Original Research Article

Phytochemical composition, antioxidant and antiproliferative activities of African Basil (*Ocimum gratissimum* L.) leaves

ABSTRACT

Aim: To determine the phytochemicals in *Ocimum gratissimum* leaves, their phenolic content, antioxidant potential and antiproliferative activity against human prostate (DU145), colon (CT26) and cervical (HeLa 229) cancer cells.

Place and Duration of the Study: Leaves of *O. gratissimum* were collected from cultivated plants in Wakiso district of Uganda.The samples were analyzed at Directorate of Government Analytical Laboratory, Kampala (Uganda) and Kenya Medical Research Institute, Centre for Traditional Medicine and Drug Research, Nairobi (Kenya)between August 2019 and January 2020.

Methodology: The leaves were separately extracted by maceration using hexane, dichloromethane, ethyl acetate and methanol. The methanolic extract was further fractionated and subjected to solid phase extraction. Antiproliferative assay was done using dimethylthiazol-2,5-diphenyl-tetrazolium bromideassay while total phenolic content and antioxidant activity were determined by Folin-Ciocalteu method and 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay respectively. Compounds were identified by Fourier transform infrared spectroscopy and liquid chromatography-tandem mass spectrometry.

Results:A total of 34 compounds were identified in the fractions. The highest mean total phenolic content was 401.07 ± 6.47 µg/ml for the methanolic extract which also had the highest antioxidant activity with minimum inhibitory concentration of 5.79 ± 0.13 mg/ml. There was a positive correlation between the antioxidant activity of the extracts and antiproliferative activity of the extracts on prostate and cervical cancer cell lines. The extracts exhibited the highest toxicity against prostate cancer cells and the least against cervical cancer cells.

Conclusion: The results of this study support the traditional use of this plant in cancer therapy in Uganda. Further research should isolate pure anticancer compounds from this plant which could act as lead candidates in the development of anticancer drugs.

Keywords: Cytotoxicity, colon cancer, cervical cancer, traditional medicine, polyphenols, prostate cancer.

1. INTRODUCTION

Cancer is one of the leading causes of mortality worldwide [1]. It is characterized by irregular proliferation of malignant cells in a series of stages with different biochemical, molecular and cellular events [2]. Cancer is caused by both internal factors (such as mutations, hormones and immune conditions)and external factors like chemicals, radiation and infectious microorganisms [3, 4]. This is attributed to changes in lifestyle such as smoking, unhealthy eating, lack of physical exercise and excessive consumption of alcohol [5].

The International Agency for Research on Cancer (IARC) reported that the incidence, mortality and prevalence of cancer worldwide was attributed to 36 different types of cancer [6]. In 2018, 18.1 million new cancer cases and 9.6 million deaths were reportedglobally[6]. Lung cancer registered 11.6% of the total cancer incidences with 18.4% mortality. It was followed by breast cancer (11.6%), prostate cancer (7.1%), and colorectal cancer (6.1%) in mortality. Colorectal cancer (9.2%), stomach cancer (8.2%), and liver cancer (8.2%) were listed among the top four deadliest tumors. Lung, prostate, liver and stomach cancers represented the most deadliest among males while among females, breast, lung and colorectal cancersdominated [7]. In Africa, cancer cases recorded are over 1 million yearly, and the most common include cervix, breast, liver and prostate cancers [5]. Despite the increasing burden, cancer management is not a priority in developing countries largely due to limited resources and other pressing public health concerns such as HIV/AIDS, malaria and tuberculosis [1].

In East Africa, Uganda recorded 32,617 cancer cases in 2018 compared to 5,000 in 2017 and 4,000 in 2016. Of the 2018 figure, 21,829 succumbed to death [8]. In Tanzania, data showed that at least 28,610 patients die of cancer every year. Kenya on the other hand reported 47,887 cancer cases in 2018, with breast, cervical and esophageal cancer incidence rates of 13%, 11% and 9% respectively. There were 15,762 cancer deaths in 2016, which rose to 16,953 in 2017 [8]. Treatment of cancer is costly, and this has been exacerbated by resistance of tumor cells to the available antineoplastic drugs. Due to their lack of specificity, the conventional cancer therapies present severe side effects and in most developing countries are inaccessible to cancer patients[9]. Thus, traditional medicine is gaining more attention in chemoprotective management of cancer in East Africa [1, 10, 11]. Over 3,000 plant species have been reported to have anticancer properties[12]. An example is the extract of *Camptotheca acuminate* from the Cornaceae family whichshowed anticancer activity against brain, rectal, liver, gastrointestinal and breast tumors and this led to isolation of an anticancer drug, Camptothecin[12]. Antitumor drugs: vinblastine and vincristine have also been developed from the periwinkle plant (*Catharanthus roseus* (L.) G. Don) [13] while *Prunus africana* (Hook.f.) Kalkman have been patented in France for the management of prostate cancer [14]. Increasing evidence suggests thatantioxidants and natural product-based compounds with antioxidant activity can effectively neutralize oxidative stress and thus suppress reactive oxygen species-mediated tumorigenesis [3].

Although a number of plants have been claimed to have antitumor properties, they have not been fully investigated for the development of novel anticancer drugs[15]. *Ocimum gratissimum* (known as *Omujaja*in the local Luganda dialect) is one of the plants used in traditional management of cancer in Uganda [16]. It was previously reported to be effective against prostate, lung, breast, colorectal and cervical cancer[17-23]. However, its safety to humans as well as identification and isolation of the main phenolic compounds as the presupposed source of anticancer activity has not been fully documented [24, 25]. In the current study, we report on the phytochemicals in the leaves of *O. gratissimum*, the total phenolic content, antioxidant potential and antiproliferative activity against human prostate (DU145), colon (CT26) and cervical (HeLa 229) cancer cells.

2. MATERIAL AND METHODS

2.1Reagents and bioassay cell lines

The chemicals and reagents used were of analytical grade and obtained from Sigma Aldrichexcept for Liquid Chromatography (LC) solvents which were of LC grade. Reagents included Culture media Rosewell Pack Memorial Institute (RPMI), dimethylthiazol-2,5-diphenyl-tetrazolium bromide (MTT) powder, doxorubicin drug, dimethyl sulfoxide (DMSO), methanol, dichloromethane, methylene chloride, silica gel, alumina, 2,2-diphenyl-1-picrylhydrazyl (DPPH), tris-Hydrochloric acid buffer, Folin-Ciocalteau reagent, rosmarinic acid, rutin, sodium carbonate, ethyl acetate, sulphuric acid, hydrochloric acid, andethyl acetate. LC grade solvents included formic acid, acetonitrile, methanol(LCMS HiperSolvChromanorm from VWR chemicals, Belgium)and water (LCMS Ultra ChromaSolv, Germany). Vero (normal) cells, human prostate cancer (DU145), cervical cancer (HeLa 229) and colon cancer (CT26) cell lines were generously supplied by Centre for Traditional Medicine and Drug Research, Kenya Medical Research Institute, Kenya.

2.2Sampling and sample preparation

Leaves of *O. gratissimum* were collected from cultivated plants in Wakiso district of Uganda ($0^0$23'36" N, $33^0$0'9" E) with permission from Uganda Natural Chemotherapeutics Research Institute, Kampala, Uganda where they were identified by Kyoshabire Medius (a taxonomist). A voucher sample (number: 50908) was deposited at Makerere University Herbarium, Kampala, Uganda on 7th August 2019.

Laboratory samples were air-dried in mesh bags and ground into fine powder using a laboratory mill. Weighed 150 ± 0.1 g of the powder were separately extracted with 775 ml of *n*-hexane, dichloromethane (DCM), ethyl acetate and methanol in 1000 ml conical flasks for 96 hours at room temperature. The crude extracts were filtered using a cheese cloth, Whatman No. 1 filter paper and concentrated to dryness on a rotary evaporator (Rotavapor BUCHIR-100, Switzerland) [26]. The extracts were transferred into sample bottles which were placed in a desiccator of anhydrous sodium sulphate. The yield of the extracts were calculated using **Equation 1**. The extracts were transferred into tightly stoppered bottles and kept in a refrigerator at 4 ℃ until further analysis.

$$\text{Percentage yield} = \left(\frac{A}{A0}\right) \times 100 \qquad (1)$$

Where A is the amount of crude extract obtained after drying and A_0 is the weight of the leaves used for extraction.

Fractionation was done for the methanol extract because it had the highest yield. The dried crude methanol extract was divided into two parts; one portion (1.5 g) was kept in the crude form and the other portion (8 g) was subjected to column chromatography fractionation.

2.3Antiproliferative activity of the extracts

The Vero, prostate, colorectal and cervical cancer cells were separately thawed in a water bath at 37 ^{0}C. Growth media (20 ml) was added to 1 ml of each of the cell lines in T-75 culture flasks and incubated at 5% carbon dioxide and 37 ^{0}C in order to revive the cells. Culturing was done for 3 days until when the cells obtained at least 80% confluence. The excess media was poured off, leaving the cells attached to the surface of the flask and the flask was washed 3 times with phosphate buffer saline (PBS). Excess PBS was poured off and then 500 µL of Trypsin-EDTA was added into the flask having cells attached to the surface. This was spread evenly on the inner surface of the flask by tilting the flask back and

forth and then incubating for 3 minutes. Trypsin was added to detach the cells off the surface of the flask. Growth media (10 ml) was added immediately to stop action of Trypsin. Growth media was purged gently to allow breaking of clumps between cells.

In vitro antiproliferative assay was done for both crude extracts and the solid phase extracted methanolic isolates using MTT assay [21]. Briefly, the cancer cells were washed 3 times with 5 ml of PBS after attainment of 100% confluence and harvested by trypsinization. The number of viable cells was determined by Trypan blue exclusion test. Approximately 2 × 10^4 cells/ml suspension of both Vero and cancer cells were seeded in 96-well plates and incubated for 24 hours.

Measured 15 µl of the drugs (extracts and the commercial drug doxorubicin) at seven different concentrations (1000, 333.33, 111.11, 37.03, 12.34, 4.11 and 1.37 µg/ml) were added from rows H to B and the plates incubated for 48 hours. Row A acted as the negative control (drugs were not added to it).

After incubation, 10 µl of MTT dye solution was added to each of the wells in the plates and incubated for 4 hours. The media was then poured off from the wells of the plates leaving cells alone attached to the surface. Measured 50 µl of DMSO was added to solubilize the formazan crystal formed by viable cells. Absorbance was then read on a scanning multi-well spectrophotometer at 562 nm [27].

Absorbance values higher than the control cells indicated an increase in the rate of cell proliferation and vice versa [28]. The percentage viability was evaluated by determining absorbance with the corresponding chemical concentrations. Linear regression analysis at 95% confidence limits and R^2 were used to define dose response curves of percentage viability of cells against concentration. Percentage cell viability was calculated using **Equation 2** and **Equation 3**.

$$\text{Percent cell viability} = 100 - \%\ \text{Cytotoxicity} \quad (2)$$

$$\%\ \text{Cytotoxicity} = \frac{A-B}{A} \times 100 \quad (3)$$

Where A is optical density of control and B is optical density of test drug.

Data was then analyzed to obtain the minimum inhibitory concentration (IC_{50}) and median cytotoxic concentration (CC_{50}) of the extracts on the cancer and Vero cells respectively [29]. The selectivity index (SI) was calculated as the ratio of CC_{50} to IC_{50}[15, 30].

2.4Determination of total phenolic content and antioxidant activity

The dried crude extracts obtained were used for determination of total phenolic content (TPC) and antioxidant activity.

The TPC of the extracts were determined using Folin-Ciocalteu reagent as described by previous authors [31, 32]. Briefly, 0.5 ml of the extract dissolved in 1 mg/L of methanol in falcon tubes. Gallic acid solutions of 0, 20, 40, 80 and 100 µg/ml were also added into the tubes in methanol : water (50 : 50 v/v) were mixed with 0.5 ml of Folin-Ciocalteu reagent diluted 10-fold in distilled water in falcon tubes and allowed to stand at room temperature for 5 minutes. Exactly 1.5 ml of sodium carbonate (20 g in 100 ml of distilled water) solution was then added, followed by 8.5 ml of distilled water. After 90 minutes, the absorbance was measured using UV-1900 UV Vis Spectrophotometer (Shimadzu Corporation, Japan) at 755 nm using Gallic acid as the standard solution [33].

Antioxidant activity was assessed using DPPH radical scavenging assay as described byAwah and Verla [31]. Briefly, 8.5 ml of methanol was added to 0.1 g of the extracts. From these, 200 µg/ml was made by transferring 0.167 ml of sample stock solutions in different falcon tubes and the volume made up to 10 ml. The solutions were then mixed with 1 ml of 0.1 mM DPPH in methanol. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 25 minutes. Blank solutions were prepared with 1 ml of methanol while the negative control was 1 ml of 0.1mM DPPH solution in 2 ml of methanol. Thereafter, the absorbance of the assay mixtures were measured at 517 nm using a UV visible spectrophotometer to measure the decolorization to yellowdiphenylpicrylhydrazine. DPPH radical inhibition was calculated using **Equation 4**.

$$\% \text{ inhibition} = \left(\frac{A_S - A_O}{A_o}\right) \times 100 \qquad (4)$$

Where A_o = the average absorbance of blank (untreated cells and A_S = absorbance of the sample (treated cells).

2.5 Characterization of compounds in *O. gratissimum* methanolic leaf extract

2.51 Fourier transform infrared spectrometry

The functional groups in the extract fractions were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) using a Shimadzu FTIR spectrometer (Nicolet NEXUS 470, Thermo Scientific, USA). Aliquots (0.1 g) of the fractions were dissolved in 10 ml of methanol. Exactly 0.6 ml of the sample solution was poured on Attenuated Total Reflection (ATR) crystal and the spectra were read at 4500 to 400 cm^{-1}. The frequencies of the different components were recorded. The resolution was 4 cm^{-1} for 20 scans on each sample [34, 35]. The analysis was repeated twice for spectra confirmation [36].

2.52 Solid phase extraction and clean up

This was done for the methanol and ethyl acetate fractions. The end capped C18 cartridge of sorbent mass, 500 mg; particle size, 50 µm; pore diameter, 48Å; surface area, 526 m^2/g was conditioned with 5 ml of 10% methanol in acidified water. Measured 20 ml of each fraction solution was loaded into a C-18 (Supelco, Sigma-Aldrich Germany) column and allowed to flow under gravity. The co-extracted substances were eluted from the sorbent with 100 ml of aqueous acetic acid (2% v/v). The column was dried using a pressure pump in the vacuum manifold for 5 minutes and total retained phenols were eluted with 1.2 ml of 0.1% formic acid acidified methanol [37]. Purified extracts were filtered through a 0.1 µm filter prior to liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analyses [38].

2.53 Liquid chromatography-tandem mass spectrometry analysis

The LC-MS/MS was used to identify compounds in the clean-up fractions. The auto-sampler LC system (Finnigan, Thermo Electron Corporation, USA) was coupled to a MS detector (Agilent Technologies, 6420 Triple Quad, USA). Sample solutions of 5 µL were injected into C-18 reverse phase column (Poroshell 120 EC-C18 3 x 50 mm, 2.7 µm, USA) at 40 °C. Data acquisition software was for 6400 Series Triple Quadrupole (Version B.08.00, Qualitative analysis software Version B.07.00 Service Pack 1). Solvent A was made of a mixture of 0.1% formic acid in water and 0.1% ammonium formate in water. This was made by adding 1 ml of formic acid (LCMS HiperSolvChromanorm from VWR chemicals, Belgium) to 1000

ml of water (LCMS Ultra ChromaSolv, Germany) and then a solution of 1.0 g of ammonium formate dissolved in 1000 ml of deionized water and the two solutions were mixed together to form solvent A. Solvent B was made of 0.1% formic acid in methanol which was made by adding 0.6 ml formic acid to 600 ml of methanol.

The elution was conducted at column flow rate 0.5 ml/min, pressure of 350 bars, column temperature of 40 ^{0}C at gradient elution for 35 minutes [39]. From 0-0.5 minutes, elution was 95% solvent A and 5% solvent B, 0.5-12 minutes was 58% A and 42% B, at 12-15 minutes was 40% A and 60% B, 15-20 minutes was 5% A and 95% B, 20-25 minutes was 5% A and 95% B, 25-25.5 minutes was 90% A and 10% B and then 25-35 min was 95% A and 5% B. The eluent was monitored at Electron spray ionization connected to an ion trap MS (ESI-MS) under negative ion mode at full scan mode of 55-500 m/z [40]. Identification of the compounds was based on retention time in reversed phase LC and MS spectral features [39].

2.6Statistical analysis

All experiments were run in triplicate and data presented as means ± standard deviations. ANOVA test was used to establish any significant differences between extracts and controls. Correlations between antioxidant activity of the extracts and antiproliferative activity were established using Pearson's correlation coefficient. All analyses were performed at *P =.05* using Minitab statistical software (Release 17, Minitab Inc., USA).

3. RESULTS AND DISCUSSION

3.1 Percentage yield

The yield of the different extracts, obtained as the percentages of initial mass of the sample macerated are shown in **Table 1**. Methanol gave the highest yield (27.66%) while *n*-hexane gave the least yield (3.19%). This is could be due to the differences in polarity as methanol being the most polar gave the highest yield. This could be because it extracted many compounds from the leaves. Differences in solvent polarities used for extraction is known to play a key role in increasing the solubility of phytochemical compounds [41, 42]. Further, differences in the structure of phytochemical compounds also determine their solubility in solvents of different polarities [43]. Indeed, the four solvents used had different polarities arranged as hexane < DCM < ethyl acetate < methanol. Therefore, the results of the current study confirmed the effect of different solvent polarities on the yield of plant extracts and confirmed the richness of *O. gratissimum* leaves in polar phytochemicals[44, 45]. The results obtained are consistent with those of Widyawati et al. [46] who assessed the effects of solvent polarity on the phytochemical yields from *Pluchea indicia* Less leaf extracts. The same observation was made by Bomma et al. [47] who concluded that methanol is a suitable solvent for extracting plant bioactive compounds.

Table 1: Organic extract yield of *O. gratissimum* leaves.

Solvent	Yield (g)	Percentage yield
Methanol	41.490	27.66
Ethyl acetate	30.945	20.63
Dichloromethane	20.610	13.74

Hexane	4.785	3.19

3.2 Antiproliferative activity of *O. gratissimum* leaf extracts and fractions

The anticancer activity was determined for both crude solvent extracts and fractionated methanol extract. The minimum inhibitory concentrations (µg/ml) required to give 50% of cell death (IC_{50}) by the crude extracts and positive control (doxorubicin) on prostate, colorectal and cervical cancer cells are shown in **Table 2**. Doxorubicin showed the highest activity on all cancer cell lines compared to the plant extracts (*P = .05*). This was evidenced by its very low IC_{50} values (4.36 ± 0.22, 6.39 ± 0.47 and 3.64 ± 0.33 µg/ml) for prostate, colorectal and cervical cancer cell lines compared to the plant extracts.

Table 2:IC_{50} values of *O. gratissimum* leaf crude extracts against prostate, colorectal and cervical cancer cell lines.

Extract	Prostate (DU145)	Colorectal (CT26)	Cervical (HeLa 229)
Crude Methanol	104.84 ± 0.44	586.68 ± 0.93	359.91 ± 0.45
Crude Ethyl acetate	158.21 ± 0.38	626.33 ± 0.50	598.48 ± 0.47
Crude Dichloromethane	967.21 ± 0.19	1094.41 ± 0.47	1761.50 ± 0.65
Crude Hexane	1259.56 ± 0.49	Inactive	2874.81 ± 0.33
Doxorubicin (positive control)	4.36 ± 0.22	6.39 ± 0.47	3.64 ± 0.33

For results of antiproliferative activity, IC_{50} < 10 µg/ml is considered potentially very toxic; IC_{50} between 10 and 100 µg/ml is potentially toxic; IC_{50} between 100 and 1000 µg/ml is potentially harmful and IC_{50} > 1000 µg/ml is potentially non-toxic [48]. As shown in **Table 2**, the methanol extracts were highly toxic on all the cancer cell lines studied. This is because they showed the least IC_{50} which means, only small concentrations of the extracts are required to reduce the number of cancer cells by 50%. Ethyl acetate extract was the second most active, followed by DCM extracts and then finally hexane extracts. This order was also recorded for the TPCs as well as the antioxidant activity of the extracts. This shows that the phenols responsible for the antioxidant activity as well as cytotoxicity of these cancer cells are polar. Correlations between antioxidant activity of the crude extract and antiproliferative activity were established using Pearson's correlation coefficient. It was found that the antioxidant activity is positively correlated with the antiproliferative activity of the crude extracts against cervical and prostate cancer cell lines. However, there was a negative correlation for colorectal cancer cell lines. The correlation was statistically significant for only cervical cancer cell lines (*P = .05*).

The results of antiproliferative activity of the fractions obtained from methanolic fraction of *O. gratissimum* leaves are shown in **Table 3**. Ethyl acetate fraction showed the highest anticancer activity with IC_{50} of 6.39 ± 0.26, 261.31 ± 0.27 and 119.34 ± 0.38 µg/ml for prostate, colorectal and cervical cancer cell lines respectively. On the other hand, hexane fraction showed no activity against cervical and colorectal cancer cell lines and had an IC_{50} of 1019.26 ± 0.28 µg/ml for prostate cancer cell line.

The fractions were comparatively more cytotoxic than the corresponding crude extracts while the solid phase extracts were less cytotoxic when compared to the crude extracts. This could be attributed to greater activity of the polyphenols than in crude extracts where they had interferences [23]. It was observed that the isolates obtained through solid phase extraction showed lower toxicity than the crude extracts and fractions. This could be due to synergistic

effects in the crude extracts [49]. Among the fractions, ethyl acetate fraction showed better activity on the cells than the methanol fraction. This could be due to the fact that ethyl acetate solvent was passed through the column before methanol and it had extracted most of the active compounds from the plant extracts. This was still carried on to the solid phase extracted isolates where it was observed that ethyl acetate isolates showed higher activity (*P< 0.05*).

Table 3: **IC_{50} values (µg/ml) of the fractions of methanolic extract of *O. gratissimum* on the cancer cell lines.**

Fraction	Prostate (DU145)	Colorectal (CT26)	Cervical (HeLa 229)
Methanolic	16.16 ± 0.14	357.39 ± 0.34	201.21 ± 0.23
Ethyl acetate	6.39 ± 0.26	261.31 ± 0.27	119.34 ± 0.38
Dichloromethane	626.13 ± 0.25	922.21 ± 0.66	833.73 ± 1.11
Hexane	1019.26 ± 0.28	-	-
Methanol (solid phase extract)	571.00 ± 0.01	666.49 ± 0.52	602.20 ± 0.34
Ethyl acetate (solid phase extract)	510.35 ± 0.33	572.54 ± 0.46	535.88 ± 0.82
Doxorubicin (positive control)	4.36 ± 0.22	6.39 ± 0.47	3.64 ± 0.33

The 50% cytotoxic concentration (CC_{50}) of the extracts and the fractions were determined for Vero cells (**Table 4**). The methanolic fraction of *O. gratissimum* showed the least CC_{50} value of 355.04 ± 0.04 µg/ml which is potentially harmful while the methanolic solid phase extract showed the highest CC_{50} value of 1897.12 ± 0.11 µg/ml which is potentially non-toxic. The results obtained showed that all the extracts under investigation were less toxic to normal Vero cells, compared to the positive control which was doxorubicin drug (6.36 ± 0.45 µg/ml) which is potentially very toxic.

Table 4: CC_{50} values of *O. gratissimum* leaf extracts and fractions on Vero cells.

Extract	CC_{50}(µg/ml)
Crude methanol	610.85 ± 0.64
Crude ethyl acetate	642.04 ± 0.07
Crude dichloromethane	>1000.00 ± 1.74
Crude hexane	-
Ethyl acetate fraction	495.60 ± 0.35
Methanol fraction	355.04 ± 0.04
Dichloromethane fraction	1694.18 ± 0.10
Hexane fraction	-
Methanol (solid phase extract)	1860.46 ± 0.40
Ethyl acetate (solid phase extract)	1749.68 ± 0.60
Doxorubicin (positive control)	6.36 ± 0.45

To further understand the cytotoxicity of the extracts when used for cancer therapy, the selectivity indices were calculated (**Table 5**). The selectivity index (SI) is the ability of an extract to inhibit the growth of cancer cells more than it does to the normal cells. An extract with the SI > 3 is considered to be highly selective and has the potential to be used in the management cancer [30].

Table 5: Selectivity indices of *O. gratissimum* leaf extracts and fractions

Extract/fraction	Prostate (DU145)	Colorectal (CT26)	Cervical (HeLa 229)
Methanolic extract	5.82	1.70	1.04
Ethyl acetate extract	4.06	1.07	1.03
Dichloromethane extract	1.03	1.00	1.00
Hexane extract	Not applicable	Not applicable	Not applicable
Methanolic fraction	30.68	1.39	2.46
Ethyl acetate fraction	55.60	1.36	2.98
Dichloromethane fraction	2.71	1.84	2.03
Hexane fraction	Not applicable	Not applicable	Not applicable
Methanolic solid phase extract	3.26	2.79	3.09
Ethyl acetate solid phase extract	3.43	3.06	3.27
Doxorubicin	1.459	0.995	1.747

Solid phase extracted clean ups had the highest selectivity index since they showed selectivity on all cells, followed by the ethyl acetate and methanolic fractions and then the crude extracts then the positive control (doxorubicin). The results showed that doxorubicin was not selective on Vero cells as its selectivity indices were lower than 3 [30].

The results of anticancer activity of *O. gratissimum* leaves recorded in this study agreed with previous reports. Methanol crude extracts showed high cytotoxicity (*P< 0.05*) on the cancer cell lines, yet they also proved to be selective on the Vero cells. Methanolic plant extracts have been previously proven to possess the best anticancer activity [50]. This is supported by the results of the antioxidant activity which showed that methanolic extracts of *O. gratissimum* leaves showed the highest antioxidant activity. Indeed, the antioxidant and antitumor activities of plant extracts have been always reported to be positively related with each other [51, 52].

In a preceding study [53], partially purified *O. gratissimum* fractions (1.61 mg/mL) were reported to be effective in inhibiting the proliferation of prostate adenocarcinoma (PC-3) cells. The fractions exhibited antiproliferative activity against PC-3 cells in a concentration dependent manner.Further, the antiproliferative activity of the organic solvent-soluble and aqueous extracts of *O. gratissimum* leaves against prostate cancer (PC3•AR)cellswere evaluated by their inhibitory effects on the Androgen Receptor and Survivin protein. Two organic solvent- soluble extracts P2 and P3–2, and a water-soluble extract, PS/PT1, were found to reduce Androgen Receptor and Survivin levels in a time-dependent manner [22]. Unfractionated aqueous leaf extracts of *O. gratissimum* was also reported to present cytostatic effects with an 80% decrease in human breast cancer cell line (MCF-7) growth at 1 mg/mL[21].Nangia-Makker et al.[20]reported that crude extracts of *O. gratissimum* and its hydrophobic and hydrophilic fractions differentially inhibited breast cancer cell chemotaxis and chemoinvasion*in vitro* and retarded tumor growth and temporal progression of MCF10ADCIS.com xenografts, a model of human breast comedo-ductal carcinoma *in situ* (comedo-DCIS).The extracts-induced inhibition of tumor growth was associated with decreases in basement membrane disintegration, angiogenesis and MMP-2 and MMP-9 activities as confirmed by *in situ* gelatin zymography and cleavage of galectin-3. There was also decrease in MMP-2 and MMP-9 activities in the conditioned media of *O. gratissimum*-treated MCF10AT1 and MCF10AT1-EIII8 premalignant human breast cancer cells as compared with control. The matrix metalloproteinases(MMP-2 and MMP-9) inhibitory

activities of the extracts were verified *in vitro* using gelatin, a synthetic fluorogenic peptide and recombinant galectin-3 as MMP substrates. These indicated that the anti-cancer activity of the extracts may in part be contributed by its MMP inhibitory activity.

Ocimum gratissimum aqueous extract was also reported to significantly and dose-dependently decrease the viability of human pulmonary adenocarcinoma (A549) cells[19]. A recent study [18] reported that *O. gratissimum* leaf extracts decreased the cell viability of hepatocellular carcinoma (HCC SK-Hep1 and HA22T) cells in a dose-dependent manner (from 400 to 800 µg/mL) while there was little effect on Chang liver cells. Cell-cycle analysis showed increased Sub-G1 cell count in SK-Hep1 and HA22T cells which was not observed in Chang liver cells. The authors suggested that the extracts-induced cell death may be mediated through proteins that regulate cell cycle and apoptosis in SK-Hep1 and HA22T cells which was confirmed by further experiments which indicated that treatment with the extracts resulted in a dose-dependent decrease in caspase 3 and PARP expressions and in CDK4and p-ERK1/2expressions. Therefore, the inhibition of cell viability and tumor growth induced by the extracts were hinted to be correlated to their alteration of apoptosis-related proteins[18].

3.3Total phenolic content and antioxidant activity of the extracts

The TPC of the extracts were determined using the Folin-Ciocalteau method. Folin-Ciocalteau reagent consists of a mixture of sodium molybdate, sodium tungstate and other reagents which when added to plant extracts react with phenolic compounds to produce a solution of a blue complex which absorbs at 760 nm. The assay relies on the transfer of electrons in alkaline medium from phenolic compounds tophosphomolybdic/phosphotungstic acid complexes [54, 55]. A calibration curve (**Figure 1**) was prepared for the quantitative analysis and the linearity for gallic acid standard was established from the range of 1 µg/ml to 100 µg/ml which was fitted on the line $y = 0.0025x$.

The methanol crude extract gave the highest TPC of 401.07 ± 6.47 µg/ml (**Table 6**). Methanol is a polar protic solvent [41, 56] and thus, it extracted more polyphenols which are inherently polar and their solubility is through hydrogen bond formation [57]. Further, ANOVA test showed that there were significant differences ($P < 0.05$) among the mean TPC of the different solvent extracts. From the results of antioxidant activity assay (**Figure 2**), the IC_{50} of methanolic extract (5.79 ± 0.13 mg/ml) was the lowest as compared to 0.06 ± 0.01 mg/ml for ascorbic acid (control). This is because most phenolic compounds responsible for antioxidant activity have polar functional groups which are easily dissolved in polar protic solvents like methanol [46]. Awah and Verla [31] reported a TPC of 839.0 ± 97.0 µg/ml and an IC_{50} of 12.3 ± 1.95 mg/ml for methanolic extract of *O. gratissimum* antioxidant activity.Similar results of TPC and antioxidant activity were reported by Ekunwe et al. [58] for *O. gratissimum* leaf extracts after fractionating ethanol extracts using different solvents (chloroform, ethylacetate and *n*-butanol). In a similar study, Venuprasad et al. [59] reported that the ethanolic leaf fraction of *O. gratissimum* had TPC of 124.3 ± 5.8 µg/ml gallic acid equivalent (GAE) and potential antioxidant activity with IC_{50} of 0.470 ± 0.286 mg/ml. The antioxidant activity of plant phenolic compounds is attributed to their redox propertieswhich allows them to act as reducing agents, hydrogen donators, singlet oxygen quenchers and metal chelators[60].The DPPH test measures the hydrogen atom or electron donating capacity of extracts to the stable radical DPPH formed in solution [61].

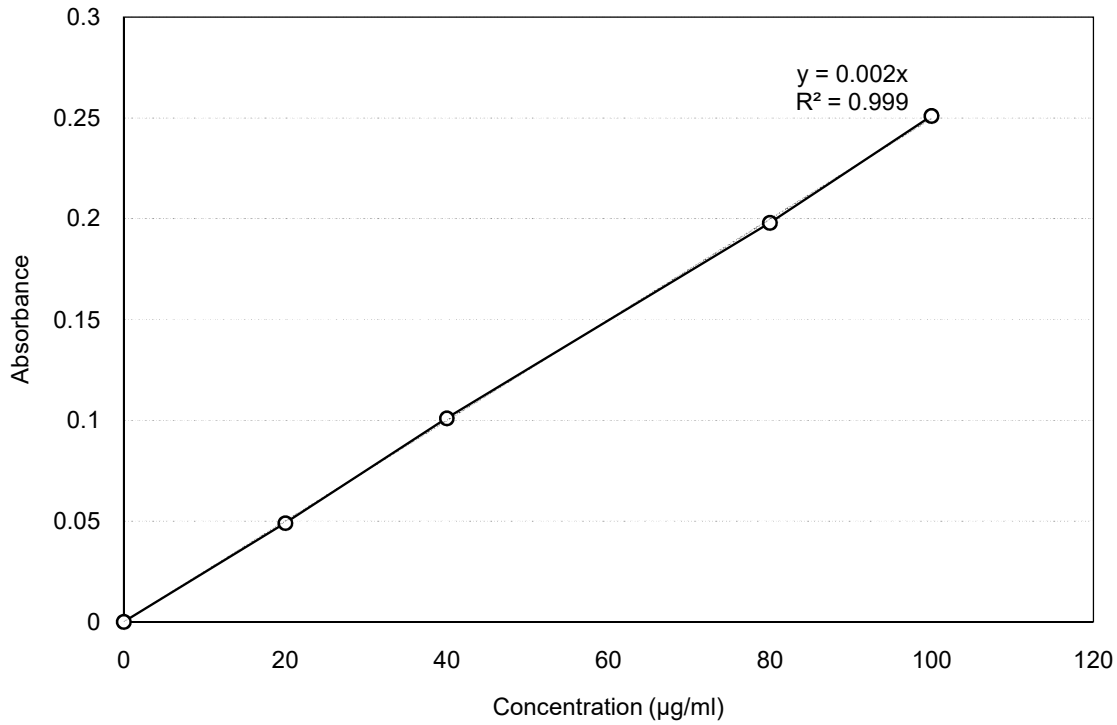

Figure 1.Calibration curve for TPC using Gallic acid standard.

Table 6: Total phenolic content of *O. gratissimum* leaf extracts.

Extraction solvent	Total phenolic content (µg/ml GAE)
Methanol	401.07 ± 6.47
Ethyl acetate	69.60 ± 1.06
Dichloromethane	24.67 ± 1.22
n-hexane	14.67 ± 1.01

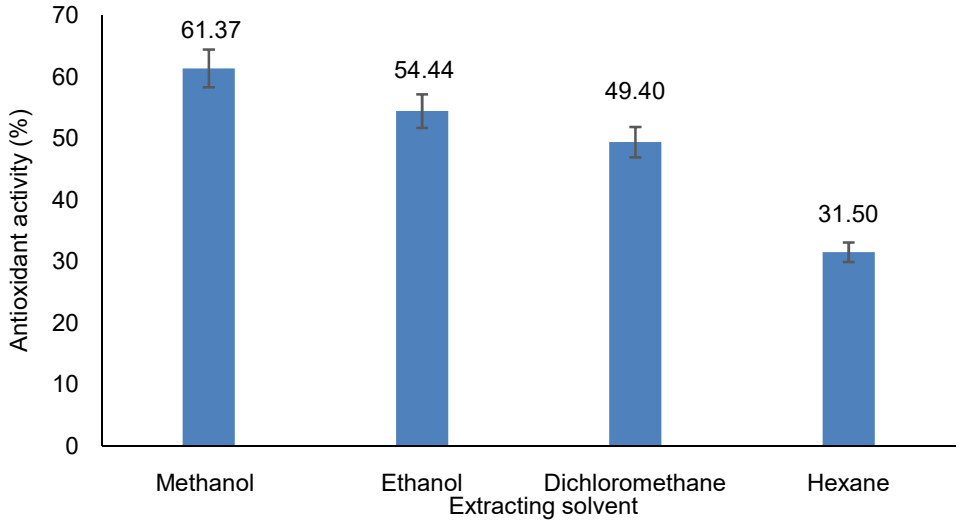

Figure 2.Antioxidant activity of different solvent extracts of *Ocimum gratissimum* leaves.

3.4 Characterization of compounds in *O. gratissimum* methanolic leaves extracts

3.4.1 FT-IR Analysis

In the FT-IR spectrum (**Figure 3**), the intense absorption at 3400 cm^{-1} was due to stretching of phenolic groups present in the extracts. The band at 2900 cm^{-1} was due to stretching of hydroxyl groups like alcohols and water while the absorption at 2800 cm^{-1} could have been due to a C-H group stretching of sp^3 hybridized (R_3C-H) portion. Absorption at 1700 cm^{-1} is due to stretching of C=O group. The bend at 1550 cm^{-1} are due to C=C bonds, typical of aromatic compounds (containing a benzene ring). Absorption at 1400 cm^{-1} was due to asymmetric in-plane bending of $–CH_3$ while at 1350 cm^{-1}, the absorption was due to symmetric in-plane bending of $–CH_3$. The stretch at 1250 cm^{-1} is due to nitro groups ($-NO_2$). The absorption at 1100 cm^{-1} was due to C-O stretching vibration. The weak bands at 1000 cm^{-1} and 900 cm^{-1} could be due to C-H bending and terminal $C=CH_2$ groups respectively. These assignments are based on previous studies on phenolic compounds in plants [34, 62, 63]. These confirmed the presence of phenolic compounds in the extract. The various functional groups observed in the extracts reflected the biochemical profile of the leaf extract which could be responsible for the various medicinal properties of this plant leaf, including antiproliferative activity. Phytochemicals such as phenolics, carotenoids, terpenoids and alkaloids from plants have been reported to be key actors in cancer therapy [1, 12, 64].

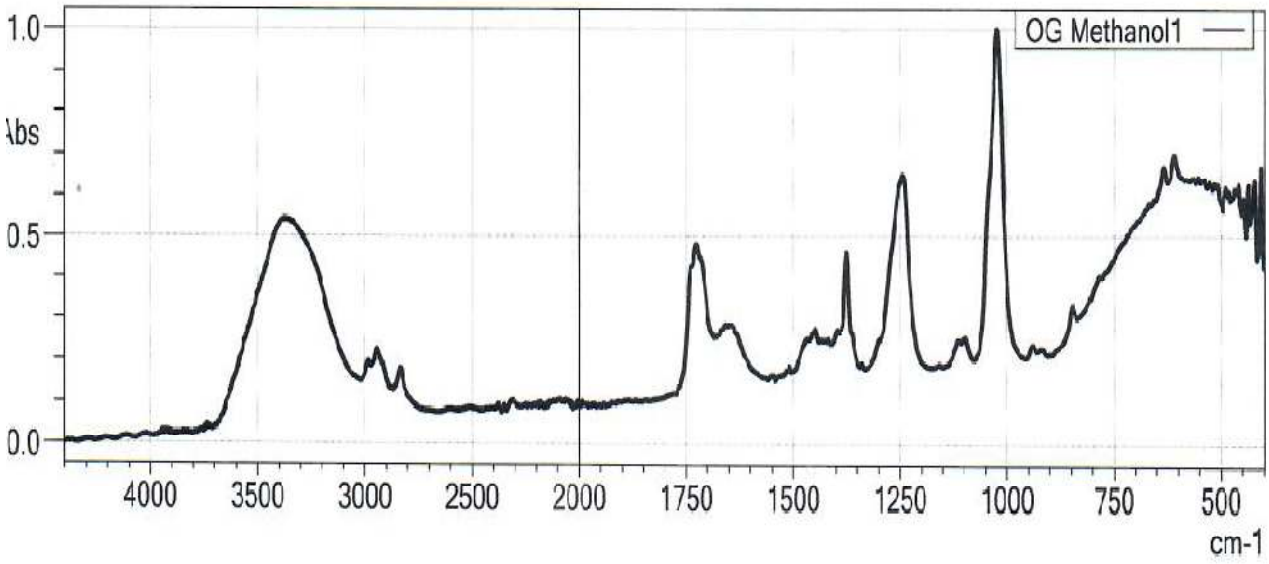

Figure 3.FTIR spectrum of the methanolic fraction of *Ocimum gratissimum* leaf extract.

3.4.2 LC-MS/MS analysis

Qualitative analysis afforded the identification of 34 compounds in *O. gratissimum* methanolic leaf extract (Table 7).

Table 7: Compounds characterized in *Ocimum gratissimum* methanolic leaf extract.

Peak	Retention time (s)	m/z	Molecular formula	Fragments (CE)	Compound	Author(s)

Peak	Retention time (s)	m/z	Molecular formula	Fragments (CE)	Compound	Author(s)
1	1.371	234.1	$C_{27}H_{32}O_{14}$	84.1 (20)	Naringin	[65]
2	2.06	191.1	$C_7H_{12}O_6$	127.0 (24), 93.0 (32)	Quinic acid	[38, 66]
3	8.90	477.1	$C_{22}H_{22}O_{12}$	315.1 (36)	Nepetrin	
4	9.79	359.1	$C_{18}H_{16}O_8$	123.0 (20),161.0(100)	Rosmarinic acid	[67]
5	11.168	179	$C_9H_8O_4$	135 (10), 134 (20)	Caffeic acid	
6	11.21	244.2	$C_{21}H_{20}O_9$	91.1 (36), 86.2 (8)	Diadzin	
7	11.269	169	$C_7H_6O_5$	125 (10), 79 (20)	Gallic acid	[37, 38]
8	11.283	318.2	$C_{21}H_{24}O_{10}$	196 (16), 82.1 (32)	Phlorizin	[37, 38]
9	11.318	208.1	$C_{21}H_{20}O_{12}$	163.1 (8), 105.1 (24)	Isoquercetin	
10	11.415	195.1	$C_{76}H_{52}O_{46}$	138 (20)	Tannic acid	[37]
11	11.429	163.1	$C_7H_8O_2$	117.1 (40)	Anustoline	[68]
12	11.433	150.1	$C_{28}H_{34}N_2O_{11}$	91.1 (20)	Carboxylstrictosidine	
13	11.440	147	$C_9H_6O_2$	103.1 (20), 91.1 (20)	Coumarin	[69]
14	11.489	261.2	$C_{15}H_{14}O_6$	176.1 (0), 55.2 (28)	Catechin	[37, 38]
15	11.610	180.1	$C_{10}H_{10}O_4$	163.1 (4), 105.1 (24)	Isoferullic acid	
16	11.760	290.2	$C_{17}H_{23}NO_3$	124.1 (24), 93.1 (32)	Atropine	[37, 49]
17	11.795	194.1	$C_{10}H_{10}O_4$	163.1 (8), 105.1 (24)	Ferrullic acid	[69]
18	12.227	304.2	$C_{15}H_{15}O_6$	182.1 (16), 82 (48)	Hydroxyphlorentin	
19	12.237	272.2	$C_{21}H_{20}O_{12}$	215.1 (20), 171.1 (40)	Hyperin	[37, 38]
20	12.279	150.1	$C_{28}H_{38}N_2O_4$	65.1 (48), 65.1 (44)	Cephalin	
21	12.310	153	$C_7H_6O_4$	109 (10), 108 (20)	Gentisic acid	[68]
22	12.345	234.1	$C_{22}H_{18}O_{11}$	56.1 (56)	Cathequin	
23	13.810	313.1	$C_{17}H_{14}O_6$	283.0 (32), 298.1 (24)	Cirsimaritin	[66]
24	13.920	345.2	$C_{20}H_{26}O_5$	283.2 (100), 301.2 (49)	Rosmanol	[65]
25	15.140	283.1	$C_{16}H_{12}O_5$	-	Genkwanin	[65]
26	16.040	487.3	$C_{30}H_{48}O_5$	-	Asiatic acid	[65]
27	21.117	150.1	$C_{28}H_{36}N_2O_4$	91 (20)	Psychotron	
28	21.570	195.1	$C_{14}H_6O_8$	83 (40)	Ellargic acid	[38]
29	21.910	471.3	$C_{30}H_{48}O_4$	-	Benthamic acid	[65]
30	22.35	471.3	$C_{30}H_{48}O_4$	-	Augustic acid	[65]
31	30.250	455.4	$C_{30}H_{48}O_3$	-	Ursolic acid	[65]
32	33.591	235.2	$C_{27}H_{30}O_{16}$	86.1 (16), 58.2 (36)	Rutin	
33	34.461	177.1	$C_{16}H_{18}O_9$	98.1 (24), 80.1 (28)	Chlorogenic acid	
34	34.517	290.1	$C_{15}H_{10}O_7$	168 (16), 77 (60)	Quercetin	[40]

The methanolic extract of *O. gratissimum* revealed the presence of polyphenols (Gallic Acid, Rosmanol, Rosmarinic acid), flavonoids (Nepetrin, Quercetin, Rutin, Hesperidin, Catechin), terpenoids, alkaloids. The standards used for quality assurance in the study (gallic acid and rutin) showed similar LC chromatograms and MS spectra with their corresponding compounds in the samples.Of the 34 that were identified, were 9 compounds reported for the first time in this plant. These included Procyanidin, Carboxystrictosinedine, Isoferullic acid, Psychotrin, Hydroxyplorentin, Cephalin, Isoquercetin, Diadzin and Hyperin (**Figure 4**). In a similar study, Venuprasad et al. Venuprasad, Kandikattu [59] reported the presence of polyphenols, flavonoids and fatty acids in ethanolic leaf fraction of *O. gratissimum* analyzed by LC-ESI–MS/MS. Oleanolic acid, Methyl acetate, Plamitic acid, 2-alpha, 3-beta-Dihydroxyolean-12-en-28-oic acid, Basilimoside, Apigenin-7,4,'-dimethyl ether, Hymenoxin, Salvigenin, Nevadensin, Xanthomicrol, Nepetoidin A, Apigenin, Luteolin, Methyl eugenol, Sinapic acid and Rosmarinic acid were reported.Some of the compounds identified such as ursolic, rosmarinic and gallic acids have been previously reported to have anticancer activity [70, 71].

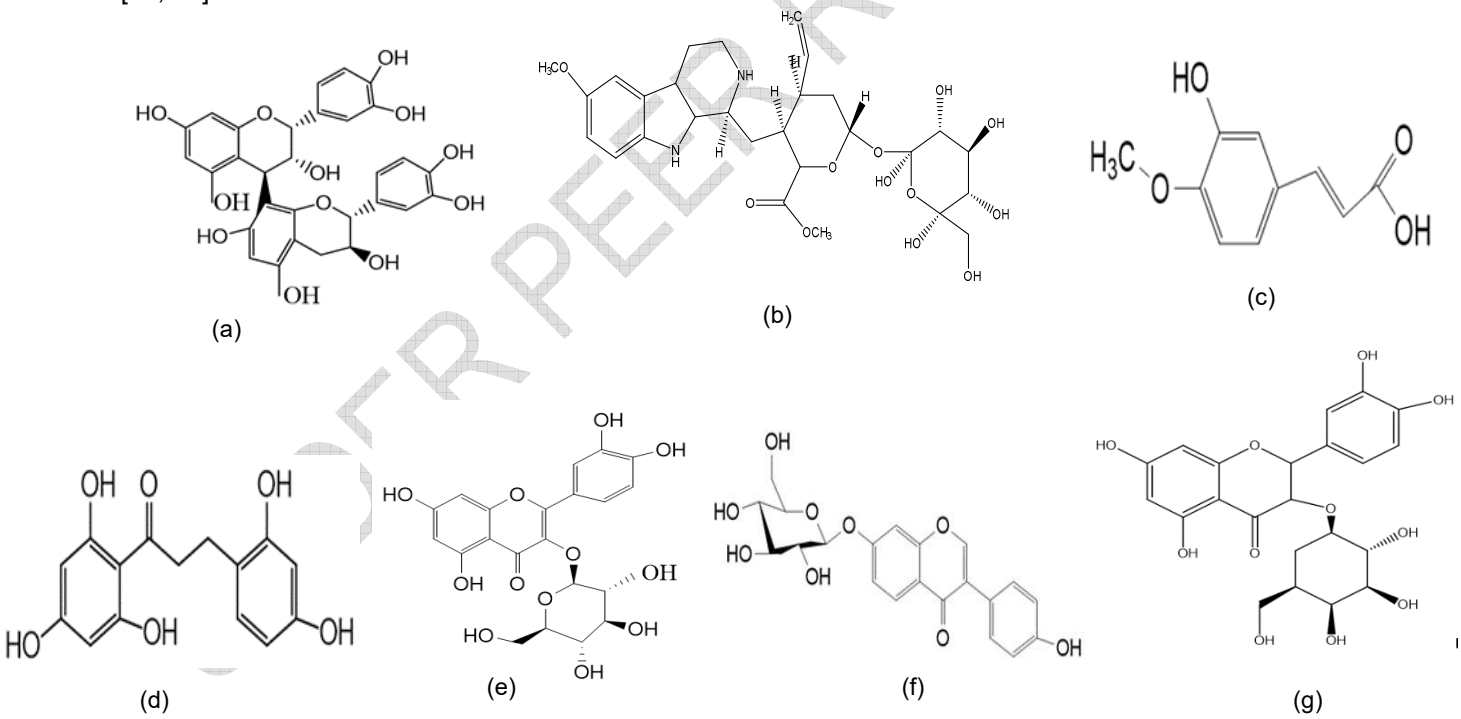

Figure 4. Structures of some of the new molecules identified in fractions of *O. gratissimum* leaves: (a) Procyanidin, (b) Carboxystrictosinedine, (c) Isoferullic acid, (d) Hydroxyplorentin, (e) Isoquercetin, (f) Diadzin and (g) Hyperin.

4. CONCLUSION

The results of this study showed that *O. gratissimum* extracts has phenolic compounds with antiproliferative activity against human prostate (DU145), colorectal (CT26) and cervical (HeLa 229) cancer cells. Further studies should evaluate the anticancer activity of the extracts on other cancer cell lines because some of the polyphenols could be inactive on the cell lines investigated in this study yet active on the other cell lines that have not been studied. Isolation of the pure compounds as well as investigation of the mechanism of cytotoxicity of compounds from the leaves of *O. gratissimum* should be undertaken. Further studies on the chemical composition and antiproliferative activity of *O. gratissimum* roots should be done.

ETHICAL APPROVAL

This study was approved by Centre for Traditional Medicine and Drug Research, Kenya Medical Research Institute Scientific and Ethics Review Unit, Kenya (Approval No. KEMRI/RES/7/3/1).

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