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Anticancer properties of recipes derived from Nigeria and African medicinal plants on breast cancer cells *in vitro*

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ABSTRACT

The study was carried out to examine the effects of two recipes prepared from Nigerian and African medicinal plants on the cell cytotoxicity, cell cycle distribution and apoptosis on breast cancer (MCF-7 and MDA-MB-231) cells and normal human fibroblasts. *In vitro* cytotoxicity and anti-proliferative effect was evaluated by 3-[4, 5-dimethylthizol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay while cell cycle and apoptosis were evaluated by flow cytometry using propidium iodide (PI) staining. The reactive oxygen species analysis by DCFDA staining and flow cytometry analysis while alterations in Mitochondrial Membrane Potential (MMP, $\Delta\Psi_m$) were determined by staining the cells with Rhodamine 123 then FACS Calibur flow-cytometry analysis. Expression of apoptotic-related proteins and key signalling transduction proteins was carried out by western blotting. MTT assay results indicated that the recipes were able to inhibit the growth and proliferation of cancer cells and fibroblasts in a dose-dependent manner ($P < 0.05$). Nevertheless, both recipes exhibited G_0/G_1 cell cycle arrest by flow cytometry. The reactive oxygen species analysis showed that the extracts did not induce free radical generation in the cancer cells whereas the $\Delta\Psi_m$ assessment showed alterations in mitochondrial membrane potential with increased expression of two apoptosis inducing protein i.e. caspase-3 and poly (ADP-ribose) polymerase. The two recipes inhibited cell proliferation through the induction of G_0/G_1 cell cycle arrest and increased expression of apoptotic inducing protein, which leads to cancer cell death. This study provides an early evidence to support the traditional use of African medicinal plant recipes in cancer treatment.

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Introduction

The use of medicinal as well as aromatic plants and its products predates written human history, and occupy an important position as sources of drugs discovery [1]. More than 122 compounds used in contemporary medicine were derived from plant sources; 80% of which have been reported earlier for ethnomedicinal use and are similar to the current use of the active principles of plant origin [2]. Numerous pharmaceuticals currently available for clinical use have a long history as herbal remedies for human use including aspirin, digitalis, quinine and opium [3]. Medicinal and aromatic herbs have been used to treat disease among developing countries and are less expensive than the modern pharmaceuticals [4,5,6]. According to World Health Organization (WHO), over 80 percent of the populations of some Asian and African countries have been reported to use medicinal plants for primary health care [1,7]. Whereas, the clinical use of medicinal plants and phytomedicines in the United States and Europe is less common, but these has been dramatically increasing in recent years as the scientific evidence on the effectiveness of herbal medicine are being increasingly explored [8,9].

Uvaria chamae, Khaya grandifolia, Xylopia aethiopica, Musa parasidiaca, Senna alata, Olax subscorpioidea and *Securidaca longepedunculata* are naturally distributed in the rain forests of the southern parts of Nigeria and many parts of Africa [10–12]. Some of these plants (*Allium ascalonicum, Citrus medica* and *Mangifera indica*) are also available in India while some exist as different species (*Antiaris toxicaria, Bridelia montana, Bridelia retusa, Kigelia pinnata* and *Xylopia parviflora*) [13,14]. Many parts of these plants and other plants have been used traditionally for their anti-cancer, antioxidants, ascaricidal, schizonticidal, antimicrobial, anthelmintic, insecticidal, anti-inflammatory, anti-diarrheal and larvicidal properties [15–18]. Similarly, different parts of these medicinal plants have been combined resulting into recipe, which has been used traditionally in the treatment and management of cancer in Nigeria and many parts of Africa because of the presence of many active compound such as antioxidants which may act synergistically [12,19].

Cancer is characterized by uncontrolled proliferation and growth of abnormal cells [20–22]. It has been reported that mortality due to cancer is about 3,500/million population annually around the world [21]. The need for cells to survive, as the cells become tumorigenic, brought about many fundamental changes in cell behavior [20]. The uncontrolled growth and proliferation leading to activation of epithelial to mesenchymal transition (EMT) is a major challenge for cancer treatment and survival of the patients [23,24]. Failure of important organs such heart, liver, kidney and lungs are the results of metastasis, which may finally leads to death. Researchers have attributed the anticancer activity of medicinal plants to the presence of antioxidants [9]. However, combination of many medicinal plants has been used traditionally to treat cancer with little or no investigation of their cytotoxic properties but rather relying on the past experience and observation passed on from generation to generation verbally and in writing [12].

Anticancer properties of several medicinal plants were characterized individually with different mechanisms to control cancer cell proliferation whereas; the anticancer properties of recipes from various medicinal and aromatic plants were not studied in detail. Based on the importance of Nigerian and African medicinal plants (NAMPs) as well as its recipes, the study was designed to characterize the anticancer properties of NAMPs recipes used traditionally for the treatment and management of cancer in Nigeria on cell lines through *in vitro* cytotoxicity, cell cycle assay and mitochondrial membrane potential. The study is of great significant as there is lack of scientific evidence on the mechanism of action of the recipes from African traditional medicine on cancer cells.

Materials and methods

Cell lines and chemicals

Cell lines were obtained from National Centre for Cell Science *(*NCCS*),* Pune, India. Dulbecco's Modified Eagle's medium (DMEM), Leibovitz's L-15 Medium (L-15), fetal bovine serum (FBS) and Trypsin are obtained from HiMedia Laboratories, Mumbai, India. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) dye, Tetramethylrhodamine Methyl Ester Perchlorate (TMRM) dye, Propidium iodide, EDTA, methanol and phosphate buffers were purchased from Sigma-Aldrich Corporation, Missouri, USA.

Plant materials

The plants namely, *Allium ascalonicum, Anchomanes difformis, Antiaris africana, Bridellia scadens, Calliadra haematocephala, Citrus medica, Garcinia kola, Kigelia africana, Mangifera indica, Nauclear latifolia, Tricalysia macrophylla, Trichilia monadelpha* and *Xylopia aethiopica* and their parts were collected fresh from farmland in Sagamu, Ogun State, Nigeria between January and December, 2015 (Table 1). The plant parts collected fresh during the daytime and grouped into two as described as previously described [12]. The plants parts possess medicinal and pharmacological properties (Supplementary Table 1)

Extraction of plant materials

Plant materials were dried in hot air oven at 40°C and powdered by grinding using Kenstar electric mill (Kitchen Appliances Indian Ltd, India). The powders were kept in an air-tight container in dark until used. The powder of the constituent (2.5 g) in each group was added together and extracted by methanol using Soxhlet apparatus (B & C Industries, Kerala, India)

Table 1
Enumeration of Recipes

Recipe composition	Voucher No	Plants parts
Recipe A		
Anchomanes difformis	AD15005	Tuber
Garcinia kola	GC15017	Bark
Kigelia africana	KA15022	Pod
Xylopia aethiopica	XA15046	Pod
Recipe B		
Allium ascalonicum	AA15004	Leaves
Antiaris africana	AA15006	Bark
Bridellia scadens	BS15008	Bark
Calliadra haematocephala	CH15009	Root
Citrus medica	CM15013	Fruit juice
Mangifera indica	MI15024	Bark
Nauclear latifolia	NL15027	Bark
Tricalysia macrophylla	TM15043	Bark
Trichilia monadelpha	TM15044	Bark

for 10 h. The resulting extract was concentrated with rotary evaporator (IKA Indian Pvt. Ltd, Karnataka, India) to about 15 ml and subsequently dried using Concentrated plus (Eppendorf Ag, Hamburg, Germany) and kept at -80°C until use.

HPLC-finger-printing of recipe extracts

The High Pressure Liquid Chromatography (HPLC) fingerprinting of the recipe extracts was performed on a HPLC system (Waters, USA) using Nucleosil® C18 HPLC reversed-phase column (250×3.2 mm) (Sigma-Aldrich Corporation, Missouri, USA) as described by Li and Wang [25]. A 100 mg/ml of the extract was separated by following conditions: 20 μl injection volume, 254 nm detection wavelength, methanol: water/50: 50 (isocratic condition) mobile phase, 22°C temperature, 28 MPa pump pressure, 1 ml/min flow rate and 12 min running time.

Cell line maintenance

Breast cancer cell lines such as MCF-7, MDA-MB-231 and fibroblasts were maintained at School of Life Sciences, Manipal University, Manipal, India according to standard cell culture procedures. MCF-7 and fibroblasts were cultured in DMEM while MDA-MB-231 was cultured in L-15 media containing 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 incubator (Thermos Fisher Scientific, Maryland, USA).

Cytotoxic assay

The cytotoxicity of the extracts was tested *in vitro* on MCF-7, MDA-MB-231 and fibroblast cells using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay [26]. Briefly, 1×10^4 cells/well in 100 µl media were seeded into 96-well cell culture plate and allowed to attach to the plate for 24 h. The cells were treated with different concentrations (0.1, 1, 10, 25, 50, 100, 200, 500 and 1000 µg/ml) in 100 µl along with 50 µg/ml mitomycin as positive control and dimethyl sulfoxide (DMSO) as negative control and incubated at 37°C in a humidified 5% CO_2 for 48 h. Following incubation, 20 µl MTT (5 mg/ml) was added to each well and incubated for another 3-4 h after which it was centrifuged at 1,000 rpm for 5 min. The formazon crystals were dissolved by the addition of 100 µl of DMSO to each well and the absorbance was measured at 540 nm using Infinite 200 PRO multimode reader (Tecan Group Ltd, Austria). The percentage cell survival was calculated by using following formula:

$$\%\ \text{Cell survival} = \frac{\text{Mean abs of triplicate drug wells}}{\text{Mean abs of triplicate control wells}} \times 100$$

A bar chat was prepared using percentage cell survival against the concentration of the recipe and the IC_{50} values of Recipe A and B extracts were calculated.

Cell cycle and apoptosis assay

Cell cycle distribution was assessed by measuring DNA content using propidium iodide (PI) as earlier described [27]. Briefly, 3×10^5 cells in 3 ml media were seeded into 6 cm petridish and allowed to attach to the plate for 24 h. For Recipe A extract, MDA-MB-231 cells were treated with 10 and 200 µg/ml, MCF-7 cells were treated with 10 and 100 µg/ml and fibroblast cell with10 and 100 µg/ml. While Recipe B extract, MDA-MB-231 cells were treated with 1 and 100 µg/ml, MCF-7 cells were treated with 10 and 200 µg/ml and fibroblast cell with10 and 200 µg/ml. The cells were incubated at 37°C in a humidified 5% CO_2 for 48 h. The cells were harvested using trypsinization, washed with phosphate buffered saline (PBS), fixed in cold 70 % ethanol at 4°C overnight, washed with PBS and centrifuge at 2500 rpm for 10 min. The cells were treated

with 20 µl RNAse (10 mg/ml) for 1 hour at 37°C, stained with PI (1 mg/ml) for 30 min in the dark and resuspended in 500 µl PBS and analyzed by FACS Calibur flow cytometer (BD, USA) using CellQuest software (BD Biosciences, USA). The experiments were performed in duplicates.

Analysis of mitochondrial reactive oxygen species (ROS) generation

Mitochondrial ROSgeneration was analysed as per the protocol published earlier [28]. Briefly, 3×10^5 cancer cells were treated with extract of 10 and 200 µg/ml of Recipe A and B for 1 h. The cells were loaded with of 5 µM of 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 30 minutes at 37°Cin, 5% CO_2 incubator. Cells were then detached by trypsinization and resuspended in HBSS (500µl) and acquired using flow-cytometer (FACS Calibur, Becton Dickinson, USA) using CellQuest software. For each sample 1×10^4 cells were counted. DCF was measured at 488 nm excitation (25 mW) and detected using a 530/30 nm bandpass filter.

Alterations in mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in $\Delta\Psi_m$ were determined by staining the cells with Rhodamine 123 as previously described [27]. Cancer cells were seeded and treated as described in ROS assay. Cells were loaded with 5µM Rhodamine 123 (Rh-123) and incubated for 30 minutes at 37°C in 5% CO_2 incubator. The cells were washed with PBS to remove excess dye, trypsinized and resuspended in 500µlPBS and acquired using FACS Calibur flow-cytometer (Becton Dickinson, USA; Ex/Em-485/570 nm) using CellQuest software.

Expression of signaling molecular protein

MDA-MB-231, MCF-7 and Fibroblast cells were treated with Recipe A and B for 48 h to determine the effect of the recipes on Caspase-3 and PARP protein expression. The expression of apoptotic-related proteins and key signalling transduction proteins were determined by Western blotting as previously reported [29]. Briefly, protein concentrations were assessed with a Lowry Method. 25µg of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes (Pierce). Caspase-3 and PARP protein expression were detected by Western blot analysis with the use of β-actin as internal control. Goat Anti-Rabbit IgG H&L (HRP) secondary antibodies were used. Signals were visualized and scanned with ImageQuant LAS4000 luminescent image analyser (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and quantified by ImageJ software (NIH, USA). The relative amounts of protein expression were quantified to those of the corresponding control, which were set to a value of 1.0.

Statistical analysis

GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and plotting of the graphs. Data are presented as mean ± SD. The $P< 0.05$ was considered as statistically significant by Student t-test.

Results

HPLC finger-printing of recipe extracts

The HPLC fingerprinting of recipe extracts (Recipe A and B) were determined to identify the major peaks (compounds) in the two extracts for the purpose of identification and reproducibility during the subsequent methanolic extract of the recipes (Fig. 1). A distinctive peak was obtained in Recipe A at 5.701 min (Fig. 1A) while three distinctive peaks was obtained in Recipe B at 5.840, 7.204 and 8.523 min respectively (Fig. 1B). These peaks indicate the presence of active compounds in the recipes. The use of HPLC as a quality control measure was performed as reported by Agyare *et al.* [30].

Cytotoxicity assay

The result of cytotoxicity evaluation of the recipes prepared from NAMPs against MDA-MB-231, MCF-7 and fibroblasts are shown in Fig. 2. The extract of Recipe A exhibited significant concentration-dependent anti-proliferative activity with an IC_{50} values of 8.00±1.25, 4.10±0.81 and 23.80±2.15 µg/ml against MDA-MB-231 (Fig. 2A), MCF-7 (Fig. 2B) and fibroblast cells (Fig. 2C) respectively. Similarly extract of Recipe B exhibited significant concentration-dependent anti-proliferative activity with an IC_{50} values of 0.40±0.01, 5.87±0.42 and 65.43±3.38 µg/ml against MDA-MB -231 (Fig. 2D), MCF-7 (Fig. 2E) and fibroblast cells (Fig. 2F) respectively (Table 2).

Values with different superscript indicates significance difference at $P<0.05$ (a<b<c)

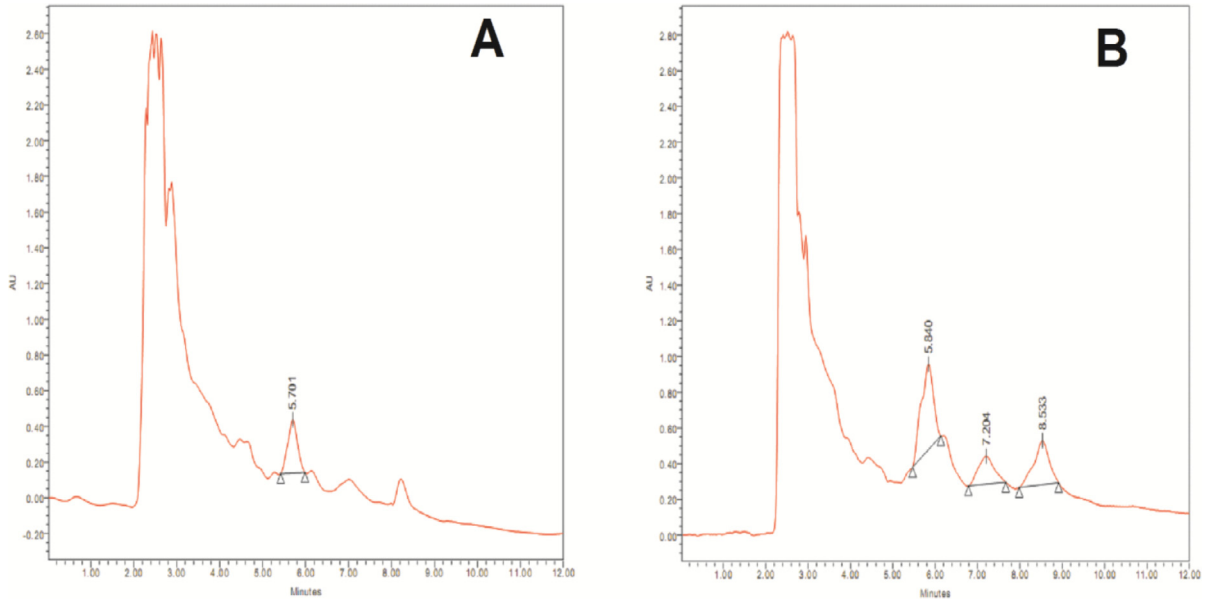


Fig. 1. HPLC chromatogram of Recipe A extracts at 5.701 min and B extracts at 5.840, 7.204 and 8.533 min respectively (positive ion mode).

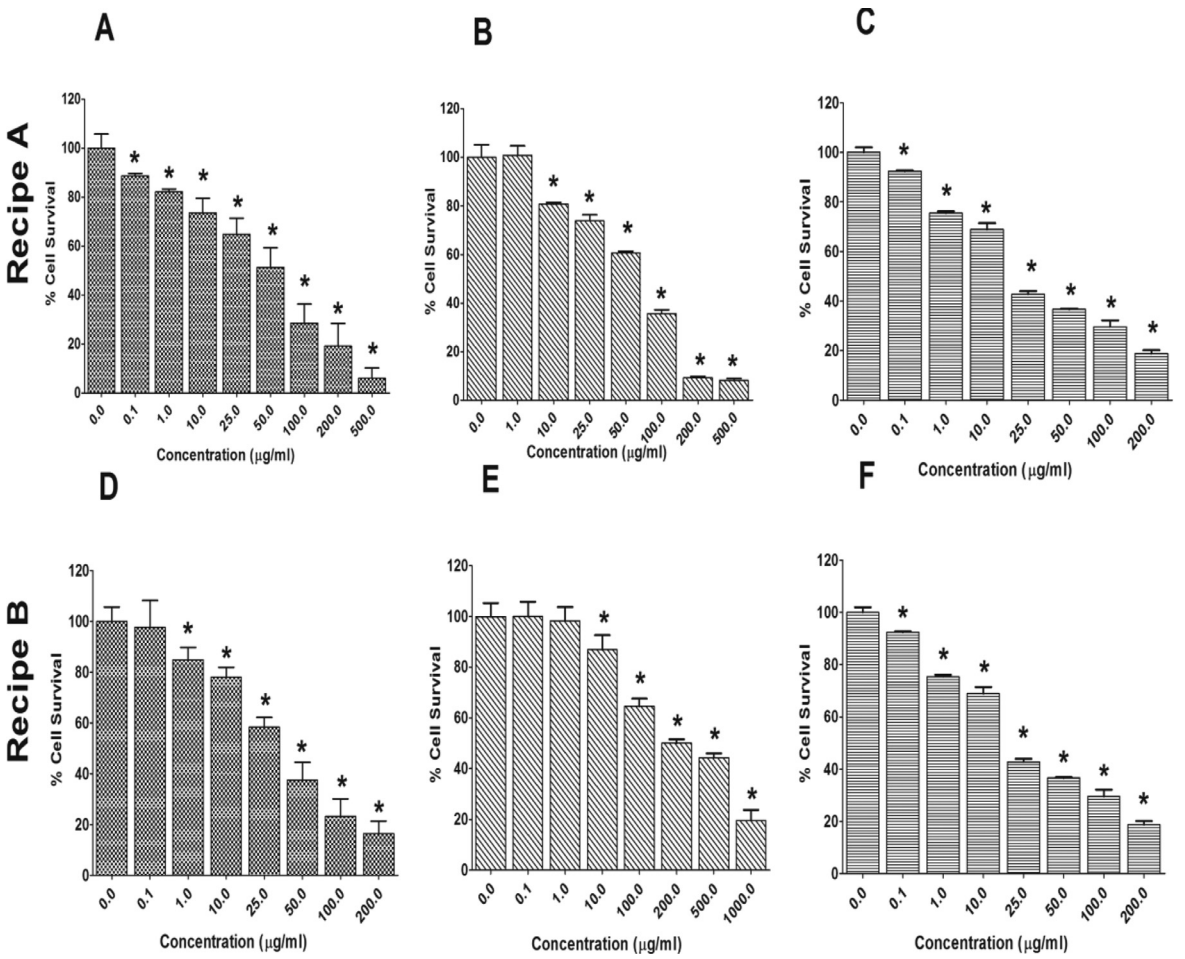


Fig. 2. Cytotoxicity effect of Recipe A and B extracts on cancer cells and fibroblast. Asterisk (*) indicates significance difference ($P<0.05$) when compared with control (0µg/ml)

Table 2
IC_{50} of the recipe extracts on cancer cells and fibroblast as determined by MTT assay.

Cell line	Recipe A	Recipe B
MDA-MB-231	8.00±1.25[b]	0.40±0.01[a]
MCF-7	4.10±0.81[a]	5.87±0.42[b]
Fibroblast	23.80±2.15[c]	65.43±3.38[c]

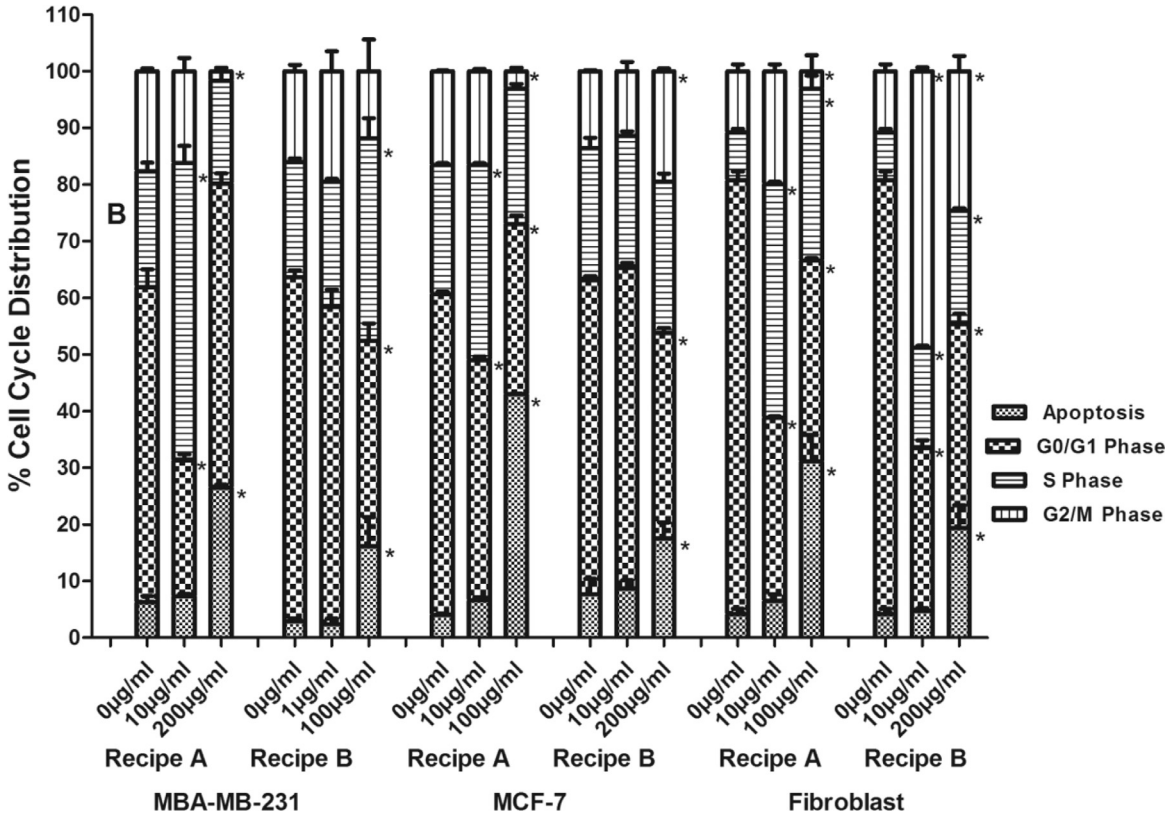


Fig. 3. Effect on cell cycle distribution of Recipe A and B extracts on cancer cells and fibroblast. Asterisk (*) indicates significance difference ($P<0.05$) for apoptosis, G0/G1, S and G2/M phases at different concentrations of recipes in cell lines when compared with control (0µg/ml)

Cell cycle and apoptosis assay

The cell cycle and apoptosis assay of the recipes extracts by flow cytometry of MDA-MB-231, MCF 7 and fibroblasts are shown in Fig. 3. The extracts caused apoptosis and altered the cell cycle phase distribution at concentration below IC_{50} and most significantly caused reduction in G_0/G_1 phase.

Assessment of mitochondrial ROS generation

The ROS assay of the cancer cells showed significant reduction in the percentage of DCF fluorescence (Fig. 4). The ROS measured was significantly reduced in MCF-7 cells when treated with 2 and 20 µg/ml of Recipe A at 1 and 24 h respectively (Fig. 4) and MDA-MB-231 cells when treated with 0.2 and 20 µg/ml of Recipe B at 1 and 24 h respectively (Fig. 4). There was only a slight increase but not significant in ROS generation at 24 h when compared with 1 h in both the cell lines.

Alterations in mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi_m$) was significantly altered as demonstrated by the percentage of Rh-123 fluorescence measurement (Fig. 5). The percentage Rh-123 fluorescence measured was significantly reduced to 82 % and 74 % in MDA-MB-231cells when treated with recipe A (Fig. 5a) and 79% and 64% in MCF-7 when treated with 10 and 200 µg/ml of recipe B (Fig. 5).

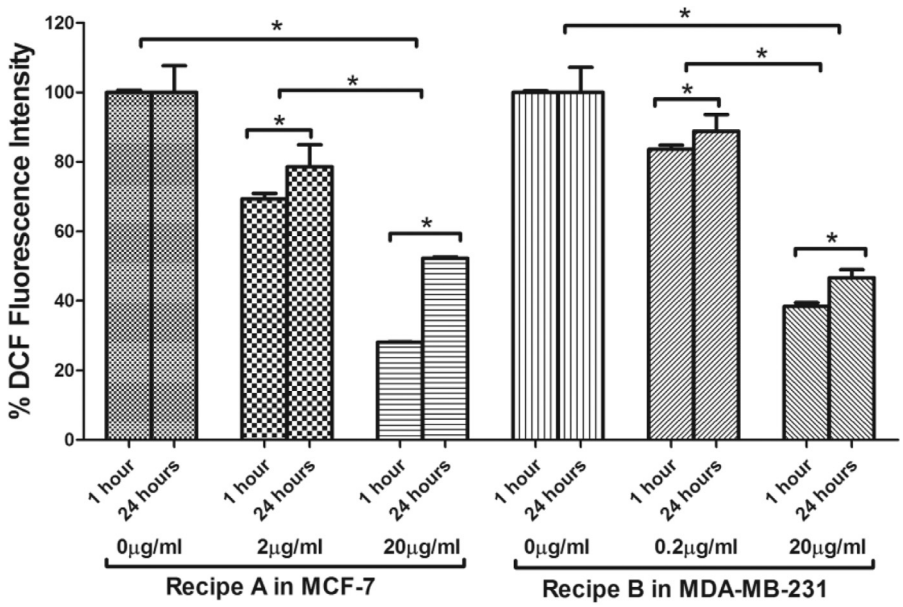


Fig. 4. The percentage of DCF fluorescence intensity. Asterisk (*) indicates significance difference ($P<0.05$) at different concentrations of recipes in cell lines when compared with control (0μg/ml).

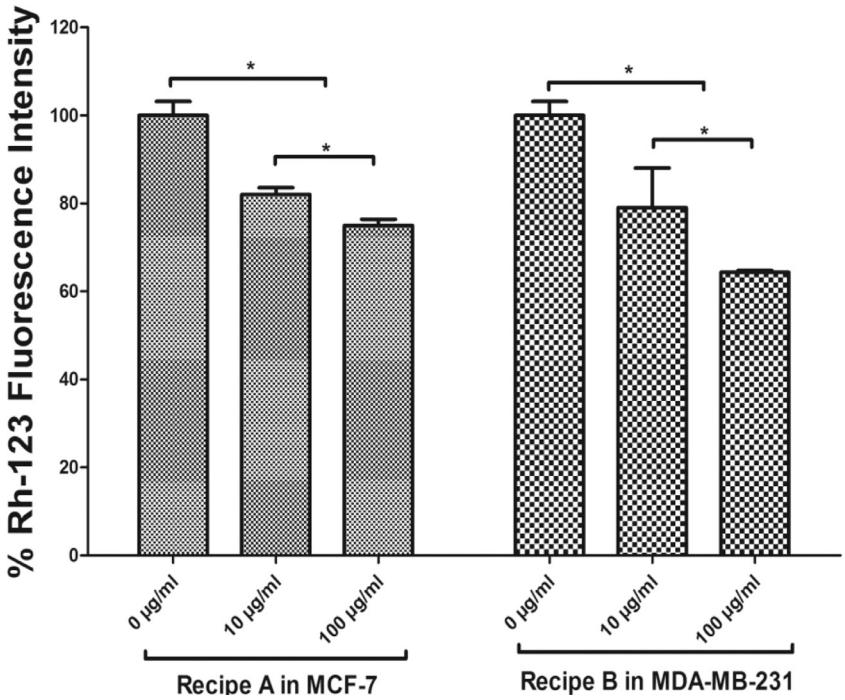


Fig. 5. The percentage of Rh-123 fluorescence intensity. Asterisk (*) indicates significance difference ($P<0.05$) at different concentrations of recipes in cell lines when compared with control (0μg/ml).

Expression of signaling molecular protein

Recipes A and B showed increased PARP and Caspase-3 expression in MDA-MB-231 and MCF-7 cells (Fig. 6). Recipe A showed 3 to 6-fold increase in PARP expression in MDA-MB-231 cells when treated with IC_{50} and half IC_{50} concentrations of both recipes (Fig. 6a and b). Similarly, there was 15 to 55-fold increase in Caspase-3 expression in MCF-7 cells when treated with IC_{50} and half IC_{50} concentrations of both recipes (Fig. 6c and d).

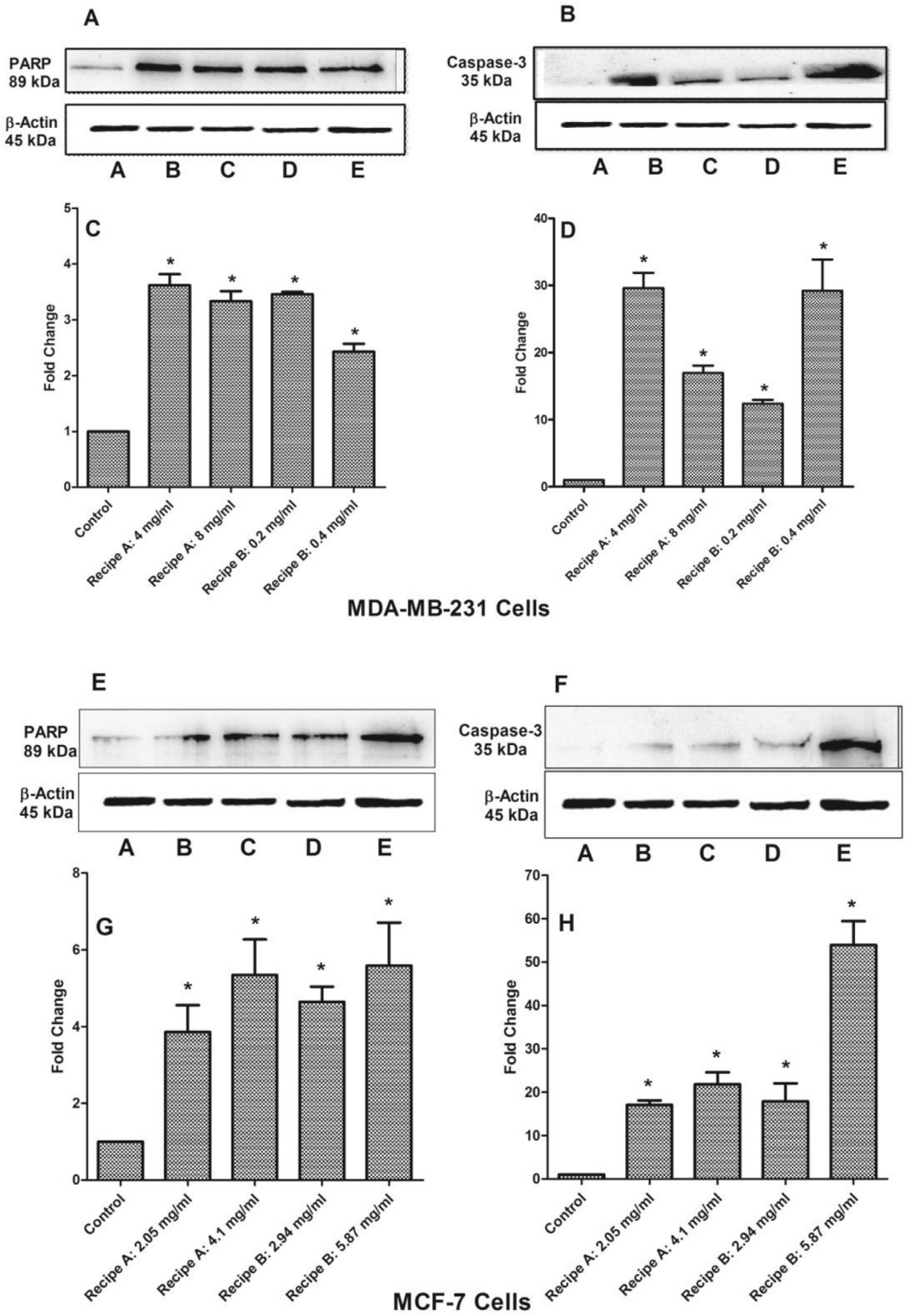


Fig. 6. Expression of PARP and Caspase-3. Asterisk (*) indicates significance difference ($P<0.05$) at different concentrations of recipes in cell lines when compared with control (0μg/ml).

Discussion

Medicinal and aromatic plants have been used in folk and traditional medicines and can prove to be beneficial for drug development programs. Therefore, the present study investigates the anticancer properties of recipes produced by combination of different parts of Nigerian and African medicinal plants (NAMP) that are used traditionally in the treatment and management of cancer in Nigeria and many parts of Africa. In recent years, medicinal plants have been the focus for the discovery of novel and effective anticancer compounds [31].

Combination therapy involving the interaction of two or more compounds with multiple targets simultaneously is considered as more efficient form of pharmacotherapy for controlling complex diseases such as cancer [32,33]. The combination therapy is gradually becoming more and more significant in the treatment of several diseases which include malaria, typhoid, acquired immune deficiency syndrome (AIDS) and tuberculosis. Recipes from different parts of plants may offer important combination therapies and provide clinical efficacy beyond the reach of single compound based drugs. The use of herb combination based formula of traditional medicine shares similar concept employed in contemporary drug therapies [34].

Many researchers consider that the medicinal plants may have the potential of addressing the association between multiple components that may lead to synergistic effects [35,36]. Moreover, systems biology and its methods have been used to explain the mystery of medicinal plants to interpret the intricate system [37,38]. Nevertheless, most researchers have reported constituents and its effects within a total extract of a single herb and combination of two or more different herbs in a formula [31.33,39]. Little is known about the interactions of different components from different plants and their relevance to treat human diseases. The ancient Indian traditional system of medicine called Ayurveda has been using several herbal or herbo-mineral combinations to treat many human ailments [30,40]. Therefore, in this study, methanolic extracts of the Recipes were evaluated *in vitro* for their anticancer potential using MTT, cell cycle/apoptosis assays, ROS assay, mitochondrial membrane potential and signaling molecular proteins. The two recipes chosen were based on the ethnobotanical survey of anticancer plants conducted by Soladoye and his co-workers [12].

The data obtained from viability assays of the cancer cells studied using both the recipes revealed that the percentage cell survival is concentration-dependent at the end of 48 h incubation as shown by the MTT assay. There was a concentration dependent increase in the inhibition of proliferation of cancer cells (MDA-MB-231 and MCF-7) and fibroblasts. This cytotoxic properties observed in the present study is in agreement with the previous work wherein individual medicinal plants induced cytotoxicity in cancer cells [31,39]. The recipes were effective below the set limit of American National Cancer Institute (NCI) guidelines for crude extracts at 50 % inhibition (IC_{50}) of proliferation with 30 µg/ml for the cancer cells except in the case of fibroblasts [41]. This could be due to interaction of the components in the extract as the reported functions of the components which varies from antioxidant, ascaricidal, schizonticidal, antimicrobial, anthelmintic, insecticidal, anti-inflammatory, anti-diarrheal to larvicidal effects [18,42].

At the concentration below IC_{50}, the extracts of both recipes cause G_0/G_1 arrest in all the cell lines tested and at concentration above IC_{50}, they caused significant apoptosis indicating a link between apoptosis and cytotoxicity as similarly reported [27]. This is in agreement with previous findings that betulinic acid inhibits growth of cultured vascular smooth muscle cells (VSMCs) *in vitro* by inducing G_1 arrest and apoptosis [43]. G_1 phase arrest after treatment, can progress normally into three possible pathways; remain in G1 arrest pending repair, repair the DNA and return to cell cycle or eliminate via apoptosis [44–46]. Induction of cell cycle arrest at G_1 phase is likely mediated by the down-regulation of proteins related to the progression to the G_1 phase. This effect at G_1 phase arrest has been supported by Yoon et al. [47] who have demonstrated that the antiproliferative effect is influenced by the activation of tumor suppressor genes p21waf1/cip1 and p27kip1.

Mitochondrial ROS generation assessment showed decrease in the percentage of DCF at the increasing concentration of the extract of the two recipes. The data demonstrated that the recipes significantly decreased the production of ROS to low levels in the tumour cells and the imbalance of ROS may promote mitochondrial dysfunction leading to increased cell death [48]. ROS is significantly associated with cell growth and that loss of ROS has been reported to trigger activation of caspase-9 and cleavage of PARP, leading to apoptosis [48].

Alterations in mitochondrial Ψ_m of cells exposed to recipe A and B indicates that the increased mitochondrial permeability transition pore (mPTP) opening along with loss of Ψ_m. Persistent opening of mPTP do not allow the accumulation of Rho-123 dye within the mitochondrial compartment. Loss of Ψ_m and prolonged mPTP opening has been reported to lead to apoptosis [27].

The over expression of PARP and caspase-3 was observed in both cell types when treated with Recipe A and B. Caspase-3 is a crucial mediator of programme cell death [49]. Activation of caspases initiate cell death by cleaving and activating effector caspases which drive the process of apoptosis while, PARP activation leads to ATP depletion and subsequent necrosis [50,51]. Caspase-3 has been reported in the cleavage of PARP and this is considered to be critical to induce apoptosis [51,52].

Conclusion

Our finding suggests that decreased proliferation of MDA-MB-231 and MCF-7 with the treatment of the recipes extract is due to cell death by compromising mitochondrial membrane potential and activation of apoptotic proteins such as PARP and caspase-3. The present research provides preliminary data demonstrating the potential effect of recipes on cytotoxic

activity against MDA-MB-231 and MCF-7 cells. It also suggests the use of the traditional medicinal plants as possible recipe for cancer treatment and management and thus indicating their potential biopharmaceutical use. Further studies including experiments in animals should be carried out in future to confirm the anticancer potential of these recipes.

Authors' contributions

MAA, AM and SPM completed the data collection and initial data analysis. MAA wrote the manuscript. OOA and KS provided assistance with analysis and contribution to writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2020.e00446.

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