radical actions of ROS in the body.

It has been reported also that ROS is known to play double roles which might be deleterious in the body depending on their concentration that are contributing to cellular death by inducing apoptosis in cells or necrosis at the point above the body's antioxidant protection mechanisms [31, 47-49]. Likewise, cancer cells possess high amount of ROS which is basic in nature owing to their high metabolic rates as well as other functions which is distinct from normal cells. In a similar case, an increase at the ROS levels is necessary for the growth of cancer cells, proliferate, and metastased [50]. Therefore, it is possible that the anti-cancer activity of the extracts exhibited in the current study is owing to the presence of certain metabolites in the pulp and seed parts of *C. lanatus* fruit which reduced the essential nutrients needed for the survival of MCF-7 and HMVII cancer cells.

From the anti-cancer study, the preliminary antiproliferative and cytotoxic evaluations of the various layers of watermelon showed that only the pulp and the seeds decreased the growth of *Sorghum bicolor*'s radicle and mortality of tadpoles in concentration-dependent manner, respectively (Fig.5). The radicles of *S. bicolor* and the cells of tadpoles were reported to possess cells which are capable of rapid growth like cancer cells [28]. In this study, the ability of these extracts to inhibit the growth of these radicles depicted that it possessed anti-proliferative effects on the radicles. Similarly, their ability to kill the tadpoles showed the cytotoxic potentials of the pulp and seed.

Moreover, the pulp indicated the most potent IC_{50} against the MCF-7 and HMVII cancer cells than the seed. Our study showed that extracts from the pulp also possessed strong selective index compared to the doxorubicin standard drug ($p < 0.05$; one-way ANOVA) (Table 3). The selectivity index (SI) which is defined as *the ratio* of the sample's toxic concentration of against its effective bioactive concentration. It indicates the ability of the extract to discriminate cancerous

cells from normal ones as seen from the obtained data of the current study (Table 3). Moreover, the evaluation of the SI values for medicinal plants or its isolated compounds is very crucial for determining whether more works can be continued on such plant. This is because for determining the anti-cancer activity of a medicinal plant, its cytotoxicity against normal cell lines should be evaluated so as to determine the SI value [51]. From the proposed study, the pulp and the seed of *C. lanatus* showed high value of SI against VeroE6 cell (Table 3). This result was comparable to that of the control drug doxorubicin ($p < 0.05$; one-way ANOVA). The pulp layer displayed significant levels of apoptosis induction in the cancer cells in concentration dependent fashion after 48 h incubation as revealed by the flow cytometry (Fig. 6).

It was reported that the apoptosis activation via the mitochondrial pathway is one of the major steps in the process of apoptosis [37, 52-53] and is it often related to the activities of caspases-3 and -8 proteins families. Thus, the pulp and seed layers of *C. lanatus* should have induced both early and late apoptosis using these pathways.

Finally, the fact that other layers of *C. lanatus* fruit (peel and rind) does not indicate significant potent antioxidant and anti-cancer activities in this present study would not be concluded that these layers may not possess known biological activities when evaluated for other diseases. The presence of some metabolites in these layers might suggest their usefulness in certain disease situation, hence further research is needed on the pharmacological relevant of these layers. Our study, therefore, showed that the pulp and seeds of watermelon fruit possessed phytochemicals which were responsible for the observed antioxidant and anticancer activities.

5. Conclusion

Our study concludes that methanol extracts of the pulp and seeds of *C. lanatus* fruit possess potent anti-oxidant and anti-cancer activities against breast cancer MCF-7 and vaginal melanoma cancer HMVII cells, with high selective indices against normal cell line VeroE6. The results obtained also indicated that the pulp and seeds significantly inhibited the proliferative activity of the cancer cells by inducing early and late cellular apoptosis as well as decreasing the activities of the ROS in concentration-dependent manner.

This special mechanism qualifies these layers of watermelon as preferable treatment alternative for oxidative stress (by ROS) as well as breast and vaginal cancers in ethnomedicine. Finally, the variation in chemical contents of these parts in the current study was due to the geographical distribution of the plant which results in these chemical races.

Statement of ethics

The animals used for this study strictly followed the guidelines approved by the Animal Ethical Committee of the University of Jos, Nigeria approval number UJ/FPS/F17-00379.

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Author's contributions

CAU, UI and AMI designed, performed the experiments and drafted the manuscript. UI and CAU performed the statistical test and analyzed the data. CAU interpret the spectra. All authors read the final version of the manuscript and approved it for submission.

Declaration of Competing Interests

We have none to declare.

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