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Research Article

Cytotoxic and Antiproliferative Properties of Methanol Extract of Stem Bark of *Adansonia digitata* Linn. in Breast Cancer Cells

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ABSTRACT

Synthetic anticancer drugs are expensive and toxic; therefore, there has been much interest in developing anticancer agents from natural resources and especially plants. The stem bark of *Adansonia digitata* (SBAD) has many traditional medicinal uses, including the treatment of cancer, but there is currently little scientific evidence to support this. The aim of this study was therefore to assess the cytotoxic and long term antiproliferative effects of a methanol extract of SBAD (MESBAD) in breast cancer cells. Cold extraction was done on SBAD using 70% methanol for 72 hrs. The extract was filtered, concentrated and lyophilized. MCF-7 breast cancer cells were cultured using an established procedure and were treated with MESBAD or DMSO as control. Cytotoxicity and long-term survival were measured using MTT and clonogenic assays respectively. Western blotting was performed with antibodies against p53 and p21. MESBAD induced short-term and long-term (13 to 15 days) cytotoxicity in breast cancer cells in a dose dependent manner and exhibited an IC_{50} of 100µM. Western blotting indicated that MESBAD activated the p53 tumour suppressor and its target, p21. MESBAD induces cytotoxicity and inhibits long-term survival of MCF-7 breast cancers cells through a mechanism involving the up-regulation of the cell cycle and cell death regulators p53 and p21.

Keywords: *Adansonia digitata, cytotoxicity, breast cancer, stem bark, antiproliferative, clonogenic assay*

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INTRODUCTION

Cancer is, for the most part, an age-related disease for which there is still an ongoing search for more effective treatment. In Nigeria, about 100,000 new cases are reported each year with breast cancer taking the lead (Adebamowo and Ajayi, 2000).

Natural products play important roles in the treatment and management of various ailments including cancers. The search for potent anticancer agents from indigenous plant origin is therefore of interest. This is particularly important because of the toxicity and high cost associated with synthetic drugs which limits their access to developing and underdeveloped countries.

Adansonia digitata (Linn) belongs to the family *Bombacaceae* and is commonly known as Baobab, Cream of Tartar Tree, Monkey Bread Tree and Lemonade Tree. It is usually found in the savanna region, but it tolerates a wide range of climate conditions (Keay, 1989). Traditional uses of *Adansonia digitata* for nutritional and various medicinal purposes have been reported (Chadare *et al.*, 2009). These medicinal uses include: treatment of intestinal and skin disorders, anti-inflammatory, antipyretic and analgesic (Shukla *et al.*, 2001; Wickens, 1979; Sibibe and Williams, 2002; Ramadan *et al.*, 1994; Palombo, 2006; Ajose, 2007). The stem bark has been shown to have antibacterial activity (Yeshua'u *et al.*, 2010), hypoglycemic activity (Tanko *et al.*, 2008). The antibacterial, antiviral and anti-trypanosomal effects of the extracts of this plant have also been reported (Anani *et al.*, 2000; Hudson *et al.*, 2000; Atawodi *et al.*, 2003; Vimalanathan and Hudson, 2009). The stem bark possesses the alkaloid "Adansonin" with strophanthus-like action, which is thought to be the active component for treating malaria and other fevers, especially in Africa and other parts of the tropics (Sibibe and Williams, 2002; Wickens and Lowe, 2008). There are also reported evidences that extracts of *Adansonia digitata* exhibit anti-cancer activity (Sundarambal *et al.*, 2015). Elsaid,

(2013) reported the antitumor effects of the extracts of seeds and fruit pulp of *Adansonia digitata* on ehrlich ascites carcinoma. A Report from Guinea revealed that baobab fruit extract possesses antitumor activities (Kerharo and Adam 1974; Jackson 2015).

There is still very limited information in the literature about the anticancer activities associated with the stem bark of *Adansonia digitata* (SBAD). This study was therefore designed to investigate the cytotoxic and antiproliferative activities of the methanol extract of SBAD (MESBAD) on oestrogen receptor positive human breast adenocarcinoma MCF-7 cells.

MATERIALS AND METHODS

Collection, Identification and Authentication of Plant materials: Samples used for this study were obtained from Ajibode extension of the University of Ibadan, Oyo State, Nigeria. Fresh leaves and fruits of the tree were used for its identification/authentication. The plant was identified by Mr. Esimekhuai Donatus, a taxonomist of the Department of Botany, University of Ibadan, and authenticated at the herbarium of Forestry Research Institute, Jericho, Ibadan Nigeria, with FHI NO. 109859.

Preparation of Methanol stem bark Extract of *Adansonia digitata* *L:* Fresh stem bark of the plant was harvested and dried at ambient room temperature to obtain a fixed weight. The dried stem bark was finely milled and extracted with 70% methanol for 72 hours at room temperature. The methanol extract was concentrated in a rotary evaporator at 40 ^{0}C, lyophilized and preserved until required for use.

Cells and culture: The oestrogen receptor positive human breast adenocarcinoma MCF-7 cells (obtained from the Department of Human Biology, University of Cape Town Medical School, Cape Town, South Africa) were maintained according to the protocols described by Celis, (1998) and Freshney, (2000). The cells were maintained in RPMI 1640 (Highveld Biological, Lyndhurst, UK) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/mL streptomycin and incubated at 37 °C in a 5% CO_2, 95% air-humidified incubator. For maintenance, cultures were passaged weekly, and the culture medium was changed twice a week. The monolayer cells were detached with trypsin - ethylenediamine tetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. Cells were seeded into 96-well plates at a density of 10,000 cells/well and incubated for at least 48 hrs to allow for cell attachment before further experiments were performed.

Cell Viability: Cell viability was assessed by the trypan blue dye exclusion method (Rosengard and Cochrane, 1983). Cells numbers were calculated as follows: cells per mL = N x 5 x D.F. / volume, where, N is the total number of cells counted in 5 squares, D.F. is the dilution factor and volume is the depth of the counting chamber (0.1 $mm^3 = 1x10^{-4}$). Therefore, cells / ml = N x 5 x D.F. x 10^4. Dilution factor was calculated by dividing the total volume used (20 µL) with the cell suspension volume (10 µL). The percentage cell viability was determined as follows: Viable cells x 100 / Total number of cells.

Cytotoxicity Study: Cells were plated to 70% confluency in 96 well plates. After 24 hrs the cells were treated with serial concentrations of MESBAD in triplicate. MESBAD was initially dissolved in dimethylsulfoxide (DMSO) and diluted to the desired final test concentrations of 50, 100, 250, 500 and 1000 µg/mL with medium and the mixture was filter-sterilized using 0.22µm syringe filter unit (Millipore, USA). Following drug addition, the plates were incubated for an additional 48 hrs at 37 °C, 5 % CO_2, 95 % air and 100% relative humidity. After 48 hrs of incubation, 15 µL of MTT (3-[4, 5-dimethyl thiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) (Roche, USA) (5mg/mL) was added to each well and incubated at 37 °C for 4 hrs. The medium with MTT was then flicked off and the formazan crystals formed were solubilized in 100 µL solubilizing solution and the absorbance measured at585 nm using a microplate reader. At least three biological repeats were conducted to determine the IC_{50} concentrations. The mean cell viability was calculated as a percentage of the mean vehicle control (Mosmann, 1983). The percentage cell inhibition was thereafter determined as follows: nonlinear regression graphs were plotted between percentage cell inhibition and Log 10 concentration and IC_{50} was determined using GraphPad Prism software. The percent inhibition was calculated using the following formula:
% inhibition = 100 - (mean of Absorbance of sample/mean of Absorbance of control) × 100

Clonogenic survival study: The clonogenic or colony formation assay is a widely used method to study the number and size of cancer cell colonies that remain after irradiation or the administration of cytotoxic agents and serves as a measure of the long-term anti-proliferative effect of these treatments. MCF-7 cells were treated with the IC_{50} and $2xIC_{50}$ concentrations of MESBAD for 24 hrs, washed, collected and re-plated at 1000 cells per well in MESBAD-free medium in 6-well plates. Cells were allowed to grow for 14 days with regular changes of medium, until the cells in the control plates formed colonies (50 cells per colony is the minimum for scoring) and surviving cells were fixed and stained with crystal violet (Sigma-Aldrich, USA). The numbers of colonies were thereafter evaluated according to Aliwaini *et al.* (2013).

Western blot analysis: MCF-7 cells were harvested, and protein prepared as previously described by Aliwaini *et al.* (2013). Equal amounts of protein were loaded in each lane and resolved on 8 % and 15 % SDS-PAGE gels and then transferred by electrophoresis to Hybond ECL nitrocellulose membranes (Amersham Biosciences, USA). Membranes were blocked for 1 hr at room temperature with PBS containing 5 % non-fat dry milk and probed with appropriate primary antibodies at 4 °C with shaking. Membranes were washed in PBS containing 0.1% Tween 20 (PBS/T) and incubated with either goat anti-mouse or goat anti-rabbit IgG peroxidase-conjugated secondary antibodies (1:5000) (BioRad, Hercules, CA, USA) in blocking solution at room temperature with shaking for 1 hr. Membranes were again washed in PBS/T and

visualised by enhanced chemiluminescence (Pierce, USA). Primary antibodies used were anti-p53 (sc-126), anti-p21 (sc-756) and anti-p38 (M0800) (Sigma, St. Louis, MO, USA).

Statistical analysis

Data presented are mean ± SEM (standard error of the means) of three independent experiments and a value of $p < 0.05$ was accepted as statistically significant. One-way ANOVA was used to compare means of more than two samples/groups and the t test was used to compare two samples/groups.

RESULTS

The cytotoxic effect of MESBAD on the human breast adenocarcinoma MCF-7 cells was examined using the MTT assay. After 48 hrs of MESBAD treatment, a dose dependent cytotoxicity was observed and an IC_{50} of 100 µM was obtained (Fig. 1). The result shows that MESBAD exerts potent cytotoxic activity against MCF-7 cells. Furthermore, MCF-7 cells were treated with MESBAD and the effect on long-term proliferation of MCF-7 cells was assessed using clonogenic survival assays. The results presented in figures 2a and 2b show that MESBAD caused a clear reduction in the proliferation of MCF-7 cells when compared with the control cells treated with DMSO. Furthermore, the antiproliferative activity of MESBAD is dose dependent as seen in the number of colonies present in the cells treated with ½ IC_{50} and IC_{50} (Fig. 2a). In order to validate this activity, multiples of the IC_{50} were used to assess the possibility of complete disappearance of colonies of the cells. The results obtained presented a similar pattern with the earlier result; the plates with the cells treated with $4xIC_{50}$ have very scanty colonies (Fig. 2b).

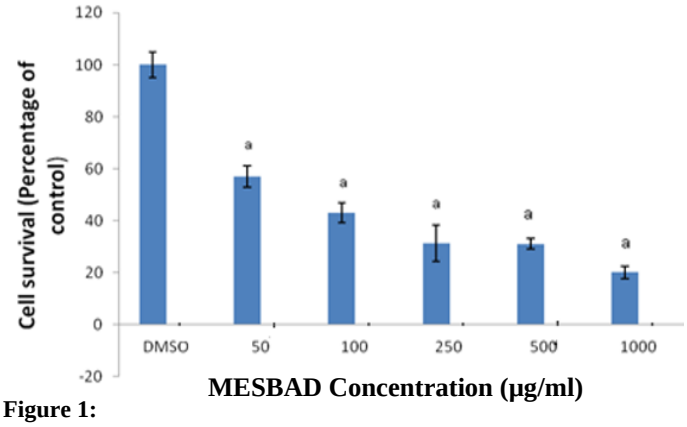

Figure 1:
Cytotoxic effect of MESBAD on MCF-7 cells using MTT assay. [a] means is a significant difference ($p < 0.05$) when compared with the DMSO group. IC_{50} = 100 µg/ml.

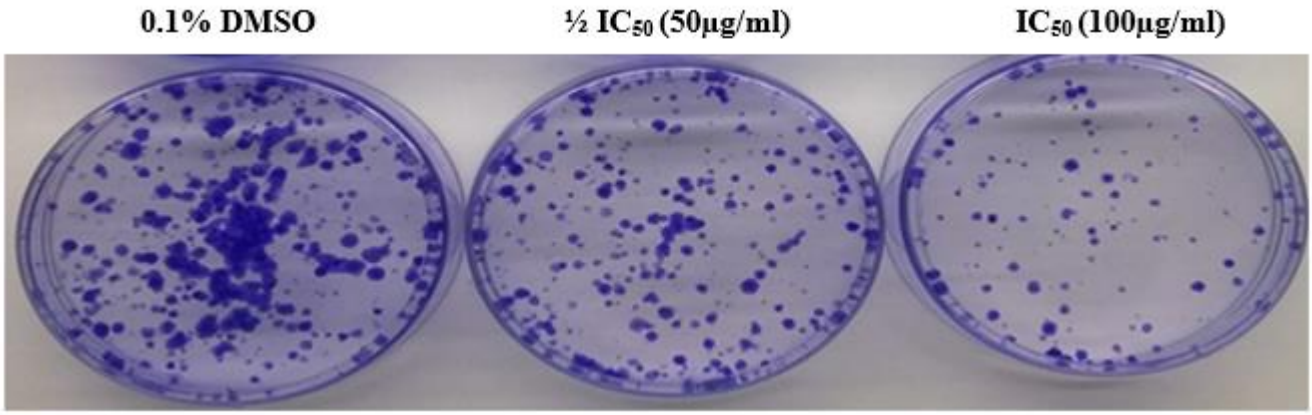

Figure 2a:
Antiproliferative and inhibitory effect of MESBAD on MCF-7 cells using the clonogenic assay at ½ IC_{50} and IC_{50} of MESBAD

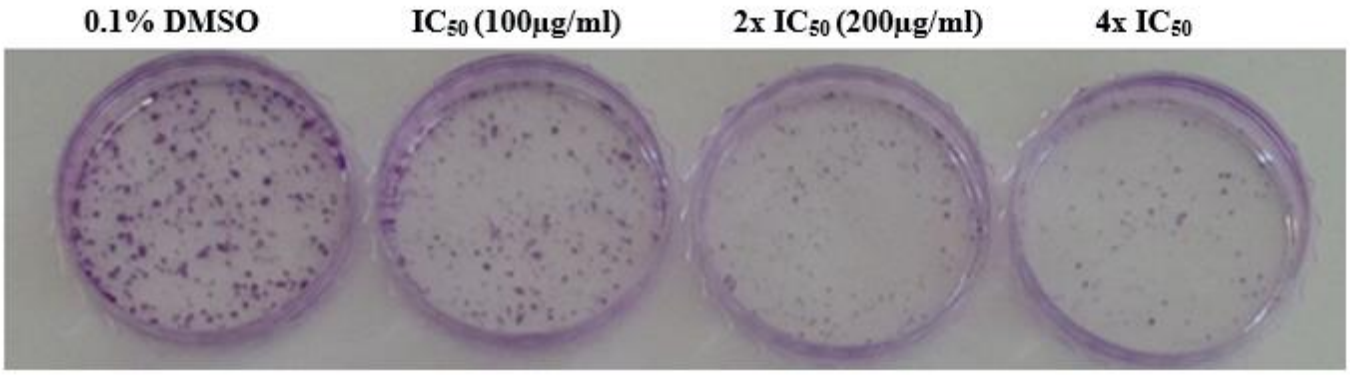

Figure 2b:
Antiproliferative and inhibitory effect of MESBAD on MCF-7 cells using the clonogenic assay at IC_{50}, $2xIC_{50}$ and $4xIC_{50}$ of MESBAD

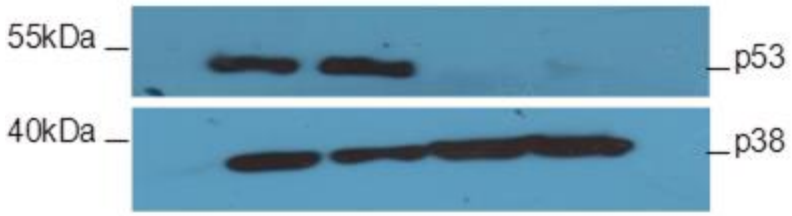

Figure 3:
p53 protein probe with 5% box milk PBS/T (MCF-7). MESBAD induces p53 activation in a concentration dependent manner. Lane 1 was designated as vehicle control (0.1% DMSO), 2. IC50x2(24hrs), 3. IC50x2(48hrs), 4. IC50x4(24hrs), 5. IC50x4(48hrs).

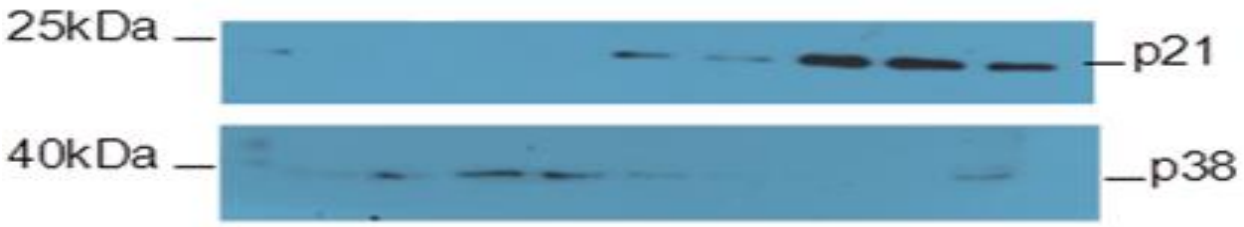

Figure 4:
p21 protein probe with 5% box milk PBS/T (MCF-7). MESBAD induces p21 activation in a concentration dependent manner. Lane 1 was designated as vehicle control (0.1% DMSO), 2. 2xIC_{50}(24hrs), 3. 2xIC_{50}(48hrs), 4. 4xIC_{50}(24hrs), 5. 4xIC_{50}(48hrs), 6. 1/2IC_{50}(24hs) 7. 1/2IC_{50}(48hs) 8. IC_{50}(24hs) 9. IC_{50}(48hs) Western blotting of protein from MESBAD treated breast cancer cells shows the levels of p53 (Figure 3) and p21 (Figure 4) and total p38 were used as loading controls. The expression of the proteins were quantified and normalised to p38 levels.

Therefore, the higher the concentration, the higher the antiproliferative activity induced by MESBAD. These data demonstrate that at the concentrations tested, MESBAD has potent cytotoxic and anti-survival/antiproliferative effects on MCF-7 cells. The tumour suppressor p53 and its target gene p21 play critical roles in regulating cell cycle progression and cell death and thus we decided to look at the effects of MESBAD on these proteins in order to predict the mechanism of cytotoxicity of MESBAD. **Fig. 3** shows that p53 levels were markedly up-regulated at 24 and 48 hrs in the 2xIC_{50} treated cells, while p21 levels were markedly increased when the cells were treated for 24 and 48 hrs with both ½ IC_{50} and IC_{50} concentrations (**Fig. 4)**. The correlation between the upregulation of both p53 and p21 would strongly suggest a p53-dependent induction of p21 in these cells. Results suggest that MESBAD cytotoxicity and antiproliferative activity may be via the upregulation of p53 and p21.

DISCUSSION

Breast cancer is the most commonly diagnosed cancer in women both in the developed and less developed countries, with 18.1 million new cancer cases of cancer projected in 2018, female breast cancer was projected to be 11.6% (Bray *et al.,* 2018). Among females, breast cancer is the leading cause of cancer death (Bray *et al.,* 2018; IARC, 2012; Azubuike *et al.,* 2018). Despite several therapeutic strategies, there is still limited success. Africa is blessed with abundant novel plant species with health-promoting compounds, many of which remain underutilized or undiscovered (Lamien-Meda *et al.,* 2008). The Baobab *(Adansonia digitata L.)* is one of such plants, it is widely distributed throughout the sub - Saharan Africa and Western Madagascar areas and has many uses, such as medicine, food, and beverages with all parts useful (Diop *et al.,* 2006; Nouruddeen *et al.,* 2014). An aqueous bark extract of *A. digitata* is traditionally used in Nigeria for treating sickle-cell anemia, the same stem-bark is also considered beneficial as a heart tonic with diuretic properties (Ashorobi and Joda, 1998). One of the widest uses in folk medicine is the use of the bark as a substitute for quinine in cases of fever or as a prophylactic (Wickens and Lowe, 2008). The many traditional uses of the stem bark of *Adansonia digitata* include among others, the treatment of cancer, for which there is very limited scientific data to validate its use. The current study describes the anti-cancer activity of MESBAD in human breast adenocarcinoma MCF-7 cells. The cytotoxic potential of MESBAD was investigated using MTT assay. The MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells (Mosmann, 1983). The cytotoxicity/anticancer activity were recorded as concentration causing 50% growth inhibition (IC_{50}) (Mosmann, 1983).

The MESBAD shows a dose dependent cytotoxic effect on MCF-7 cell line, after three consecutive experiments. The higher the concentration of MESBAD, the higher the cytotoxic effect and *vice versa*, as shown by the intensity of the purple formazan formed after the addition of the MTT salt to the cells. This experiment revealed that MESBAD possesses anticancer activity against MCF-7 cell line. In order to further establish the short-term cytotoxic effect of MESBAD, observed, clonogenic cell survival assay was employed to evaluate its long term antiproliferative potential on the cell line. The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone. The ability of a single cell to grow into a large colony, colonies are an indication that the cells have tumorigienicity; this is what is determined by the clonogenic assay. The MESBAD caused a clear reduction in the proliferation of human breast adenocarcinoma MCF-7

cells, when compared with the control that was treated with DMSO. The antiproliferative activity is also dose dependent as seen in the number of colonies present in the cells treated with ½ IC_{50} and IC_{50}. In order to be sure of the reproducibility of the observed effect, multiples of the IC_{50} were used to assess the possibility of complete disappearance of colonies. The result presented a similar pattern as the first set of results, with the cells treated with $4xIC_{50}$ having very scanty colonies. MESBAD shows a dose dependent long term antiproliferative effect on human breast adenocarcinoma MCF-7 cells. The result is in agreement with the earlier study of Elsaid *et al.* (2013), which demonstrated that the extracts of seeds and fruit pulp of *Adansonia digitata L* possess anti-tumor action and attenuated the *p*53 and *Bcl*-2 gene expression as pro-apoptic and anti-apoptic genes leading to management of tumor growth.

The mechanism by which MESBAD exerts its cytotoxic activity was therefore the next investigation. The expression patterns of certain apoptotic markers (p53 and p21) were examined. The most commonly mutated gene in human cancers is the p53 and over 50% of human cancers contain mutated form of p53, it is very useful as a therapeutic target for anticancer drug discovery (George, 2011). p21, a well-established cyclin-dependent kinase (cdk) inhibitor, was found to play an important role in controlling cell cycle progression; p21 induction can also lead to a cell cycle arrest (Harper *et al.*, 1993). p21 was introduced as a tumor suppressor in brain, lung, and colon cancer cells; it was shown that p21 induces tumor growth suppression through wild type p53 activity (El-Deiry *et al.*, 1993; Shamloo and Usluer, 2019). There is a relationship between p21 expression and mutation of the p53 tumor suppressor gene in normal and malignant ovarian epithelial cells. In many cell types, p53-mediated growth inhibition is dependent on induction of p21. There was a robust p53 response in human breast adenocarcinoma MCF-7 cells treated with MESBAD, which in general correlated with an increase in levels of the cell cycle regulator p21. p53 levels were markedly increased at 24 and 48 hrs in the $2xIC_{50}$ treated cells while their p21 levels were also increased at 24 hrs and 48 hrs of ½IC_{50} and at IC_{50} both at 24 and 48 hrs treatment, suggesting a p53 dependent induction of p21 in these cells. These suggest that MESBAD may bring about a p53 dependent apoptosis. The results suggest that the anticancer activity of MESBAD may be p53 dependent, owing to its up-regulation. MESBAD also up-regulated the expression of p21 which is an early apoptotic response suggesting the possible role of p21 in the anticancer activity of MESBAD. Further experiments would be carried out on the effect of MESBAD on other anti-cancer modulatory genes and the isolation and characterization of the active principles.

In conclusion, the MESBAD is shown to induce cytotoxic and antiproliferative activities which are mediated by the p53 and p21 responses. This study provides evidence that MESBAD is a potential anticancer and antiproliferative agent against oestrogen receptor positive human breast adenocarcinoma MCF-7 cells.

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