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Full Length Research Paper

In vitro antioxidant and cytotoxic activity of the root extract of *Aspilia mossambicensis* (Oliv) Wild (Asteraceae)

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***Aspiliamossambicensis*(Oliv) Wild is used by Traditional Health Practitioners in northeastern Tanzania, for treatment of cancers. In order to evaluate these claims root powder of the plant was extracted with dichloromethane: methanol (1:1), followed by vacuum liquid chromatography fractionation to obtain dichloromethane, ethyl acetate and methanol fractions which were screened for brine shrimp toxicity and antioxidant activity using DPPH and FRAP assays. The ethyl acetate fraction exhibited higher toxicity on brine shrimp larvae (LC50 = 12.87 µg/ml) than cyclophosphamide (LC50 = 16.12 µg/ml), and antioxidant activity with an EC50 of 200 µg/ml for DPPH and 53.92 µM ECGC equivalent/g dry weight for FRAP assay. The ethyl acetate fraction was cytotoxic against HeLa cancer cells (IC50 50.77 ± 1.69 µg/ml), causing cell cycle arrest at the M phase, phosphatidylserine (PS) externalization and activation of caspase 3 and 8. Four compounds were isolated from this fraction; (-)-Angeloylgrandifloric acid and 16α–hydroxykauran-19-oic acid, which were cytotoxic to the HeLa cervical cancer cells with IC50 = 27.75 and 40.19 µg/ml, respectively, and 16αHydroxy-9(11)-kauren-19-oic acid and grandifloric acid which were non-toxic to the HeLa cells. Further research is recommended to establish the clinical significance of the current findings.**

Key words: *Aspiliamossambiscensis*, cytotoxic activity, brine shrimp toxicity, antioxidant

INTRODUCTION

Cancer has been a major health problem throughout the history of human civilizations. Currently, it is the second disease responsible for human death all over the world after cardiovascular diseases (Ali et al., 2011) with more

than 10 million new cases and more than 6 million deaths each year worldwide, that makes it responsible for about 20% of all deaths in high income countries and 10% in low-income countries (Petersen, 2009). The increasing number of cancer patients not only becomes a burden on the society but also destroys the economy of the country (Ali et al., 2013). The common treatments for cancer that include chemotherapy, radiation and surgery have been facing challenges of inefficiency, inadequacy, cost and side effects. These factors compel the scientific community all over the world to stand united so that new potent drug molecules can be made available (Lu and Lu, 2019).

The discovery of nanoparticles has expanded the scope of search for anticancer drugs. Among the important examples is the discovery of cis-platin and its second and third generation analogues which created hope in cancer chemotherapy (Ali et al., 2013) and magnetic nanoparticles such as SPIONs which are considered as most promising materials because of their multi modal functions (Palanisamy and Wang, 2019). Others are dithiocarbamate complexes of different transition metal ions including copper and ruthenium (Nagy et al., 2012) and the synthesis of copper (II), nickel (II) and ruthenium (III) complexes of a thalidomide based dithiocarbamate ligand that have been reported to show less toxicity to RBCs as compared to the standard drug doxorubicin (Nagy et al., 2012). While efforts are going on to search for new sources of active molecules, it may be important to bear in mind that herbal medicines, natural chemical drugs, microbial, plants and animals are interrelated and thus this goal can be achieved through integrations of existing knowledge, as well as experience and innovations.

Aspilia mossambicensis (Oliv) Wild, of the family Compositae (Asteraceae), also known as the Wild Sunflower, is a perennial herb that is found in Central and Eastern tropical Africa spreading all the way from Ethiopia, through East Africa, Congo, Zambia, Zimbabwe, Malawi, Mozambique and South Africa (Kapinga et al., 2018). It is widely distributed in Tanzania, from north to south and east to west, and it is traditionally used for treatment of various ailments, including venereal diseases, pain, fever, backache, wounds, tumours and cancer (Chhabra et al., 1993). Female chimpanzees consume leaves of *A. mossambicensis* more than the males, an observation which led to investigation and the isolation, from the leaves, of two diterpenes, kaurenoic and grandiflorenic acid, which are powerful uterine stimulants (Page et al., 1997). The dried leaves are used in folk medicine to alleviate menstrual cramps, as antipyretic, anti-ancylostomiasis and for treatment of malaria and hookworm infestation (Page et al., 1992). The roots are used to increase human milk flow (Samuelsson et al., 1991). Extracts of *A. mossambicensis* have been previously reported to have exhibited antibacterial and antihepatotoxic activities (Page et al., 1992; Musyimi et al., 2008). An extract of the aerial parts *of A. mossambicensis* demonstrated significant hypoglycaemic activity at low doses in alloxan-diabetic mice, while at higher doses above 670 mg/kg body weight, apart from being hypoglycemic, the extract also exhibited toxic effects (Njangiru et al., 2019). The objective of this study was to evaluate claims by Traditional Health Practitioners (THPs) that roots of *A. mossambicensis* are useful for treatment of cancer. Therefore, root extracts and isolated compounds were tested for antioxidant activity, toxicity against brine shrimp larvae (*Artemia salina*) and cytotoxic activity on HeLa human cervical cancer cells.

MATERIALS AND METHODS

Plant material

The plant was identified by Mr. Haji Selemani, a botanist in the Department of Botany, University of Dar-es-Salaam, and a voucher specimen, No. DZM 4, was deposited in the Herbaria of the Department of Botany, University of Dar es Salaam and Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences, Tanzania. Roots of *A. mossambicensis* were collected in August 2015, from same District, Kilimanjaro Region, Tanzania.

Chemicals and reagents

Chemicals and reagents used (with their sources) included: Dimethyl sulphoxide (DMSO), epigallocatechin gallate (Sigma: Poole, Dorset, UK); dichloromethane, ethanol, ethyl acetate, methanol, petroleum ether, (CARLO ERBA, Van de Reut, France); acetic anhydride, acetic acid, ammonia solution, toluene, sulphuric acid (Sigma Aldrich Chemie GmbH, Germany), Trypsin-EDTA, Dulbecco's phosphate buffered saline (DPBS) with Ca^{2+} and Mg^{2+} and Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} and Mg^{2+} (Lonza, Walkersville, MD, USA), Trypan blue, bis-benzamide H33342 trichloride (Hoechst 33342), penicillin/streptomycin and bovine serum albumin fraction V (BSA) (Sigma- Aldrich, St. Louis, MO, USA), Tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes® - Life Technologies – Thermo Fischer Scientific, Logan, Utah, USA). Annexin V-FITC/PI kit (MACS Mitenyi Biotec, Germany), Cleaved caspase 3 (Asp 175) rabbit mAb, cleaved caspase 8 (Asp 391) rabbit mAb and Anti-rabbit IgG (H+L) F(ab')2 fragment (Alexa fluor® 647 conjugate) (Cell Signaling Technology, Massachusetts, USA). Brine shrimp eggs were obtained from Aquaculture innovations (Grahamstown, South Africa), HeLa cervical cancer cells from Cellonex, South Africa, RPMI 1640 cell culture medium and foetal bovine serum (FBS) from GE Healthcare Life Sciences (Logan, Utah, USA).

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Extraction

Grinded dry whole roots (500 g) were macerated with dichloromethane: methanol (1:1) for 24 h and filtered. The obtained filtrate was subjected to rotary evaporation (Heidolph instruments GmbH &Co. KG, Schwabach - Germany) at 40°C, under reduced pressure to obtain the extract. Any traces of water were removed from extract using a freeze drier (Edwards, BOC Ltd. Crawley Sussex- England). The dried crude extract was kept in a vial in a freezer at - 20°C until when needed for further processing.

Fractionation

The dried extract was mixed with silica gel (70 - 230 mesh) at a ratio of 1:5 and homogenized. The mixture was subjected to vacuum liquid chromatography (VLC) fractionation and elution was performed sequentially, with petroleum ether, ethyl acetate and methanol. Solvents were removed using a rotary evaporator to obtain dried fractions of petroleum ether (0.023 g), ethyl acetate (21.37 g) and methanol (0.98 g). Resulting fractions were freeze-dried to remove any remaining traces of water and kept in a freezer at - 20°C until they were needed for tests.

Brine shrimp lethality testing (BST)

The test was conducted as described by Meyer and colleagues (Meyer et al., 1982), with some modifications (Innocent et al., 2010). Solutions of the extracts were made in DMSO, at varying concentrations, and incubated in duplicate vials with the brine shrimp larvae in a total volume of 5 ml. Ten brine shrimp larvae were then placed in each of the duplicate vials. Cyclophosphamide, an anticancer drug, was used as a positive control, while 0.6% DMSO in seawater was used as a negative control. After 24 h the nauplii were examined against a lighted background, with a magnifying glass and the average number of surviving larvae was determined.

Data analysis

The mean percentage mortality of brine shrimp larvae was plotted against the logarithm of each concentration using the Fig P computer program (Biosoft Inc, USA), which also gives the regression equations. The regression equations were used to calculate LC_{50} values and confidence intervals (95% CI) according to the previously reported method (Litchfield and Wilcoxon, 1949). Extracts with activity on brine shrimps were considered for further tests.

Determination of antioxidant activity by DPPH assay

The violet coloured free radical 2, 2 - Dipheny-1 - Picryl Hydrazyl (DPPH) reacts with a hydrogen donor (antioxidant) to generate DPPH which is accompanied by the gradual disappearance of colour from deep violet to light-yellow. The changes are measured using UV/visible spectrophotometry. The stock solution of test sample was prepared at 100 mg/ml in DMSO. Working concentrations of 25, 50,100 and 200 µg/ml, were prepared from the stock solution by dilution with 50 mM phosphate buffer (pH = 7.4), and 5 µl of the test sample was mixed with 120 µl of reagents (Tris HCL) and 120µL of 0.1mM DPPH radical. Scavenging action was validated by the parallel setting of a positive control (10µM epigallocatechin gallate). The final concentration of DMSO did not exceed 0.2% and vehicle control results were the same as control wells. The microtiter plate was incubated at 37°C for 30 min and absorbance was measured at 513 nm, using a spectrophotometer (BioTek Power Wave XS- USA). The antioxidant activity was calculated as % DPPH radical scavenging activity using the following equation:

$$\%\ \text{DPPH Radical Scavenging Activity} = \frac{\text{Abs. Control} - \text{Abs. Extract}}{\text{Abs. control}} \text{X } 100$$

The EC_{50} values (concentrations required to obtain 50% antioxidant effect) were calculated as % DPPH scavenging activity in average of four replicates of the sample (Kong et al., 2012).

Antioxidant activity by ferric reducing antioxidant power (FRAP) assay

The total antioxidant potential of a sample was determined using the FRAP assay (Benzie and Strain, 1999). In this method, a potential antioxidant reduces ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) which when complexed with ferric tripyridyltriazine (Fe (III)-TPTZ) results in a blue colour with an absorption maximum at 593 nm. The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in water at 10:1:1 (v/v/v). Samples (50 µl) were added to each of the 96-well microtitre plate, followed by the addition of 200 µL of the FRAP reagent. The plate was incubated for 30 min at room temperature and the absorbance was measured at 593 nm using a BioTek Power wave XS spectrophotometer (Winooski, VT, USA). The ferric reducing activity was determined from a standard curve of $FeSO_4$ concentration (ranging between 3.906-125.3 µM) as a function of absorbance at 593 nm (R^2 = 0.995). Epigallocatechin gallate (10 µM) was used as the positive control.

Screening for cytotoxic activity on HeLa cervical cancer cells

Cytotoxicity was determined by Hoechst 33342 / Propidium iodide (PI) that led to establishment of IC_{50} of the extract and isolated compounds. HeLa cancer cells were routinely maintained in 10 cm culture dishes with antibiotic free RPMI 1640 cell culture medium, supplemented with 10% foetal bovine serum (FBS) in a humidified 37°C incubator supplied with 5% CO_2. HeLa Cells were seeded using 100 µl aliquots in 96 well microtitre plates at 4000 cells per well and left overnight to attach. An additional 100 µl of medium containing a predetermined concentration of extract as (0, 20, 40, 60, 80,100 and 120 µg/ml) of the plant extract/compound was added prior to incubation at 37°C in a humidified 5% CO_2 incubator for 48 h. After 48h the treatment medium was removed and replaced with 100 µl phosphate buffered saline (PBS) with Ca^{2+} and Mg^{2+} containing Hoechst 33342 at a concentration of 5 µg/ml. Propidium iodide (PI) was added at a concentration of 10 µg/ml using 10 µL aliquots per well of a 110 µg/ml stock. Cells were then imaged using an Image Xpress Micro XLS Wide field High-Content Analysis System (Molecular Devices ®). The IC_{50} value was calculated using graph pad prism software version 5.1.

Cell cycle analysis

HeLa Cells were seeded using 100 µL aliquots in 96 well microtitre plates at 4000 cells per well and left overnight to attach, then treated with the ethyl acetate fraction, at its IC_{50} (50.77 µg/ml) for 24 and 48 h. Treatment medium was removed and cells were stained by adding 50 µl aliquots of a mixture of Annexin V-FITC (20 µl) and Hoechst 33342 (1 µl) in 2 ml PBS (with Ca^{2+} and Mg^{2+}). Cells were then incubated in the dark for 15 min at room temperature followed by image acquisition. Similar treatment was done to Melphalan at a pre-determined IC_{50} value of 40 µM. that served as the positive

control.

Determination of Caspase 3 and 8 activation

HeLa Cells were seeded using 100 µl aliquots in 96 well microtitre plates at 4000 cells per well and left overnight to attach. The treatment medium was removed, and the cells were washed with PBS (with Ca^{2+} and Mg^{2+}). Cells were then fixed with 4% paraformaldehyde in PBS (with Ca^{2+} and Mg^{2+}) and permeabilized by adding 80% ice cold methanol and incubating at - 20°C for 10 min. Permeabilized cells were washed twice with PBS (with Ca^{2+} and Mg^{2+}) and then blocked using PBS (with Ca^{2+} and Mg^{2+}) containing 0.5% BSA for 30 min at room temperature. The cells were incubated for 1 h with cleaved Caspase 3 or cleaved Caspase 8 rabbit monoclonal antibody at the recommended working dilutions. Cells were washed to remove excess primary antibody. Alexa 647 - conjugated goat anti-rabbit secondary antibody was added at the recommended working dilution then incubated for 30 min. Cells were washed again to remove the secondary antibody and Hoechst added as a counterstain at 5 µg/ml.

Phosphatidyl serine (PS) translocation

Annexin V is used to detect apoptotic cells due to its ability to bind to phosphatidylserine, when it is on the outer leaflet of the plasma membrane (externalized) and thus a marker of apoptosis. HeLa Cells were seeded using 100 µl aliquots in 96 well microtitre plates at 4000 cells per well and left overnight to attach. The treatment medium was removed and cells were treated by adding 50 µl aliquots of a mixture of Annexin V-FITC (20 µl) and Hoechst 33342 (1 µl) in 2 ml PBS (with Ca^{2+} and Mg^{2+}). Cells were then incubated in the dark for 15 min at room temperature followed by addition of Propidium iodide (1.5 µl per well) prior to acquisition of images.

Isolation of compounds

The ethyl acetate fraction, which was the most toxic against the brine shrimps, was selected for bioassay-guided isolation of active compounds. Isolation was carried out using methods initially described and slightly modified by other researchers (Handa et al., 2008; Sasidharan et al*.,* 2011). The ethyl acetate fraction was subjected to open column chromatographic separation using gradient elution with solvent systems of increasing polarity. Silica gel (50 g) was well mixed in dichloromethane: petroleum ether (1:1) and packed in an open column of 2 cm internal diameter to the length of 25 cm. The dry fraction (5 g) was made into slurry using the solvent and silica gel and added to the column. In the first phase, elution was done with 100ml of dichloromethane: petroleum ether (1:1) and eluates were collected in 5ml portions. The second phase was done by elution with 100ml of dichloromethane (100%) and collected in fractions of 5ml each. The third phase was done using 100ml of 10% ethyl acetate in dichloromethane. Thin Layer Chromatography (TLC) profiles were established before and after each elution

Statistical analysis

For biological activity tests, each test was done in triplicate resulting to three average observations and hence final values were Mean ± SD (n = 3). Cytotoxic studies made use of three different transfer numbers of HeLa cells. Data were analyzed using Graph pad prism version 5.1. Statistical analysis of cytotoxicity, cell cycle and apoptosis induction were determined using the two-tailed Student t-test; differences were considered significant at $P \leq 0.05$. Data for antioxidant activity was determined by One Way ANOVA followed by Dunnett post-hoc test and differences were considered significant at $P \leq 0.05$.

RESULTS

Brine shrimp lethality test

The ethyl acetate fraction from the root extract of *A. mossambicensis* exhibited higher toxicity on the brine shrimp larvae with LC_{50} = 12.87 µg/ml (95% confidence interval of 9.48 - 17.49 µg/ml) than the standard anticancer drug, cyclophosphamide which gave an LC_{50} = 16.12 µg/ml (95% confidence interval of 10.32 – 24.95 µg/ml). The methanol fraction was non - toxic to the nauplii with LC_{50} = 823.00 µg/ml (95% confidence interval of 552.99 – 1224.8 µg/ml). The petroleum ether fraction was not tested because the amount recovered was too small for the test.

Antioxidant activity

The ethyl acetate fraction exhibited a dose dependent antioxidant activity in both the DPPH and FRAPS assays (Figure 1). The EC_{50} value for the DPPH assays was 200 µg/ml and for FRAP the EC_{50} was 53.92 µM Fe^{2+} [EGCGeq/g.DW].

Effect of ethyl acetate fraction on HeLa cervical cancer cells

Figure 2 shows the dose-response curve for the cytotoxic effect of the ethyl acetate fraction against HeLa cervical cancer cells after 48 h exposure. This fraction exhibited cytotoxic activity against the HeLa cells with an IC_{50} value of 50.77 ± 1.69 µg/ml. Treatment of cells with ethyl acetate fraction led to an increase in the number of apoptotic cells, reduced number of cells transitioning from G_0 to G_1 phase ($P \leq 0.05$) (Figure 3A).

Similarly, there was significant reduction in the synthesis of nucleic acids (S phase), and reduced number of cells transitioning through the G_2 phase. While there was apparent increase in cells in early mitosis, there were insignificant numbers of cells undergoing completion of mitosis. Melphalan which was used as a positive control significantly increased the percentage of apoptotic cells, it did not significantly increase the number of cells in G0/G1 transition and those in the S phase, but increased the number of cells in G_2 and early M phase of the cell cycle ($P \leq 0.05$), and there was complete suppression of cells completing the late M phase. Figure 3B shows that the ethyl acetate fraction significantly increased the number of apoptotic, late apoptotic/necrotic and necrotic cells after 48 h, compared to the untreated control. Evidence of apoptosis is supported by loss of membrane asymmetry and externalization of phosphatidylserine (PS) from the inner to the outer leaflet

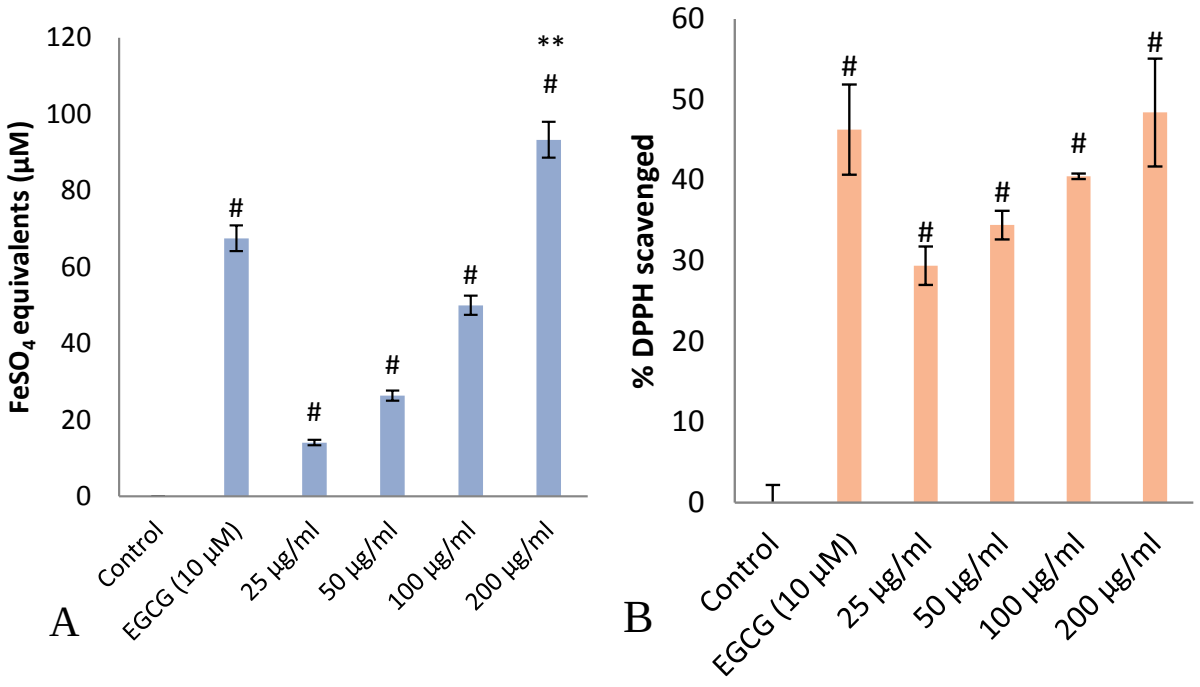

Figure 1. Antioxidant potential of ethyl acetate fraction of roots of A. mossambicensis by (A) FRAP and (B) DPPH. Results are reported as means ± SD for three independent experiments performed in quadruplicate. # $p < 0.05$ relative to control; **$p<0.005$ relative to EGCG (10 µM = 4.58 µg/ml).

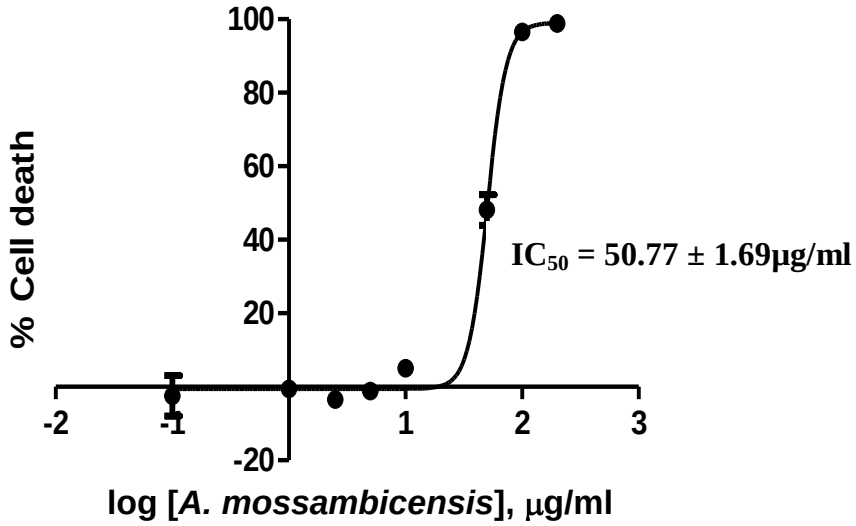

Figure 2. Cytotoxicity of ethyl acetate fraction of *A. mossambicensis* on HeLa cervical cancer cells.

of the cell membrane. The results in Figure 3B suggest that both apoptosis and necrosis (necroptosis) were involved in the cytotoxic effect of the ethyl acetate fraction. Figure 3B also shows that melphalan, which inhibits both RNA polymerase and DNA topoisomerase II significantly inhibited nucleic acid synthesis, and the number of cells exhibiting externalization of membrane phosphatidyl serine ($P \leq 0.05$). Figure 3C shows that the ethyl acetate fraction did not significantly activate caspase 3 at 24 h but its activity was slightly and significantly increased after 48 h ($P \leq 0.05$). On the other hand, Melphalan caused a large and significant release of activated caspase 3 after 24 and 48 h, respectively ($P \leq 0.05$).

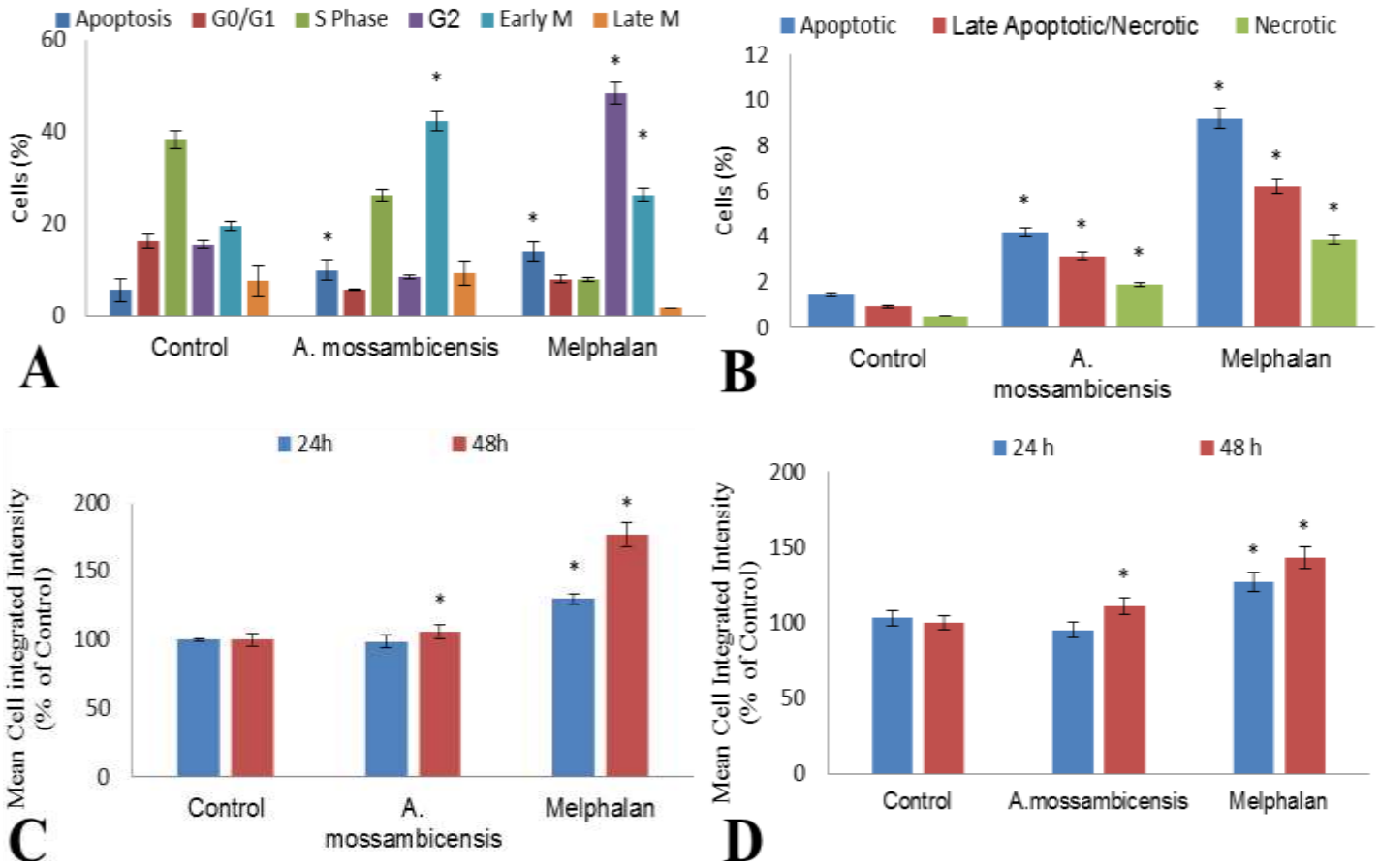

Figure 3. Effect of *A. mossambicensis* ethyl acetate fractiom at its IC50 on HeLa cells. (A) Cell cycle analysis, (B) Phosphatidylserine translocation, (C) Caspase 3 and (D) Caspase 8 activation. Error bars indicate SD of three individual experiments, each performed in quadruplicate. *P < 0.05 compared to Control, Control = Untreated cells.

Figure 3D shows the results for activation of caspase 8 by the ethyl acetate fraction of *A. mossambiscensis* and melphalan. This fraction did not activate caspase 8 at 24 h, but it seems to have shown a small activation at 48 h. On the other hand, melphalan showed significant activation caspase 8 after incubation for 24 and 48 h, respectively.

Isolation of compounds from the ethyl acetate fraction

Compounds 1, 2, 3 and 4 (Table 1) were isolated from the ethyl acetate fraction of the root extract of *A. mossambicensis*. Chemical structures were elucidated, using ^{1}H NMR and ^{13}C NMR and High-resolution electron spray - Mass spectrometry (HRESI-MS). Chemical shifts and couplings were virtually identical for all carbon positions in base structure with the exception of a few positions with double bonds or hydroxyl groups as highlighted in Table 2. In the first Compound (1), ^{1}H NMR (CD_2Cl_2, δ): H-15 (5.32, 1H), H-17 (5.07, 2H) and H-3' (6.02, 1H) and ^{13}C NMR chemical shifts (δ) for C-16 (153.7), C-17 (110.4), C-1' (167.2), C-2' (128.2) and C-3' (138.6) are characteristic of double bonds. The base ion at *m/z 83* was diagnostic for an angelic ester attached to the base structure. The (HRESI-MS) revealed the molecular ion (M^+ = 400.26 m/z) as shown in Figure 4. In the second compound (2), the ^{1}H NMR (CD_2Cl_2, δ): H-11 (5.29, 1H) and ^{13}C NMR chemical shifts (δ) for C-9 (157.5), C-11 (120.5), and C-19 (183) are characteristic of double bonds. The (HRESI-MS) analysis showed the molecular ion (M^+ = 318.22 m/z) as shown in Figure 5. In the third compound (3), the ^{13}C NMR chemical shifts (δ) revealed high shift at C-19 (183), for a carbonyl group. The (HRESI-MS) analysis showed the molecular ion (M^+ = 321.24 m/z) as shown in Figure 6. In the fourth compound (4), the ^{1}H NMR (CD_2Cl_2, δ): H-17 (5.29, 5.04, 2H) is characteristic of a double bond. ^{13}C NMR revealed high chemical shifts (δ) at C-16 (159.6) and C-17 (110.4) as the effect of double bond and high shift at C-19 (183) for the carbonyl group. The (HRESI-MS) analysis showed the molecular ion (M^+ = 318.22 m/z) as shown in Figure 7

The literature shows that compounds 1, 2, 3 and 4 are

Table 1. Compounds isolated from ethyl acetate fraction of *A. mossambicensis*.

Compound	EI-MS (M^+ = m/z)	Formula	Name of compound
1	400.26	$C_{25}H_{36}O_4$	(-)-Angeloyl grandifloric acid
2	318.22	$C_{20}H_{30}O_3$.	16-Hydroxy-9(11)-kauren-19-oic acid
3	321.24	$C_{20}H_{32}O_3$	16α -hydroxykauran-19-oic acid
4	318.22	$C_{20}H_{30}O_3$	Grandifloric acid

Table 2. ^{1}H and ^{13}C NMR data for compounds 1-4 (600 MHz; $CDCl_2$ for 1and 2 and MeOD for 3 and 4).

C- No.	^{1}H–NMR (δ)				^{13}C–NMR (δ)			
	1	2	3	4	1	2	3	4
C- 1	0.86,1.89(2H)	1.73,1.53	1.56,1.31	1.56,1.31	39.3	39.3	39.3	39.3
C- 2	1.57,1.63(m, 2H)	1.53,1.43	1.53,1.43	1.53,1.76	18.6	18.6	18.6	18.6
C- 3	1.03,2.12(m, 2H)	2.01,1.76	2.01,1.76	2.01,1.76	37.4	37.4	37.4	37.4
C- 4	0	0	0	0	43.9	43.9	43.9	43.9
C- 5	1.12(dq, 1H J=7.0,1.0Hz)	1.76	1.7	1.72	56.3	56.3	56.3	56.3
C- 6	1.52,1.27(m, 2H)	1.38,1.13	1.52,1.27	1.52,1.27	20.6	38.2	20.6	20.9
C- 7	1.26,1.64(m, 2H)	1.38,1.13	1.56,1.31	1.56,1.31	36	27.9	40.8	35.8
C- 8	0	0	0	0	46.1	27.9	43.5	48
C- 9	1.28(1H)	0	1.39	1.39	56.5	157.5	55.1	49.7
C- 10	0	0	0	0	38.3	20.5	38.3	38.6
C- 11	1.43,1.85(m, 2H)	5.29	1.52,1.27	1.52,1.27	18.1	120.5	20.8	18.4
C- 12	1.50,1.63 (m, 2H)	2.04, 1.79	1.52,1.27	1.58,1.31	32.7	27.5	26.4	33
C- 13	2.78(m,1H)	1.61	1.5	1.12	42.2	49.8	49.8	42
C- 14	1.45,1.98(2H)	1.55, 1.30	1.45,1.20	1.49,1.24	36.6	37.7	37.7	36.7
C- 15	5.32(m, 1H)	1.71,1.46	1.67,1.42	3.88	82.6	58.6	58.6	82.6
C- 16	0	0	0	0	153.7	81	81	159.6
C- 17	5.07,5.11(mm, 1H)	1.29	1.29	5.29,5.04	110.4	28	25.4	110.4
C- 18	1.22(s,3H)	1.33	1.33	1.33	28	15.8	28	28
C- 19	0	0	0	0	183	183	183	183
C- 20	0.96(s,3H)	1.29	1.04	1.04	15.8	25.4	15.8	15.8
C- 1’	0				167.2			
C- 2’	0				128.2			
C- 3’	6.02(q, 1H,J=1.0)				138.6			
C- 4’	1.86(d, 3H,J=1Hz)				15.3			
C – 5’	1.94(s, 3H,J= 1Hz)				18.8			

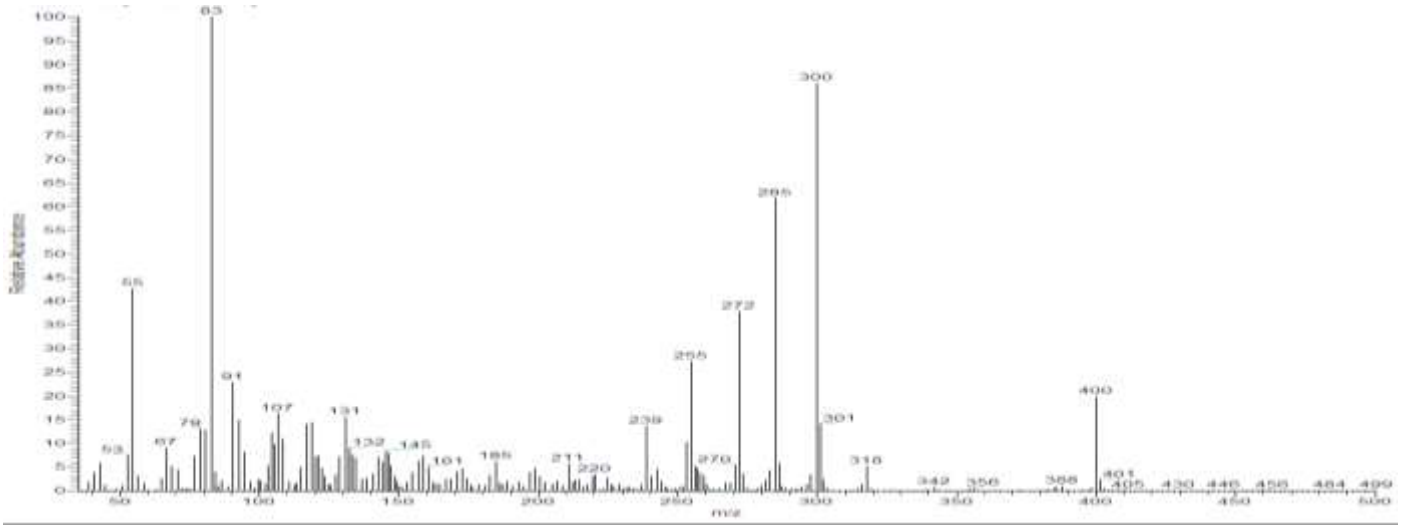

Figure 4. Mass Spectrum of (-)-angeloyl grandifloric acid (**1**).

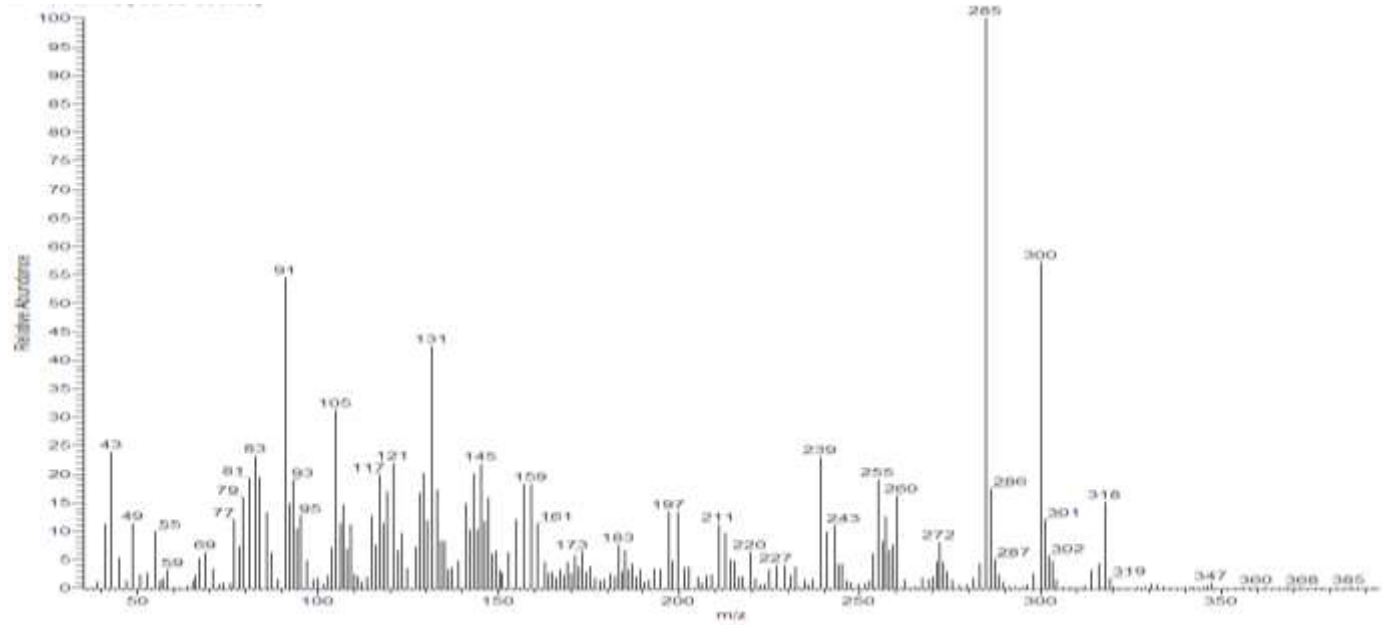

Figure 5. Mass Spectrum of 16-Hydroxy-9(11)-kauren-19-oic acid (**2**).

not new since they have been isolated from other plants Ohno and Mabry, 1980; Martin et al., 1997; Lee et al., 2015) although they have not been screened previously, for cytotoxicity and anticancer effects. Based on spectroscopic analysis, structures of the four compounds were generated as shown in Figure 8 and identified through comparison with known compounds, as (-)-Angeloyl grandifloric acid (1), 16α-Hydroxy-9(11)-kauren-19-oic acid (2), 16α-Hydroxykauran-19-oic acid (3) and Grandifloric acid (4). Characteristics of isolated compounds that include carbon position, chemical shifts obtained from ^{1}H-NMR and ^{13}C-NMR are presented in Table 2. Spectroscopic and chromatographic analyses with literature reports were finally used for confirmation of structures of the isolated four compounds as shown in Figure 8.

Cytotoxicity of isolated compounds

Cytotoxicity of isolated compounds were determined by the same procedure applied in cytotoxicity of extract The

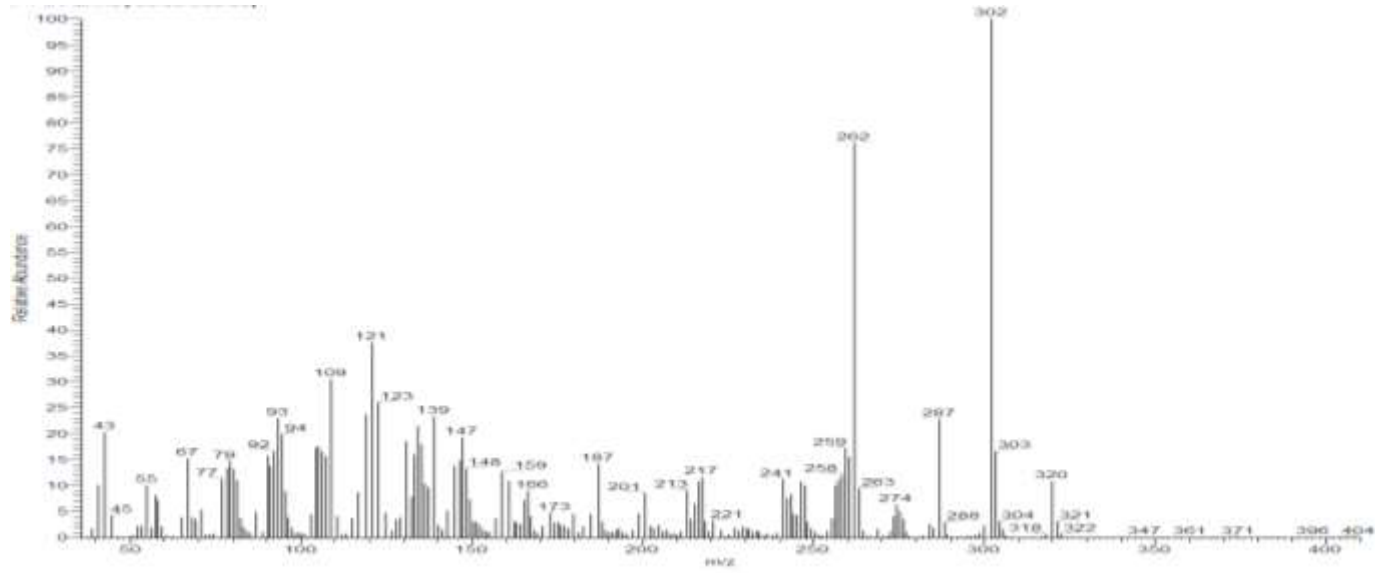

Figure 6. Mass Spectrum of 16α -Hydroxykauran-19-oic acid (**3**).

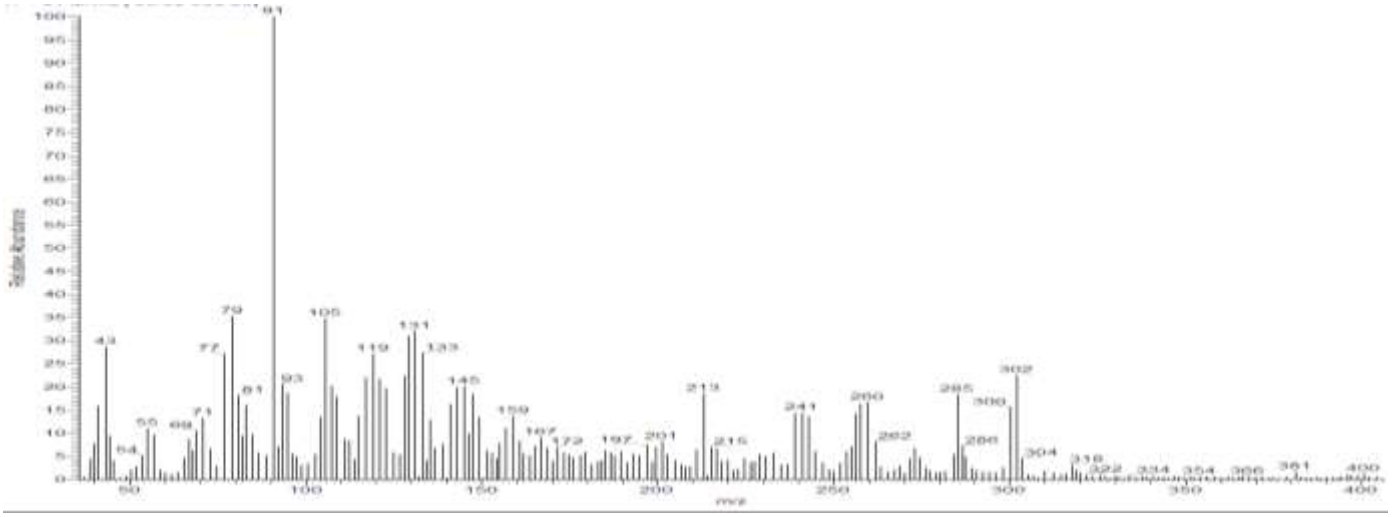

Figure 7. Mass Spectrum of Grandifloric acid (**4**).

results show that two of the isolated compounds from the ethyl acetate fraction; (-)-Angeloyl grandifloric acid (IC_{50} = 27.75 ± 1.92) and 16α -hydroxykauran-19-oic acid (IC_{50} = 40.19 ± 2.28) were cytotoxic to the HeLa cervical cancer cells following an exposure for 48 h. The other two compounds 16α-Hydroxy-9(11)-kauren-19-oic acid (2) and Grandifloric acid had IC_{50} values which were above 300 µg/ml; indicating that they are not cytotoxic to the HeLa cancer cells.

DISCUSSION

We have shown in our previous studies that the brine shrimp lethality test does, to some degree, identify plant extracts which also show cytotoxic activity against cancer cell lines (Innocent et al., 2010), although this assertion is not universally true because some brine shrimp inactive extracts have been shown to have cytotoxic activity on cancer cell lines (Eboji et al., 2017a). Notwithstanding, in this study, the observed effect of the ethyl acetate fraction on brine shrimp larvae correlates well with the observed cytotoxic activity against the HeLa cancer cells. Furthermore, another pre-screening was done using two antioxidant experiments, assays which suggest presence of a positive correlation with the cytotoxic activity against the HeLa cancer cell lines. The existence of a correlation between antioxidant activity and cytotoxic activity on cell

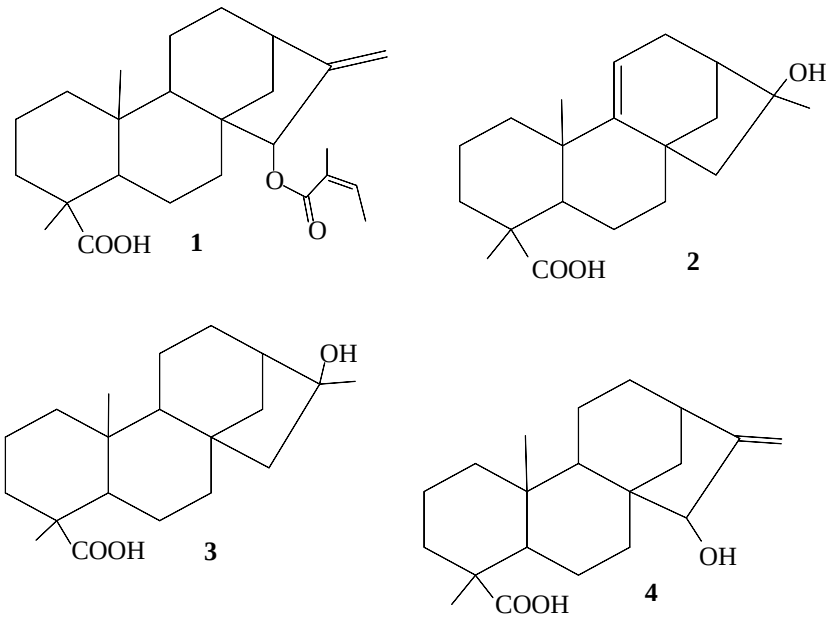

Figure 8. Compounds isolated from ethyl acetate fraction of *A. mossambicensis*.

lines has been reported before (Li et al., 2007). It is not being suggested that there is a definitive likelihood that positive/negative antioxidant or brine shrimp results will predict what will happen to cancer cell lines, but at least there is an anecdote that is worth following up with more research to better understand the frequently reported observation.

The reported toxicity of *A. mossambicensis* ethyl acetate fraction, with an IC_{50} = 50.77±1.69 µg/ml, is comparable to that of some other studied plant extracts, such as the ethanolic extracts of *Euphorbia grandidens* (LC_{50} = 57µg/ml and *Euphorbia grandicornis* (LC_{50} = 89 µg/ml, which ultimately showed anticancer activity (Whelan and Ryan, 2003; Patel and Gheewala, 2009). The cytotoxic activity is probably due to inhibition of mitosis, because, from the results, the number of cells re-entering the cell cycle at the G_0/G_1 are significantly reduced and also cells on the Late M phase of the cell cycle, are almost depleted. This may be interpreted that cells treated with the ethyl acetate fraction failed to fulfil requirements of cell cycle checkpoint number two for M phase transition (Lara-Gonzalez et al., 2012; Wang and Higgins, 2012).Cells arrest at M phase may be due to activation of the spindle-assembly checkpoint (SAC), which restricts cells with incomplete or abnormal mitosis to cross the interphase (Musacchio and Salmon, 2007). In addition to other factors, the prolonged arrest may ultimately end up with cell death by apoptosis.

Evidence of phosphatidylserine (PS) translocation to the outer leaflet of the cell membrane confirms occurrence of apoptosis, although there is possibility that the sample also causes necrosis to some of the HeLa cells as supported by Annexin V and Propedium Iodide (PI) stains, respectively (Marchette et al., 1996; Eboji et al., 2017). The results indicate evidence of activation of Caspase 3 after 24 and 48 h, but for Caspase 8 there was activation after 48 and not after 24 h. Caspase 3 is an executioner Caspase and it is involved in the central caspase system and its signal is activated by either the mitochondrial or receptor mediated pathways (Eboji et al., 2017b), but one would not have expected activation of Caspase 8, which is involved in the receptor pathway of apoptosis. Similarly, the ethyl acetate fraction seems to have caused both apoptosis and necrosis in the cytotoxic activity against the HeLa cancer cells, hence creating more questions than answers which require additional research to try to better understand the mechanism by which the *A. mossambicensis* ethyl acetate fraction causes cytotoxicity to the HeLa cancer cells.

In the ^{1}H NMR spectrum of compound 1, chemical shifts (δ) at 5.32 (1H), 5.07 (2H) and 6.02 (1H) indicated the presence of double bonds. In ^{13}C NMR chemical shifts (δ) for C-16 (153.7), C-17 (110.4), C-1' (167.2), C-2' (128.2) and C-3' (138.6) were evident characteristic of double bonds. The compound gave MS - m/z (rel.int.): 400 (20), 318 (5), and 300 (85), 285(62), 83(100) and a calculated M^+ 400.5509 as shown in Figure 4. The base ion at m/z 83 was diagnostic for an angelic ester attached to the base structure predicting the chemical formula $C_{25}H_{36}O_4$ (Table 1). Final identification of the compound (-)-angeloyl grandifloric acid (1) was confirmed through comparison with spectroscopic data of a previously reported compound (Ohno et al., 1979).

In the second compound (2), the ^{1}H-NMR chemical shift (δ) for H-11 (5.29, 1H) was relatively high probably due to the presence of a double bond. In ^{13}C-NMR

chemical shifts (δ) for C-9 (157.5), C-11 (120.5), and C-19 (183) were high and characteristic of double bonds. MS- m/z (rel. int.): 318(15), 300(55), 285(100), 131(42), 91(55) and a calculated M+ = 318.4504. The base ion at m/z 300 and 285 indicates a consecutive loss of molecules of water (H_2O) and a methyl group (CH_3) (Figure 5). Preliminary assessment of MS predicted the chemical formula $C_{20}H_{30}O_3$. Final identification of the second isolated compound was made by comparison with a previously isolated compound and reported as 16-Hydroxy-9(11)-kauren-19-oic acid (2). The compound has a generic structure similar to a known compound which was isolated from the leaves of *Piliostigma thonningii* (Martin et al., 1997), with exception that this compound has a double bond at C-9(11), which is contrary to the known ent-16α-hydroxykauran-19-oic acid.

In the third compound (3), chemical shifts in ^{1}H NMR were virtually identical for all positions. The ^{13}C NMR chemical shifts, revealed high shift at C-19(∂183) indicating the presence of a carbonyl group. A MH^+ peak was recorded at m/z 321.2424 in the high-resolution chemical ionization mass spectrum corresponding to the molecular formula $C_{20}H_{32}O_3$. An intense peak at m/z 303.2317 related to MH – H_2O (Figure 6). The compound was identified as 16α- Hydroxy-ent-kauran-19-oic acid (3) which is similar to the compound previously isolated from *Wedelia trilobata* (L) Hitchc (Ren et al., 2015).

The fourth compound (4) showed relatively high chemical shifts (δ) in the ^{1}H NMR spectrum (5.29 and 5.04, 2H) and the ^{13}C NMR shifts (δ) at 159.6 (C-16) and 110.4 (C-17); this is due to the presence of a double bond. The chemical shift at 183 was characteristic of a carbonyl group (C-19) and a peak at m/z 318.4504 (Figure 7) in the mass spectrum is a molecular ion. The compound was eventually identified as Grandifloric acid and the spectral data were identical with those of a compound previously isolated from various species of *Helianthus,* including *H. niveus* and *H. debelis* (Ohno et al., 1979; Ohno and Mabry, 1980).

Two of the isolated compounds, (-)-Angeloylgrandifloric acid (1) and 16α- hydroxykauran -19-oic acid (3) demonstrated toxicity on HeLa cells, with IC_{50} values of 27.75 and 40.19 µg/ml, respectively. However, they were less active when compared to the ethyl acetate fraction, which indicates that there are other more active compounds in the fraction which could not be isolated. This seems to be the case because a previous study reported cytotoxic activity of compounds isolated from *Aspilia* species in which compounds; 12α-methoxy-ent-kaur-9(11),16-dien-19-oic acid and 9β-hydroxy-15α-angeloyloxy-ent-kaur-16-en-19-oic acid were cytotoxic against hepatocellular carcinoma (Hep-G2) cell line with IC_{50} = 27.3 ± 1.9 µM and IC_{50} = 24.7 ± 2.8 µM respectively; while 15α-angeloyloxy-16β,17-epoxy-ent-kauran-19-oic acid was cytotoxic against adenocarcinomic human alveolar basal epithelial (A549) cells with IC_{50} = 30.7 ± 1.7 µM (Yaouba et al., 2018). The current results are a new contribution showing that there are two more compounds in *A. mossambicensis* that have been previously isolated but their cytotoxic activity on cancer cell lines has not been reported. The current results have added another cell line to the list of already reported cancer cell lines which are killed by extracts of *A. mossambicensis* and hence, form a basis for planning more studies to further elucidate the mechanism of anticancer activity and potential therapeutic application.

Conclusion

Through bioassay-guided isolation, two compounds with cytotoxic activity against HeLa cervical cancer cells were isolated from roots of *A. mossambicensis* in support of claims by traditional health practitioners who are using preparations from the root for treatment of cancer. Further studies are needed to elucidate the mechanisms of anticancer activity and the therapeutic potential of the plant.

Data availability

Data for this study are obtainable at the Department of Natural Products Development and Formulations, Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences, P. O. Box 65001, Dar es Salaam, Tanzania; and at the Government Chemist Laboratory Authority of Tanzania. P. O. Box 164, Dar es Salaam – Tanzania.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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