Detarium microcarpum Stem-Bark Extracts Induce Apoptosis in Human Breast Adenocarcinoma MDA-MB 231 Cells via cJNK Activation and Mitochondrial Cytochrome C Release

ABSTRACT

Detarium microcarpum stem-bark extract (DMSE) is one of the medicinal herbs used by the traditional medical practitioners in North East Nigeria to treat or manage various ailments such as wounds, inflammation and some form of malignancy. This study is an attempt to assess the anti-tumor activity of DMSE on human breast adenocarcinoma MDA-MB 231 cells and the underlying mechanism involved in the anticancer activity. The survival rate of MDA-MB 231 cells was reduced in a dose-dependent manner as assessed by MTT assay. Mitogen-activated protein kinase (cJNK) was phosphorylated shortly 1 h after treatment. The translocation of mitochondrial cytochrome C to the cytosol was observed after 3 h post-treatment. In conclusion, these results suggest that DMSE possibly causes mitochondrial damage leading to cytochrome release in cytosol and the activation of c-JUN kinase resulting in apoptosis of human breast adenocarcinoma MDA-MB 231 cells.

KEYWORDS  detarium microcarpum, stem-bark extract, apoptosis

INTRODUCTION

Cancer is a significant worldwide health problem generally due to lack of comprehensive early detection methods, the associated poor prognosis of patients diagnosed in later stages of the disease and its increasing incidence on the global scale. Indeed, the struggle to combat cancer is one of the greatest challenges of mankind. Breast cancer is one of the five leading causes of death amongst humankind especially females. The growing trend indicates a deficiency in the present cancer therapies which includes surgical operation, radiotherapy and chemotherapy. Since the average survival rate has remained essentially unchanged despite such aggressive treatment, there is a need for anticancer agent with higher efficacy and less side effect that can be acquired at affordable cost.

Herbal therapies cure the disease without undesirable side effect even after prolonged application or usage with a wide spectrum of activity. They are also known to be cheaper and more affordable compared to the conventional medicine. D. microcarpum is a perennial plant, non-climbing tree belonging to the Fabaceae family (legumes). It is a fruit-bearing shrub common to Sahel regions of west and central Africa. Many different vernacular names exist for this species, including the English sweet dattock or tallow tree, ‘tauhra’ (Hausa). This plant is known to be beneficial in the treatment and management of a wide variety of ailments, including some form of cancers and other inflammatory diseases in folklore medicine. Previous studies in our laboratories and others indicate that extracts from different part of the plant possess hepatoprotective activity on mycotoxin-induced liver damage in rats, and bactericidal effects against both gram positive and
gram negative clinical isolates. In this present study, we investigated the antitumor activity of *D. microcarpum* stem bark by evaluating antiproliferative activity and the underlying mechanism of action by using MDA-MB 231 adenocarcinoma breast cancer cell line.

**MATERIALS AND METHODS**

**Extract preparation**

The plant material was collected from Biu town in Northeast Nigeria during the months of May to June. It was identified as *D. microcarpum* by a plant taxonomist, and its sample was preserved and documented in the herbarium department of botany, University of Maiduguri Nigeria. The stem barks were washed with distilled water, shade dried then powdered with mechanical grinder, passing through a sieve and stored in an airtight dark container. Powdered stem bark weighing 200 g was mixed with 1000 ml of distilled water and macerated for 24 h, and the mixture was filtered using Whatman filter paper. Both the water and ethanol extracts obtained were dried under reduced pressure at room temperature not exceeding 40°C. Then, it was kept in an airtight dark bottle, and was reconstituted when required. The residue was used for aqueous and ethanol extractions.

**Preparation of cell lines**

The human breast cancer cell lines MDA-MB 231 was obtained from the Templeton/Cross Laboratory, Pathology Department, University of Virginia, U.S.A. The cells were thawed and grown in Dulbecco’s minimum essential media supplemented with 10% fetal bovine serum, MEM Non-essential amino acid, 1% penstrep and 2 mM L-glutamine maintained at 37°C in 5% CO₂ humidified sterilized incubator prior to assessing cytotoxicity of the extracts. The culture medium represents a basic medium supplemented with 10% fetal bovine serum. The basic medium was RPMI 1640 medium containing extra L-glutamine (300 µg/ml), sodium pyruvate (110 lg/ml), HEPES (15 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml), as described previously. The cells were harvested employing trypsin (0.2%) + EDTA (0.02%) in PBS prior to all experiments performed.

**Stock extract preparation**

The stock solutions of crude ethanol extracts of *D. microcarpum* were prepared by dissolving 200 mg of each sample in 1 ml of dimethylsulfoxide (DMSO). Stock crude aqueous extract was prepared by dissolving 50 mg of the sample in 1 ml of distilled water. Treatment concentrations of 1, 0.5, 0.4, 0.35, 0.3, 0.25, 0.2, 0.15, 0.125, 0.1 and 0.05 mg/ml were prepared from the stock solutions by dilution in sterile distilled water.

The MTT assay was used to assess the apoptotic effect of the extracts on MDA-MB 231 cells

This experiment was based on the conversion of the yellow tetrazolium salt-MTT, to purple-formazan crystals by metabolically active cells as previously described by Mossman. This provides a quantitative determination of viable cells. The cells were plated on to 9-well plates at a cell density of 2 × 10⁵ cells in 100 µl of RPMI 1640 and allowed to grow in CO₂ incubator for 24 h (37°C, 5% CO₂). The medium was removed and replaced by fresh medium containing different concentrations of the *D. microcarpum* extracts. The cells were incubated for 72 h (37°C, 5% CO₂). 20 µl MTT ([3-(4,5-dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide]) stock solution (5 mg/ml in PBS) was added to each well and incubated for 5 h. The medium was removed and 200 µl DMSO was added to each well to dissolve the MTT metabolic product. Then, the plate was shaken at 150 rpm for 5 min and the optical density was measured by using Biorad plate reader at 560 nm. Untreated cells (basal) are used as a control of viability (100%), and the results are expressed as % viability (log) relative to the control.

Cell viability/survival = absorbance of treated cells/ absorbance of untreated cell × 100

The effect of the extracts on kinase activation in MDA-MB 231 cells by using the densitometry analysis

The MDA-MB 231 cells at density of 2 × 10⁵ per well were seeded in a 12-well culture plate and allowed to adhere onto plate for 24 h prior to treatment. Ethanol and water extracts of *D. microcarpum* at concentration range of 250 and 500 (g/ml were added onto the cells and incubated at 37°C, 5% CO₂ for 1 h. TPA (200 ng) served as a positive control.

**Western blot**

Activation of MAP Kinases by phosphorylation was measured by western blot analysis using phospho-specific antibodies that detect the dual phosphorylated (activated) form of SAPK/JNK and ERK. Treated or untreated cell lysate were prepared by lysis in the MLB complete buffer and proteins denatured by boiling in 2 × DSB for 5 minutes. Proteins were separated by SDS gel electrophoresis (10% gels) and transferred onto PDVF membrane using Bio-Rad miniprotein-III wet transfer unit. Nonspecific binding of proteins on the membrane was blocked with 5% non-fat dry milk in Tris buffered saline TBS/0.05% tween 20 for 1 h at RT. Phosphorylation of kinases was analysed using pERK 1/2 and pJNK primary antibody (1:1000, 3% BSA in TBST and overnight incubation at 4°C). Blots were then washed and incubated with secondary horseradish–linked anti-mouse or – rabbit antibody for 1 h at RT (1:5000). The membranes were incubated with the horseradish peroxidase-conjugated anti-rabbit...
IgG secondary antibody for 1 h at room temperature followed by three washings. The signals of phosphorylated kinases were detected with a chemiluminescence ECL reagent and quantified by densitometry using a gel visualize (Alpha Innotech, CA, USA). Actin and tubulin served as the loading control and the results were expressed as the percentage of control.

Mitochondrial cytochrome c translocation assay

Three sets of 6-well culture plates were set up and to each plate $2 \times 10^4$ MDA-MB 231 cells per well were seeded and allowed to adhere onto a plate for 24 h prior to treatment. Water and ethanol extracts of the plant at concentration range of (125 and 500 g/ml) were added onto the cells in each plate and incubated at 37°C, 5% CO₂, for 4 h. Straurosporine (500 nM) served as a positive control.

Statistical analysis

All tests are reported as means ± SD of at least triplicates of two independent experiments. The difference in activities between the samples was analyzed by ANOVA and Turkey multiple comparison using the Graph pad Prism software Version 6.

Results

Aqueous and ethanol extracts of D. microcarpum inhibited MDA-MB 231 cell proliferation/viability

The effect of aqueous and ethanol extract of D. microcarpum stem bark on the viability of MDA-MB 231 cells is presented in Fig. 1a, b. The MDA-MB 231 cells treated with the extracts for a period of 72 h showed a significant apoptotic features compared to control cells. Both the water and ethanol extracts inhibited the cell proliferation in a dose range of 0.05–0.25 mg/ml with inhibitory value of 16.83 ± 6.14% to 99.76 ± 4.41%. However, the extracts showed different inhibitory activity (Fig. 2a, b), the water extract exhibited much lower IC₅₀ and had completely inhibited cell growth at the concentration of 110 µg/ml whilst the ethanol extract shown to be having IC₅₀ of 125 µg/ml. Inhibitory activity at the concentration of 125 µg/ml as calculated by graph pad prism non linear regression fit. The inhibition of cell growth and proliferation in response to the extract treatment is however in a dose-dependent manner.

Effects of the ethanol and aqueous extracts of D. microcarpum on cellular JNK kinase activation in MDA-MB 231 cells

Western blot analysis revealed phosphorylation of pJNK in D. microcarpum treated MDA-MB 231 cells at concentrations of 125 and 500 g/ml after 60 minutes. Phosphorylation of pERK was increased in D. microcarpum treated cells when compared to controls. Figure 3a, b shows an increase in the phosphorylation of pJNK and pERK normalized to tubulin control.

Cell death induced by D. microcarpum stem-bark extract is preceded by cytochrome C release

One of the upstream events in the apoptotic process is the release of cytochrome C from the mitochondrion to the cytoplasm, where it engages in a cascade of interaction that leads to execution stage of programmed cell death. This is exactly what happened in this experiment. Mitochondrial cytochrome C was released to the cytosol of MDA-MB 231 cells in response to D. microcarpum stem-bark extract treatment at a dose range of 0.125 mg/mL and 0.5 mg/mL for 3H(Fig. 3a, b). Western blot analysis showed a marked translocation of cytochrome C from
the mitochondria to the cytosol which is evident with a significant increase in the proportion of cytoplasmic protein compared to untranslocated pellets.

This is a western blot indicating the translocation of cyt c from the mitochondria of extract treated MDA-MB 231 cells. Labels point at protein bands of DM1 (0.5 mg/mL) and DM2 (0.125 mg/mL) and negative control C (no treatment). The pelleted cells of each treatment were labelled P next to the cytoplasmic fractions. STS was the positive control whilst tubulin served as loading control.

The release of cytochrome c from the mitochondria of MDA-MB 231 cells treated with D. microcarpum (DM2 (0.125 mg/mL) and DM1 (0.5 mg/mL)) for 3H is shown cytochrome c was translocated in mitochondria of D. microcarpum treated cells as seen in the proportion of cytoplasmic to untranslocated (pelleted) fractions.

**DISCUSSION**

Cancerous cells are characterized by indefinite cell proliferation, metastasis, angiogenesis and reduced apoptosis. Natural agents that target these abnormal characteristics exhibited by neoplastic cells are explored with the aim of identifying metabolites that provide cheap, safe and effective anticancer drugs. In this study we investigated the antitumor effects of D. microcarpum a medicinal plant commonly used in folkloric medicine for the treatment and management of inflammatory and tumor related ailments.

The methyl tetrazolium assay is a colorimetric assay for determining viable cell number in proliferation and cytotoxicity studies. The method is based on the cleavage of yellow tetrazolium salt (MTT) to form a blue formazan product by mitochondrial enzymes, and the amount of formazan produced is proportional to the number of living not dead cells present during MTT exposure. Findings from this study showed D. microcarpum stem-bark extracts inhibited proliferation of MDA-MB 231 cells in a dose-dependent manner (Fig. 1a, b). This observation is in accordance with an earlier report by, that D. microcarpum stem-bark extract had cytotoxic and antiviral activities. In this study both aqueous and ethanol extracts exhibited a minimal inhibitory concentration of 110 µg/ml and 250 µg/ml respectively. This observation confirmed that both the water and ethanol extracts are moderately active according to the USA National Cancer Institute (NCI) guideline. The aqueous extract of D. microcarpum was relatively more cytotoxic than its ethanol counterpart. This difference in activity of the extracts could be attributed to the fact the water might possibly be a better solvent than the ethanol in the solubility of the phyto-components. And moreover, the higher cytotoxic activity of the water extract is important because traditional administration of
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D. microcarpum and most herbal preparations are often prepared by soaking in either hot or cold water. This present study showed that activation of SAPK/JNK by crude extract of D. microcarpum in MDA-MB 231 cells resulted in increased phosphorylation of p48 MAPK (Figs. 4, 5). Upon activation, JNKs translocate from the cytoplasm to the nucleus up-regulating apoptotic signals like APaf-1 and cyt c. This activation of JNKs maybe the crucial step of cell death, in response to genomic or structural damages after treatment with apoptotic agents.

To further understand the molecular mechanism behind the extract-mediated apoptotic activity on human MDA-MB-231 breast adenocarcinoma cells, translocation of cytochrome c from mitochondria into the cytosol of treated cells was assessed. A pivotal event in the process of apoptosis is the permeabilisation of the mitochondrial outer membrane to release cyt c and other proteins. Cyt c is a 10.5 kDa protein whose release from the mitochondria to the cytosol is a central event in apoptotic signaling. In this study, D. microcarpum stem-bark extracts markedly facilitated the translocation of mitochondrial cytochrome c to the cytosol in MDA-MB-231 cells (Fig. 6). This observation may possibly explain the apoptotic effect of some phytochemicals such as flavonoids and polyphenols present in the extract. Also extract treatment triggers a rapid release of cytochrome c within half an hour before cell death was detected by using MTT assay, suggesting that it is an early event in the course of both cell death process induced by the phytocompounds present in the extract. However, the mechanism of cytochrome c release is not clear. The phytochemicals might in part, induce mitochondrial membrane permeability transition (PT) or perturb the outer mitochondrial membrane. Our experiment with rhodamine 123 showed that cytochrome c release might be accompanied by apoptotic changes such as membrane blebbing and nucleus shrinkage which were observed under the microscope whilst loss of mitochondrial activity was evident from the MTT assays. The results of kinase phosphorylation and mitochondrial cyt c permeabilization when put together suggests that crude D. microcarpum stem-bark extract induces apoptosis in MDA-MB-231 cells via the activation of SAPK/JNK and a subsequent release of cytochrome c from the mitochondria.

CONCLUSION

This study had confirmed D. microcarpum an indigenous plant from Northernen Nigeria has ability to induced cytotoxicity in human breast cancer cell line. The result from the study have therefore demonstrated the reliance on ethnomedicinal information to screen for anticancer remedy from plant source. Extensive further analysis on the anticancer properties of D. microcarpum compared with those of anticancer drugs as positive control is currently underway. Alongside with bio-assay guided purification to elucidate the active anticancer phytocomponent from the crude extract.

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