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In vitro cytotoxicity of recipes derived from Nigerian medicinal plants (NMPs) on breast cancer cells

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Abstract
Treatment and management of cancer in Nigeria and many parts of Africa has included the use of combination (recipe) of defined medicinal plants of different genera and family. There has been little research work to validate the *in vitro* cytotoxicity of recipes derived from the combination of these plants. Therefore, this research work was carried out to investigate the cytotoxic properties of five (5) recipes prepared from the combination of different parts of Nigeria medicinal plants (NMPs) on breast cancer cells (MCF-7 and MDA-MB-231) and normal human fibroblasts. *In vitro* cytotoxic and anti-proliferative effect was evaluated by 3-[4, 5-dimethylthizol-2-yl]-2, 5-diphenyltetrazolium bromide **(**MTT) assay. The MTT assay result showed that the recipes were able to inhibit the growth and proliferation of MCF-7, MDA-MB-231 and fibroblast cells in a dose-dependent manner ($P < 0.05$). Amongst which Recipes A and D were within the set limit of American National Cancer Institute (NCI) guidelines for crude extracts at 50 % inhibition (IC_{50}) of proliferation with the concentration of 30 µg/ml for the cancer cells. This study provides an early evidence to support the traditional use of medicinal plant recipes in the treatment of breast cancer. Further studies on the animal models and molecular biology are recommended.

Keywords: cancer, cytotoxicity, medicinal plants, recipe, 3-[4, 5-dimethylthizol-2-yl]-2, 5-diphenyltetrazolium bromide assay

1. Introduction
Medicinal and aromatic plants and their products have been used from time immemorial and are important sources of drugs discovery [1]. Many compounds used in modern medicine were derived from plant sources; many of which have been described earlier for ethnomedicinal use and are similar to the current uses of the active principles of plant origin [2]. Several drugs currently available for clinical use have a long history as herbal remedies for human use and include aspirin, digitalis, quinine and opium [3]. Medicinal and aromatic herbs are used to treat diseases in developing countries because they are less expensive than the contemporary drugs [4, 5, 6]. World Health Organization (WHO) reported that over 80 percent of the populations of Asian and African countries rely on medicinal plants for primary health care needs [1, 7]. Although the use of medicinal and aromatic plants in the developed country was less common, an intense increase has been documented recently due to scientific proof of their effectiveness [8, 9].
Medicinal plants are naturally distributed in the rain forests of the southern parts of Nigeria and many parts of Africa [10, 11, 12]. Some of these plants exist in different species [13]. Many parts of these plants have been used traditionally for their anti-cancer, antioxidants, ascaricidal, schizonticidal, antimicrobial, antihelmintic, insecticidal, anti-inflammatory, anti-diarrheal and larvicidal properties [14, 15, 16, 17, 18]. Likewise, specific parts of these plants have been combined into recipes, which are been used traditionally for the treatment and management of cancer in Nigeria and many parts of Africa [12, 19].
Cancer is a collection of diseases illustrate by uncontrolled growth and multiply of abnormal cells [20, 21, 22]. If such uncontrolled multiplications are not restricted, it can lead to death. Annual mortality rate of cancer is about 3,500/million population around the world [9]. Many fundamental changes in cell behaviour is brought about by the need for cells to survive, as the cells become tumorigenic [1]. Activation of epithelial to mesenchymal transition (EMT) by the uncontrolled growth and proliferation of cells is a major challenge for treatment and survival of cancer patients [23, 24]. Metastasis leads to important organs failure such heart, liver, kidney and lungs, which may finally leads to death.
The anticancer activity of medicinal plants has been attributed to the presence of antioxidants [9]. However, combination of several medicinal plants has been used traditionally in the management and treatment of 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]. Therefore, the study was designed to investigate *in vitro* cytotoxic properties of recipes derived from the combination of several plants traditionally used for the treatment and management of breast cancer in Nigeria.

2. Materials and Methods

2.1 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. Dimethyl sulphoxide, EDTA, methanol and phosphate buffers were purchased from Sigma-Aldrich Corporation, Missouri, USA.

2.2 Plant Materials, Collection and Extraction

The plants (Table 1) and its parts were collected in Sagamu, Ogun State, Nigeria between January and June, 2015.The plant parts were grouped into five as described by Soladoye *et al*. [12] Plant materials were then dried in a hot air oven at 40 °C and powdered by grinding using Kenstar electric mill (Kitchen Appliances Indian Ltd, India). The powders were kept in air-tight containers in the dark until used. The powder (2.5 g) of each constituent in each recipe was added together and extracted by methanol using Soxhlet apparatus (B & C Industries, Kerala, India) 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.

2.3 High Pressure Liquid Chromatography (HPLC) Finger-Printing of Recipe Extracts

The HPLC fingerprinting of the recipe extracts was performed on a HPLC system (Waters, USA) using Nucleosil® C18 HPLC reversed-phase column (250 x 3.2 mm) (Sigma-Aldrich Corporation, Missouri, USA). 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.

2.4 Cell Line Maintenance

Breast cancer cells such as MCF-7, MDA-MB-231 and fibroblasts were maintained at School of Life Sciences, Manipal University, 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).

2.5 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 [25]. Briefly, 1 x 10^4 cells/well in 100 µl media were seeded into 96-well 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 with50 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 recipeand the IC_{50} values of Recipe A and B extracts were calculated.

2.6 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.

3. Results

3.1 HPLC Finger-Printing of Recipe Extracts

The HPLC fingerprinting of recipe extracts (Recipe A, B, C, D and E) were determined to identify the major peaks (compounds) in the extracts for the purpose of identification and reproducibility during the subsequent methanolic extract of the recipes (Fig. 1). The use of HPLC as a quality control measure was performed as reported by Agyare *et al*. [26]

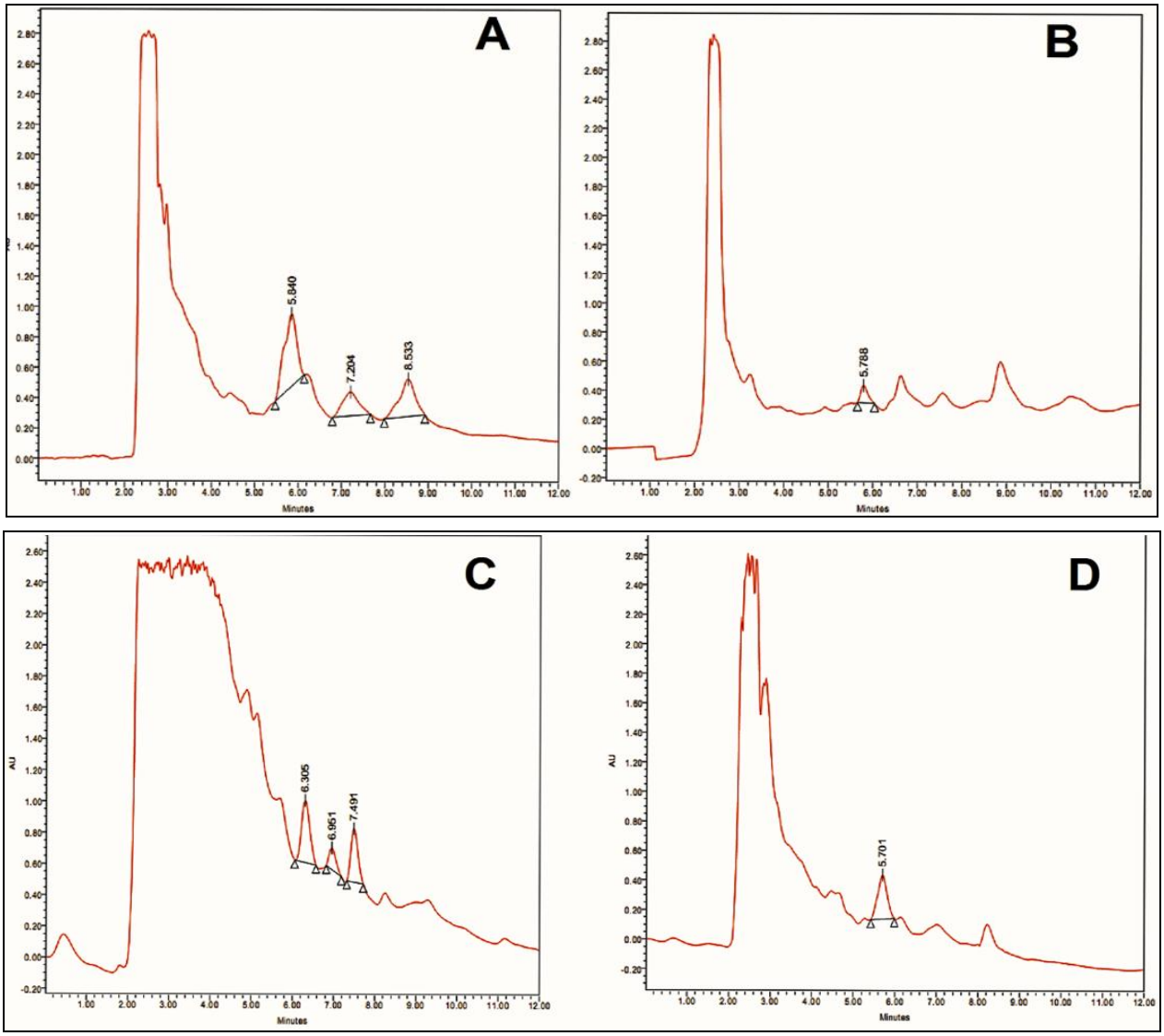
3.2 Cytotoxicity Assay

The result of cytotoxicity evaluation of the recipes prepared from NMPs against MDA-MB-231, MCF-7 and fibroblasts are shown in Fig. 2 – 6. The extract of all the recipes exhibited significant concentration-dependent anti-proliferative activityagainst MDA-MB-231, MCF-7 and fibroblast cells respectively (Table 2).

Table 1: Enumeration of Recipes

Recipe	Voucher No	Plants parts
Recipe A		
Uvaria chamae	UC15045	Bark
Khaya grandifolia	KG15020	Bark
Xylopia aethiopica	XA15046	Pod
Recipe B		
Garcinia kola	AA15004	Bark
Morinda lucida	AA15006	Leaves
Terminalia avicennioides	BS15008	Leaves
Psorospermum febrifugum	CH15009	Leaves

Antiarisafricana	CM15013	Bark
Harungana madagascarense	MI15024	Bark
Recipe C		
Alafia barteri	AB15002	Leaves, Root
Lannea engregia	LE15023	Leaves
Securinega virosa	SV15039	Leaves, Root
Xylopia aethiopica	XA15046	Pod
Recipe D		
Musa parasidiaca	MP15026	Tuber
Senna alata	SA15040	Root
Uvaria chamae	UC15045	Root
Olax subscorpioidea	OS15030	Root
Securidaca longepedunculata	SL15038	Root
Xylopia aethiopica	XA15046	Pod
Recipe E		
Nyphaea lotus	NL15029	Leaves
Pistia stratiotes	PS15033	Leaves
Saccharum offinarum	SO15036	Crushed Stem
Morinda lucida()	ML15025	Bark
Citrus aurantifolia ()	CA15012	Juice
Xylopia aethiopica	XA15046	Pod



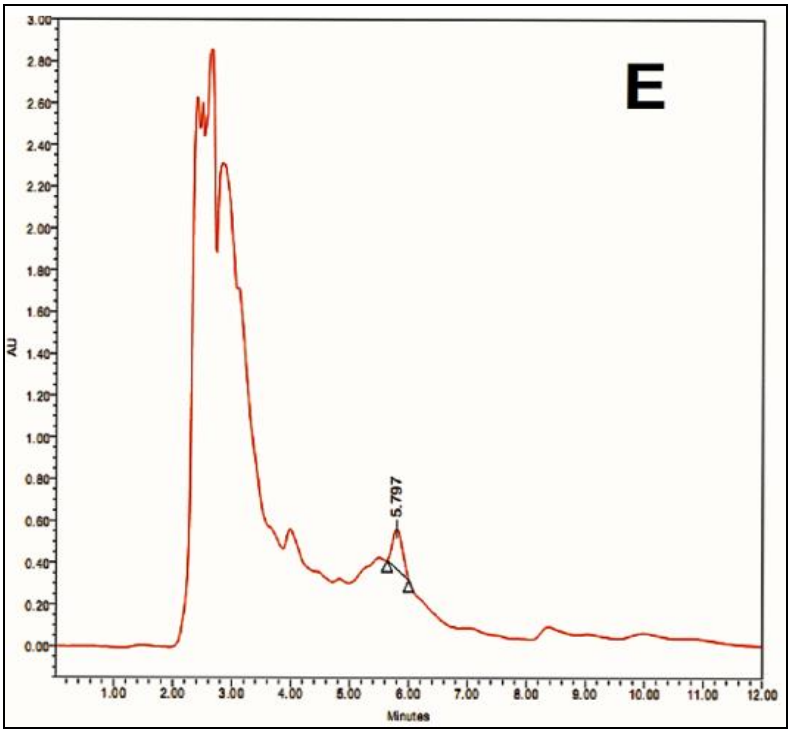


Fig 1: HPLC finger-printing chromatogram of Recipe A, B, C, D and E extract at λ 254nm

Chromatogram of complex mixture of methanolic extract of the Recipes showing distinctive peaks and thus serving as quality control.

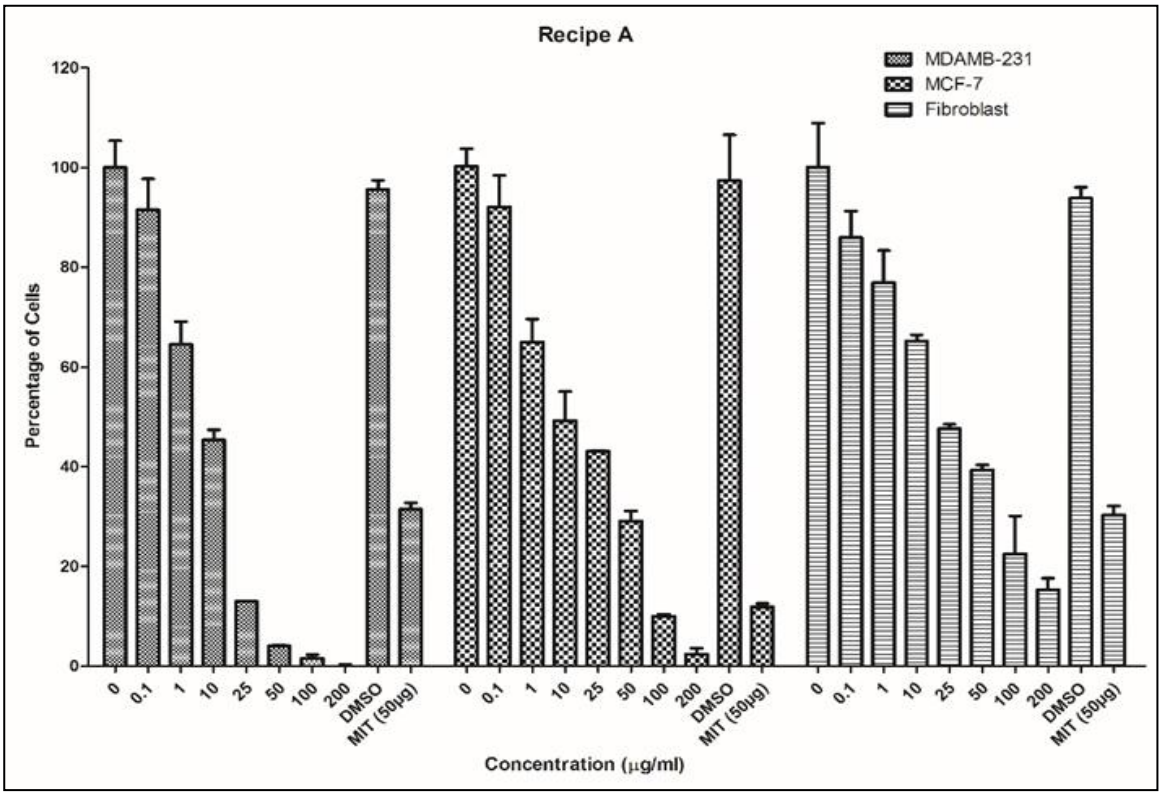


Fig 2: Cytotoxicity effect of Recipe A extract on cancer cells and fibroblast

MDA-MB-231, MCF-7 and Fibroblast cells were grown for 24 h and treated with different concentrations of Recipe A (0.1 - 200 µg/ml). The cells were then subjected to MTT assay after 48 h of treatment. The graph displays the means ± SD of three independent experiments.

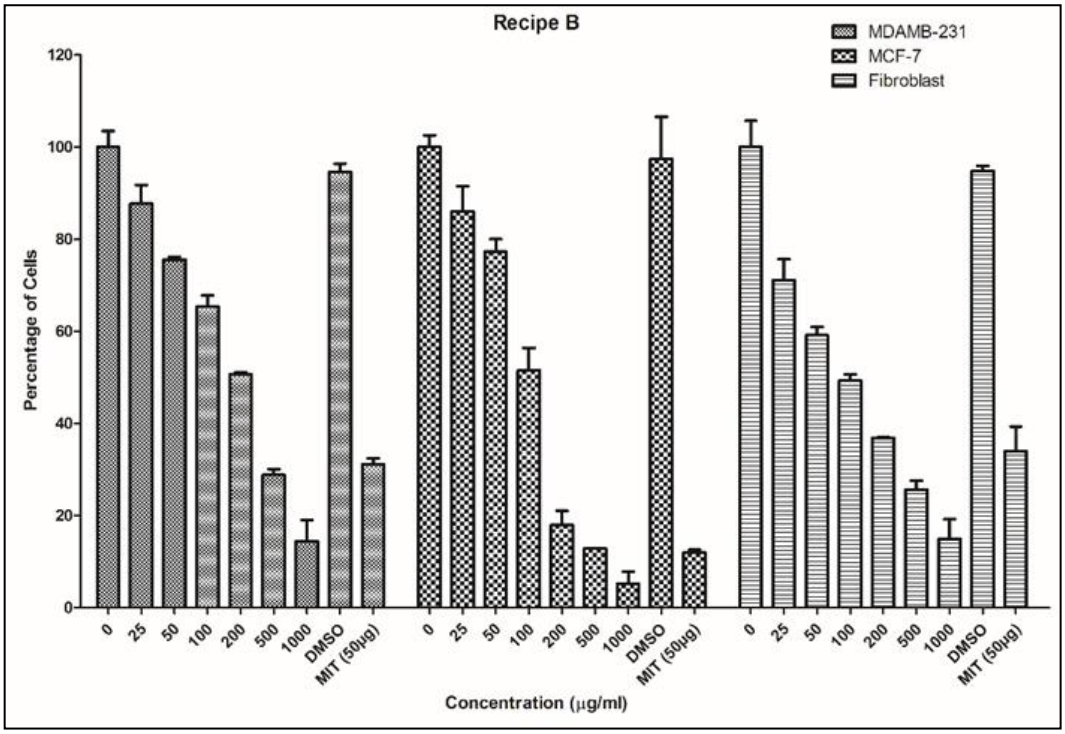


Fig 3: Cytotoxicity effect of Recipe B extract on cancer cells and fibroblast

MDA-MB-231, MCF-7 and Fibroblast cells were grown for 24 h and treated with different concentrations of Recipe B (0.1 - 1000 µg/ml). The cells were then subjected to MTT assay after 48 h of treatment. The graph displays the means ± SD of three independent experiments.

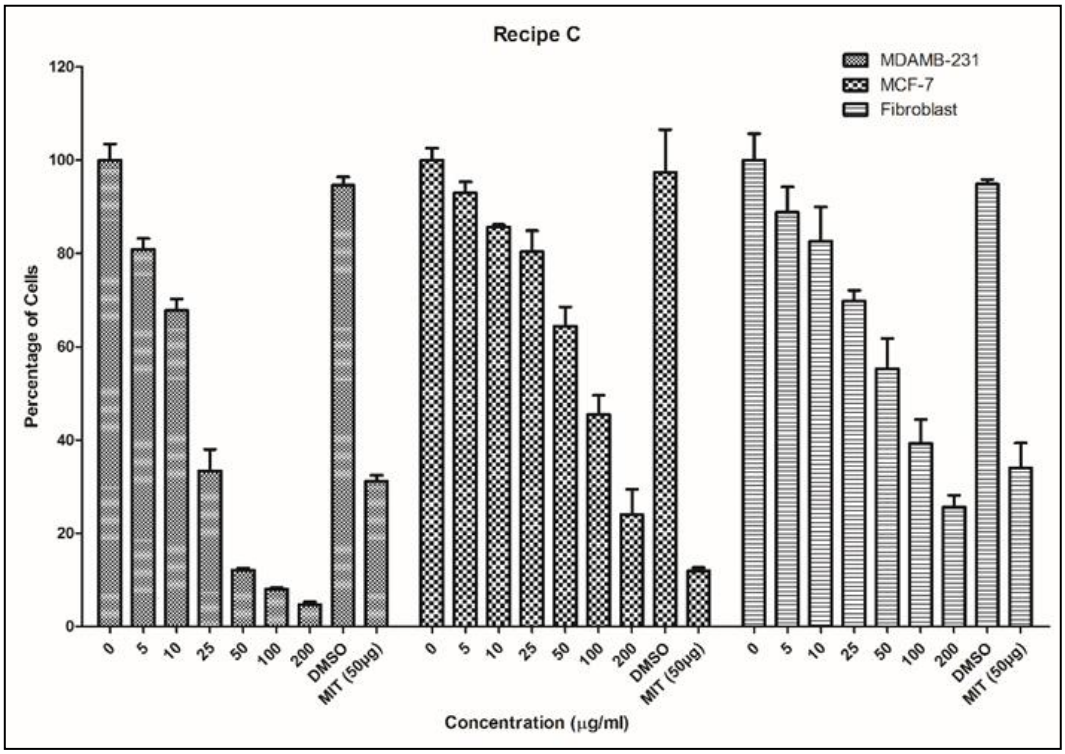


Fig 4: Cytotoxicity effect of Recipe C extract on cancer cells and fibroblast.

MDA-MB-231, MCF-7 and Fibroblast cells were grown for 24 h and treated with different concentrations of Recipe C (0.1 - 200 µg/ml). The cells were then subjected to MTT assay after 48 h of treatment. The graph displays the means ± SD of three independent experiments.

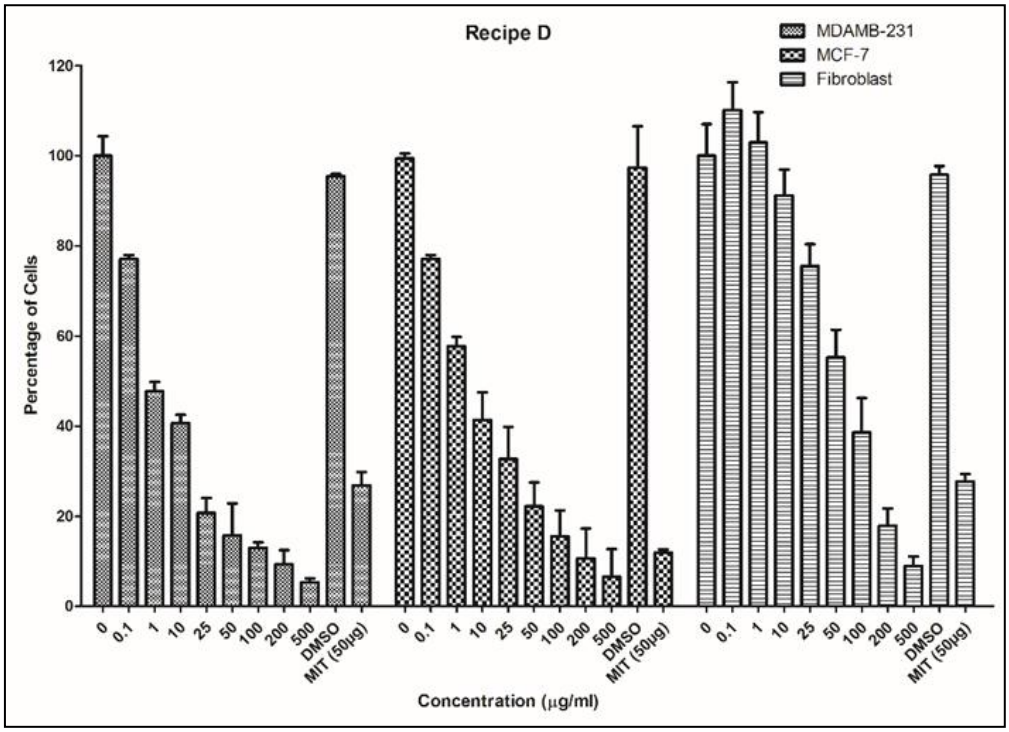


Fig 5: Cytotoxicity effect of Recipe D extract on cancer cells and fibroblast

MDA-MB-231, MCF-7 and Fibroblast cells were grown for 24 h and treated with different concentrations of Recipe D (0.1 - 500 µg/ml). The cells were then subjected to MTT assay after 48 h of treatment. The graph displays the means ± SD of three independent experiments.

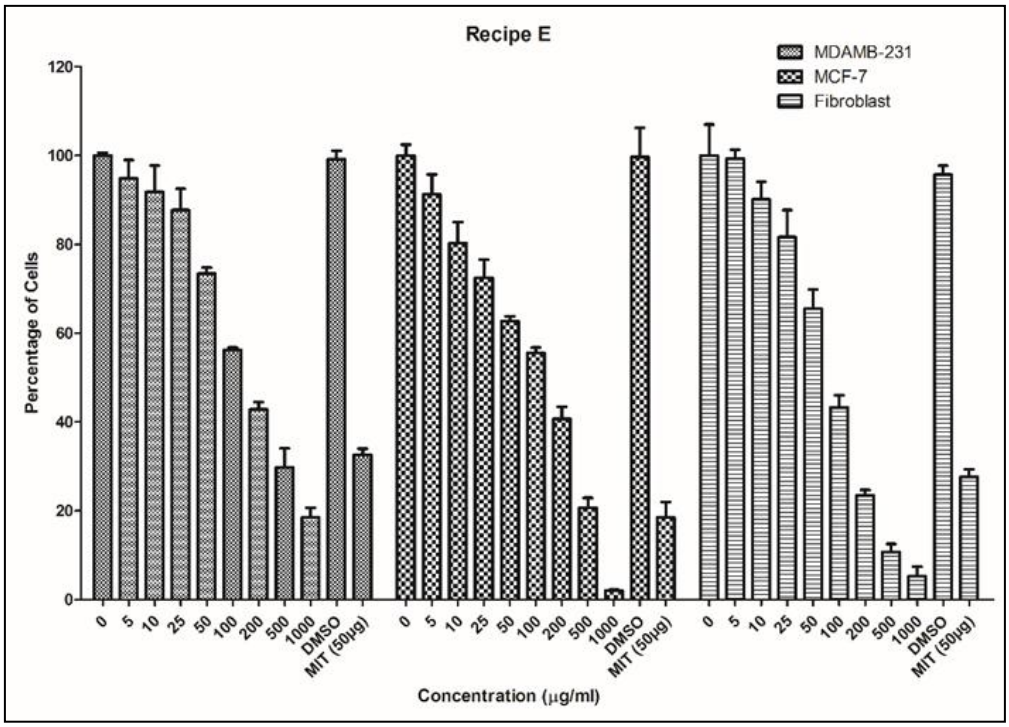


Fig 5: Cytotoxicity effect of Recipe E extract on cancer cells and fibroblast

MDA-MB-231, MCF-7 and Fibroblast cells were grown for 24 h and treated with different concentrations of Recipe E (0.1 - 1000 µg/ml). The cells were then subjected to MTT assay after 48 h of treatment. The graph displays the means ± SD of three independent experiments.

Table 2: Cytotoxicity of the recipe extracts on cancer cells and fibroblast as determined by MTT assay

Recipe	MDA-MB-231	MCF-7	Fibroblast
A	8.00±1.25	4.10±0.81	23.80±2.15
B	210.56±10.82	108.35±5.84	95.38±4.88
C	17.35±2.15	88.31±5.57	66.38±4.42
D	0.40±0.01	5.87±0.42	65.43±3.38
E	145.82±6.78	138.58±5.87	82.58±4.48

4. Discussion

Nigerian Medicinal plants (NMPs) have been used in folk and traditional medicines for the management and treatment of cancer [12]; and can prove to be beneficial and less expensive than conventional drugs but there has been no report to authenticate the cytotoxicity of these recipes. Therefore, the present study investigates in vitro cytotoxic properties of recipes produced by combination of different parts of Nigerian medicinal plants (NMPs) that are used traditionally in the treatment and management of cancer in Nigeria and many parts of Africa. Recently, medicinal plants have been the focus for the discovery of novel and effective anticancer compounds [27].

Combination therapies are increasingly becoming more important in the treatment of several diseases which include malaria, typhoid, acquired immune deficiency syndrome (AIDS) and tuberculosis. The therapy involves the interaction of many compounds having multiple targets concurrently and is considered a more efficient form of pharmacotherapy for controlling complex diseases including cancer [28]. Recipes from different parts of plants may offer important combination therapies and provide clinical efficacy beyond the reach of single compound based drug. Herbal combination based formula used in traditional medicine shares similar concept as employed in contemporary drug therapies [29].

It has been considered that medicinal plants may have the potentials of addressing the relationship between many components that may lead to synergistic effects [30, 31]. Furthermore, the mystery of medicinal plants to interpret the intricate system has been explained by the use of systems biology and its methods [32, 33]. However, the constituents and its effects within a total extract of a single herb and combination of two or more different herbs in a formula have been reported [27, 28, 34]. 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 [35, 36]. Therefore in this study, methanolic extracts of the Recipes were evaluated *in vitro* for their cytotoxic potential using MTT assays. The five recipes chosen were based on the ethnobotanical survey of anticancer plants conducted by Soladoye and his co-workers [12].

The reverse phase High Pressure Liquid Chromatography (HPLC) fingerprinting of extracts of Recipe A, B, C, D and E were determined to identify the major peaks (compounds) in the extracts for the purpose of identification and quality control (Fig. 1). To demonstrate anticancer properties *via* cytotoxic effect of the recipes, two breast cancer cell lines, MDA-MB-231 and MCF-7 were selected while, the fibroblast served as control in the present study.

The result showed viability of the cancer cells studied using 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 this study is in agreement with the previous work wherein individual medicinal plants induced cytotoxicity in cancer cells [27, 34]. Recipe A and D in MDA-MB-231 and MCF-7 and Recipe C in MDA-MB-231 were effective within 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 but above this limit in the case of fibroblasts [37].

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 [14, 15].

5. Conclusion

In conclusion, our finding showed that decreased proliferation of MDA-MB-231 and MCF-7 with the treatment of the recipes extract is due to cell death. The study provides preliminary data indicating the cytotoxic potential of recipes on cancer cells. It advocates the use of the traditional medicinal plants as possible recipes for cancer treatment and management and thus indicating their potential biopharmaceutical use. Further studies including animal studies should be carried out to establish the anticancer potential of these recipes.

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