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Mitochondria-dependent apoptogenic activity of the aqueous root extract of *Croton membranaceus* against human BPH-1 cells

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ABSTRACT. *Croton membranaceus* aqueous root extract (CMARE) is among the widely used phytotherapeutics in Ghana for the management of benign prostatic hyperplasia (BPH) and prostate cancer. However, the mechanism of action of CMARE remains to be elucidated. This study aimed to establish whether apoptosis is involved in the antiproliferative effect of CMARE on human BPH-1 cells. We determined the effect of treatment with 0, 1, 3, and 5 mg/mL CMARE for 24, 48, and 72 h on the viability and morphology of BPH-1 cells using the MMT assay and phase-contrast microscopy, respectively. We examined the apoptosis-inducing effects of CMARE after 48 h at the cellular level using Hoescht 33258 and JC-1 dye staining and flow cytometry analysis. We performed reverse transcription polymerase chain reaction and Western blotting to confirm the apoptotic effects of CMARE at the molecular level. CMARE induced a significant dose-dependent inhibition in the proliferation of BPH-1 cells ($P < 0.05$) and an alteration in their

morphology and a reduction their density. Furthermore, CMARE induced dose-dependent staining of the nuclear chromatin, significant DNA fragmentation with G_0/G_1 sub-diploid cells ($P < 0.01$), and loss of the mitochondrial membrane potential in the treated cells compared to the controls after 48 h ($P < 0.01$). Additionally, while CMARE induced a significant upregulation of the mRNA and protein levels of Bax, those of Bcl_2 did not change significantly. Therefore, induction of mitochondria-dependent apoptosis of BPH-1 cells may be a possible mechanism of action of CMARE.

Key words: *Croton membranaceus*; BPH-1 cells; Proliferation; Cell cycle; Apoptosis

INTRODUCTION

Benign prostatic hyperplasia (BPH) and prostate cancer have a high impact on public health; BPH is observed mostly in men older than 50 years. Approximately 42% of men aged 51-60 years, 70% of men aged 61-70 years, and almost 90% of men aged 81-90 years have BPH (Tanguay et al., 2009). Enlargement of the prostate often results in lower urinary tract symptoms, which have a negative impact on the quality of life of patients (Kramer et al., 2007). Despite the increase in the incidence and prevalence of BPH, its etiology and pathophysiology remain to be clarified. The growth of prostate cells is governed by a delicate balance between cell proliferation and apoptosis (Kyprianou and Jacobs, 1996). Although the normal stroma to epithelium ratio of the prostate is 2:1, in patients with BPH, this ratio is 5:1 (Prabhav and Bairy, 2009). A previous study showed that excessive proliferation of the stromal and epithelial cells (37-fold and 9-fold, respectively) was observed in some patients with BPH (Claus et al., 1993). This finding indicates that an imbalance of the molecular mechanisms of proliferation and apoptosis is involved in the development of BPH and prostate cancer.

Apoptosis or programmed cell death, which occurs as a normal and controlled part of growth, is generally observed in eukaryotic cells. This process is genetically controlled, and a defect in the apoptotic process may lead to the development of neoplasms. Apoptosis can be induced by a variety of intrinsic and extrinsic stimuli such as hormones, DNA damage, growth factors, chemotherapeutic agents, and ionizing radiation. Many proteins are involved in the cellular apoptosis pathway, and the Bcl-2 family proteins have emerged as vital regulators of the intrinsic apoptotic pathway (mitochondria-mediated apoptosis) by functioning as either promoters (e.g. Bax and Bak) or inhibitors such as Bcl-2 and Bcl-xL (Chao and Korsmeyer, 1998).

Studies on cell viability, proliferation, and apoptosis are vital for evaluating the response of a cell population to chemotherapeutic agents and growth factors for the BPH drug development (Hong et al., 2012). Furthermore, therapeutic agents that can inhibit cell growth and/or induce apoptosis have emerged as potential targets for optimizing the treatment of BPH and prostate cancer. However, management of cancers require novel therapies that are affordable, non-toxic to normal cells, and can delay the onset and/or progression of the condition with minimal side effects (Singh et al., 2005). Currently, phytotherapeutic agents have attracted tremendous attention for the treatment of BPH and prostate cancer, because they are safer, more cost-effective, and have fewer side effects than conventional alternatives (Thompson, 2001; Arora et al., 2003).

Croton membranaceus Mull. Arg (Euphorbiaceae) is a medicinal plant that has been used for decades in Ghana for the management of BPH and prostate cancer. Previous *in vitro* studies revealed that the ethanolic root extract of *C. membranaceus* was cytotoxic to the normal and cancerous cells of the human prostate (Bayor et al., 2008). Preclinical toxicity studies indicate that the ethanolic root extract of *C. membranaceus* is generally non-toxic (Asare et al., 2011; Afriyie et al., 2013a). Recent *in vivo* studies showed that the aqueous root extract of *C. membranaceus* causes a significant inhibition in the proliferation of glandular epithelial cells and a decrease in the serum prostate-specific antigen levels (Afriyie et al., 2013b). However, the mechanism underlying the therapeutic effect of *C. membranaceus* on the prostate cells has not been elucidated thus far. Additionally, histological examination of the *C. membranaceus*-treated prostate shows growth inhibition (Afriyie et al., 2013b). We therefore hypothesized that the cytotoxic effect on the prostate cells may be because of induction of apoptosis. In this study, we investigated the effects of the aqueous root extract of *C. membranaceus* (CMARE) on the viability and apoptosis of human BPH-1 cells.

MATERIAL AND METHODS

Reagents

Rosewell Park Memorial Institute Medium 1640 (RPMI 1640), trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethybenzimidazolylcarbocyarine iodide (JC-1), bis-benzimidazole dye Hoescht 33258 assay kit, and TriZol reagent were purchased from Invitrogen (California, USA). Bcl-2, Bax, β-actin antibodies and secondary horseradish peroxidase (HRP)-conjugated antibodies were purchased from Santa Cruz Biotechnology (Texas, USA). Cell cycle assay kit was purchased from BD Biosciences (California, USA). Penicillin-streptomycin, 10% fetal bovine serum (FBS), and methythiazolyltetrazolium (MTT) were obtained from Sigma Chemicals (St. Louis, USA). Other reagents used were phosphate-buffered saline (PBS; Thermo Scientific, Florida, USA), 0.25% trypsin (Hyclone, Utah, USA), and Promega SuperScript II reverse transcriptase (Wisconsin, USA). All other chemicals, unless stated, were obtained from Sigma Chemicals (St. Louis, USA).

Cell culture

Human BPH-1 cells were kindly provided by Professor Zhengfeng Hong (Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, China). Cells were cultured in RPMI 1640 medium (L-glutamine) containing 10% (v/v) FBS, 100 Units/mL penicillin, and 100 μg/mL streptomycin and were maintained at 5% CO_2 in a humidified incubator at 37°C.

Extraction of CMARE

The roots of *C. membranaceus* were harvested in December 2012, authenticated, and the aqueous extract was obtained as described by Afriyie et al. (2013a). The freeze-dried extract was weighed and stored in a sealed container in a refrigerator at a temperature of 5° ± 3°C until use.

Preparation of phosphate-buffered solution of CMARE

To enhance complete dissolution of the CMARE powder, PBS was used as a solvent to prepare a stock solution of 200 mg/mL. The CMARE was diluted further using the RPMI 1640 medium (L-Glutamine) in 10% FBS to obtain the required working concentrations.

MTT assay for cell viability

BPH-1 cells were seeded on 96-well plates at a density of 1.0 x 10^5 cells/well in 0.1 mL medium in 10% FBS. Then, the cells were treated with various concentrations of CMARE (0, 1, 3, and 5 mg/mL) and incubated for 24, 48, and 72 h. At the designated times after drug treatment, 10 µL MTT (5 mg/mL in PBS) was added to each well, and the plates were incubated for 4 h at 37°C. Subsequently, the plates were removed, and 100 µL dimethyl sulfoxide was added to dissolve the purple-blue MTT formazan crystals. The absorbance was read at a wavelength of 570 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Model EXL 800; Bio-Tek, Florida, USA). All experiments were performed in triplicates. The percentage cellular viability was calculated with the appropriate controls taken into consideration as follows: Cell viability (%) = average $OD_{treatment\ group}$/average $OD_{blank\ group}$ x 100%.

Assessment of changes in cell morphology

BPH-1 cells were seeded on 6-well plates at a density of 1.0 x 10^5 cells/well in 2 mL culture medium (with 10% FBS) and were incubated for 24 h. After the designated time, the cells were treated with the increasing concentrations of CMARE (0, 1, 3, and 5 mg/mL) for 48 h. Cell morphology was observed using a phase-contrast microscope (Olympus, Tokyo, Japan). Photomicrographs were taken at a magnification of 200X.

Detection of apoptosis using Hoescht 33258 staining

BPH-1 cells were seeded on two 6-well plates at a density of 1.0 x 10^5 cells/well in 2 mL culture medium (with 10% FBS). After incubation for 24 h, the cells were treated with various concentrations of CMARE (0, 1, 3, and 5 mg/mL). After 48 h, BPH-1 cells were fixed in 4% neutral formaldehyde for 30 min, washed with PBS for 5 min, and stained with 10 µL Hoescht 33258 dye at 37°C for 30 min in the dark. The Hoescht 33258 exhibited high fluorescence on binding to the double-stranded DNA. The shape of the nucleus and chromatin condensation were detected in the live and apoptotic cells using Hoescht 33258 and were examined using fluorescence microscopy. Then, photomicrographs of BPH-1 cells were taken at a magnification of 200X under a fluorescent phase-contrast microscope.

Examination of mitochondrial membrane potential using JC-1 staining

BPH-1 cells were seeded on 6-well plate at a density of 1 x 10^5 cells/well in 2 mL medium in 10% FBS. After incubation for 24 h, the cells were treated with various concentrations of CMARE (0, 1, 3, and 5 mg/mL) for 48 h. BPH-1 cells (1 x 10^5) in each group were "collected" after trypsinization with 0.25% trypsin and were incubated with 10 µg/mL JC-1 dye (Invitrogen) at 37°C and 5% CO_2 for 30 min. To examine the loss of mitochondrial membrane

potential, we stained the cells with fluorescent JC-1 dye. The JC-1 dye is a cationic dye that indicates mitochondrial potential during the apoptosis of cells by shift of fluorescence emission from green to red. The mitochondrial membrane potential was assessed by analyzing the red and green fluorescence after using flow cytometry analysis via fluorescence-activated cell sorting (FACS) caliber (Becton Dickinson, California, USA).

Detection of background aggregate and debris using flow cytometry analysis

BPH-1 cells were seeded on 6-well plates at a density of 1.0 x 10^5 cells/well in 2 mL culture medium in 10% FBS. After incubation for 24 h, the cells were treated with various concentrations of CMARE (0, 1, 3, and 5 mg/mL) for 48 h. The cells were treated further with 0.25% trypsin and washed with PBS. We observed the progression of cells through the different phases of the cell cycle phases, and determined the proportion of BPH-1 cells undergoing apoptosis, which is the percentage of background aggregate and debris (BAD) in sub-diploid G_0/G_1 peak, using Cycle-test Plus DNA assay kit (BD Biosciences, NJ, USA) using a flow cytometer (BD Biosciences) according to manufacturer instructions.

RNA extraction and RT-PCR analysis

To determine RNA expression, we seeded the BPH-1 cells into 25 cm^2 flasks (1.5 x 10^5/mL) in 5 mL culture medium (with 10% FBS). After 24 h, the cells were treated with various concentrations of CMARE (0, 1, 3, and 5 mg/mL) for 48 h. We isolated total RNA from the BPH-1 cells using the TriZol reagent. Oligo (dT)-primed RNA (1 μg) was reverse-transcribed using SuperScript II reverse transcriptase according to manufacturer instructions. The cDNA obtained was used to determine the mRNA expression levels of Bax and Bcl-2 using PCR with Taq DNA polymerase (Fermentas). GAPDH was used as an internal control. Primer sequences used for the amplification of Bax, Bcl-2, and GAPDH transcripts were as follows: Bax forward, 5'-TGC TTC AGG GTT TCA TCC AGG-3' and reverse 5'-TGG CAA AGT AGA AAA GGG CGA-3'; Bcl-2 forward, 5'-CAG CTG CAC CTG ACG CCC TT-3' and reverse 5'-GCC TCC GTT ATC CTG GAT CC-3'; GAPDH forward, 5'-GTC ATC CAT GAC AAC TTT GG-3' and reverse 5'-GAG CTT GAC AAA GTG GTC GT-3'.

Western blot analysis

BPH-1 cells (1.5 x 10^5/mL) were seeded into 25-cm^2 culture flasks in 5 mL medium for 24 h. After the designated time, the cells were treated with the various concentrations of CMARE for 48 h and lysed with mammalian cell lysis buffer containing protease and phosphatase inhibitor cocktails. The lysates were separated using 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gel (SDS-PAGE). The proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes and then blocked using 5% skimmed milked at room temperature for 2 h. Then, the membranes were washed in TBS with 0.25% Tween-20 (TBST) and probed with primary antibodies against Bax, Bcl-2, and β-actin (1:1,000) overnight at 4°C. β-Actin was used as an internal control. The membranes were washed in TBST, and secondary HRP-conjugated anti-rabbit antibodies (Cell Signaling Technology, Oregon, USA) were added at a dilution of 1:2500, and the membranes were incubated for 2 h at room temperature. The membranes were finally washed in TBST, analyzed with

ECL Plus reagents and scanned using Storm PhosphorImager (ChemiDoc XRS+; Bio-Rad, California, USA).

Statistical analysis

Statistical analysis was performed using the Graph Pad Software version 5.0 for windows (Graph Pad Software, California, USA), and data are reported as means ± SE of at least triplicate experiments. Significant differences between the treated groups and control were evaluated by performing a one-way analysis of variance (ANOVA). Where ANOVA showed significant differences, *post-hoc* analysis was performed using Bonferroni multiple comparison test. $P \leq 0.05$ was considered to be statistically significant.

RESULTS

CMARE inhibits the proliferation of BPH-1 cells

We determined the viability of BPH-1 cells after treatment with various concentrations of CMARE (0, 1, 3, and 5 mg/mL) for 24, 48, and 72 h using the MTT assay; we compared the relative number of cells in the BPH-1 monolayer treated with CMARE with those in the untreated controls. Cultures with more than 90% viable cells were considered unaffected, those with 80-90% viable cells were modestly affected, and cultures with <80% viable cells were considered to have cytotoxic effects of the compound (Hostanska et al., 2007). Cells treated with 1 mg/mL CMARE for 24-72 h showed 95-84% viability compared to that of the control cells. Furthermore, treatment with 3 and 5 mg/mL CMARE for 48-72 h decreased the BPH-1 cell viability to 79-42% and 61-25%, respectively, compared to that of the controls ($P < 0.05$ and 0.001, respectively). CMARE induced a dose- and time-dependent decrease in BPH-1 cell viability (Figure 1). Examination of the BPH-1 cells under a phase-contrast microscope showed that treatment with CMARE for 48 h induced a significant dose-dependent alteration in the morphology and a reduction in the density of BPH-1 cells (Figure 2).

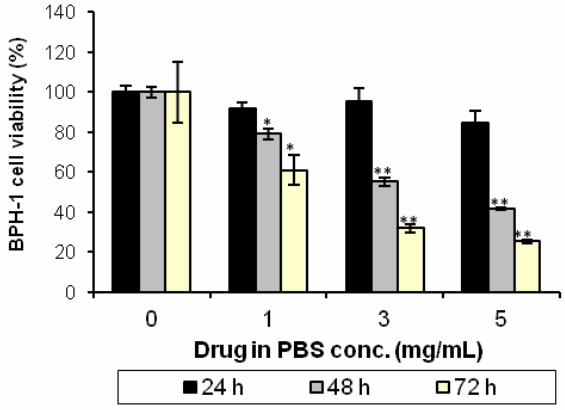

Figure 1. Graph showing the effect of *Croton membranaceus* aqueous root extract (CMARE) on the viability of BPH-1 cells at 24, 48, and 72 h. Data are reported as means ± standard error (SE) from at least three independent experiments. *$P < 0.05$ compared to control (untreated). **$P < 0.001$ compared to control (untreated).

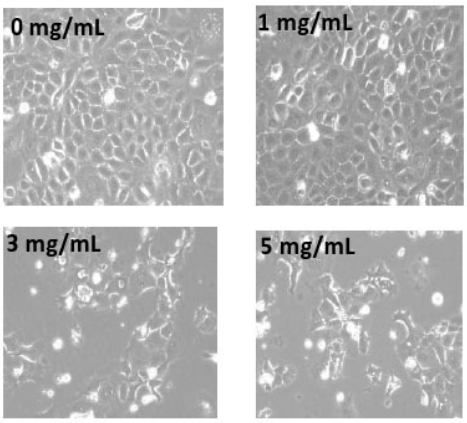

Figure 2. Photomicrographs obtained at 200X magnification showing dose-dependent effects of various concentrations of *Croton membranaceus* aqueous root extract (CMARE) on the confleuncy of BPH-1 cells after 48 h. Images are representative of three independent experiments.

CMARE induces an alteration in the nuclear morphology of BPH-1 cells

To ascertain whether apoptosis was involved in the antiproliferative activity of CMARE, we analyzed nuclear chromatin condensation in BPH-1 cells using Hoescht 33258. After 48 h of exposure of BPH-1 cells to various concentrations of CMARE (0, 1, 3, and 5 mg/mL), the cells were stained using the Hoescht 33258 dye. The number of brightly stained blue apoptotic cells was significantly higher in the BPH-1 cells treated with 1 mg/mL CMARE than the control cells, which displayed weak fluorescence (Figure 3). BPH-1 cells treated with 3 and 5 mg/mL of CMARE showed a significant decrease in the number of cells after 48 h, and compared to the untreated control cells, the treated cells showed brightly stained blue condensed chromatin and fragmented nuclear morphology.

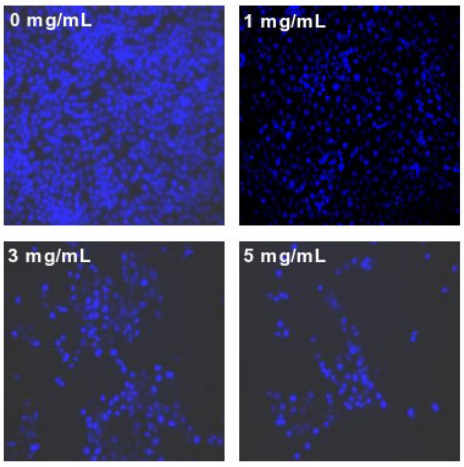

Figure 3. Hoescht 33258 staining showing brightly blue stained condensed chromatin in BPH-1 cells treated with various concentrations of *Croton membranaceus* aqueous root extract (CMARE) after 48 h.

Effect of CMARE on the mitochondrial potential (Δψm) of BPH-1 cells

We examined the characteristic loss of the mitochondrial potential in the apoptotic cells in relation to the high mitochondrial potential of healthy cells using FACS analysis with JC-1 dye staining. JC-1 selectively enters the mitochondria of healthy cells to form complex aggregates that emit intense red fluorescence (590 nm, FL2); a shift from the intense red to bright green fluorescence (525 nm, FL-1) is an indication of the loss of membrane potential. Our data showed that the percentages of JC-1 green bright BPH-1 cells treated with 1, 3, and 5 mg/mL CMARE were significantly higher, 13.1 ± 1.5%, 32.4 ± 5.1%, and 36.1 ± 1.7% ($P < 0.01$), respectively, than the untreated cells 5.6 ± 1.3% (Figure 4A and B).

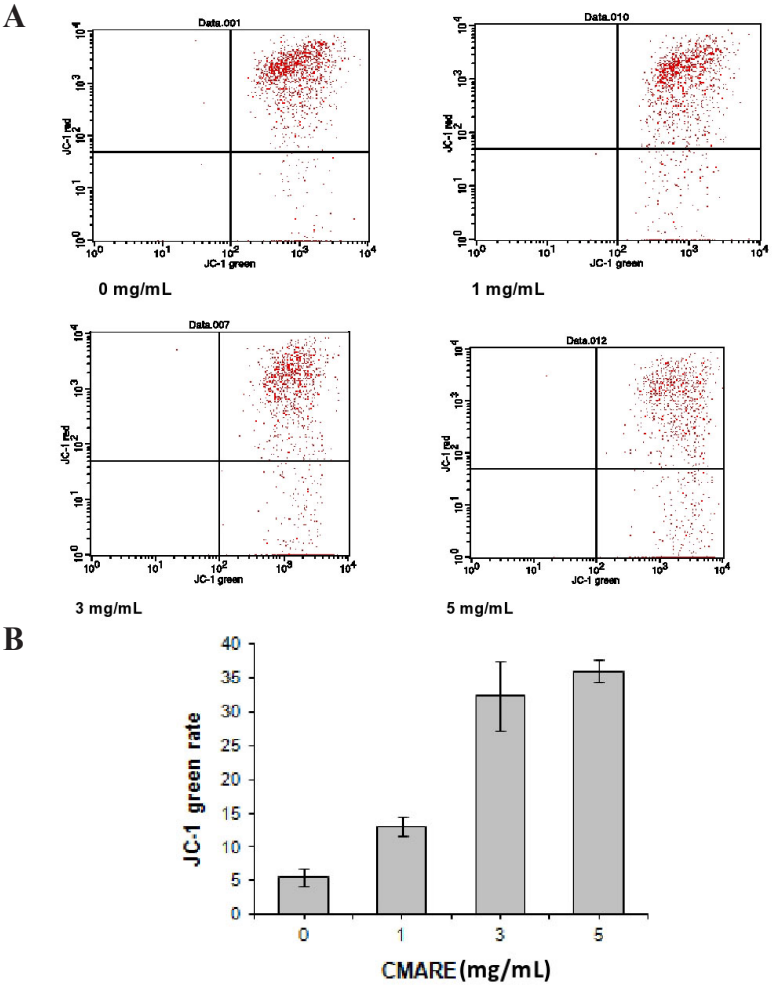

Figure 4. A. Effect of various concentrations of *Croton membranaceus* aqueous root extract (CMARE) on the mitochondrial membrane potential of BPH-1 cells after 48 h. Data are representative of three independent experiments. **B.** Bar charts showing dose-dependent loss of mitochondrial membrane potential in BPH-1 cells treated various doses of *Croton membranaceus* aqueous root extract (CMARE) after 48 h. CMARE induced background aggregate and debris (BAD) in BPH-1 cells.

CMARE induces BAD in BPH-1 cells

To further ascertain whether the antiproliferative activity of CMARE was caused by DNA fragmentation induced by apoptosis, we analyzed the proportion of BAD during BPH-1 cell cycle progression after treatment with various concentrations of CMARE. After 48 h of treatment with 1, 3, and 5 mg/mL CMARE, the proportion of BAD in the cells was 2.63 ± 0.56, 4.68 ± 0.52, and 6.0 ± 0.21%, respectively, which was significantly higher ($P < 0.01$) than that present in the untreated control cells, 3.48 ± 0.56%, except that present in the 1 mg/mL dose group. We observed a dose-dependent increase in the induction of apoptosis as shown by the presence of sub-diploid peaks (G_0/G_1) in only the treated cells. The proportion of BAD in the BPH-1 cells is a measure of the rate of apoptosis (apoptosis index) (Figures 5 and 6).

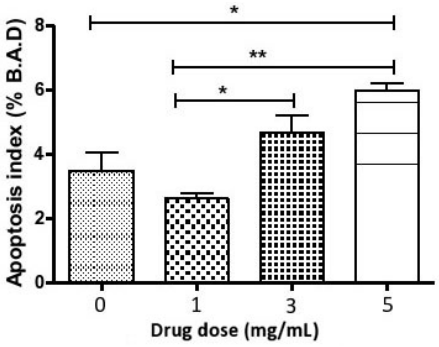

Figure 5. Bar chart showing flow cytometry analysis of percent background aggregate and debris (BAD) in *Croton membranaceus* aqueous root extract (CMARE)-treated BPH-1 cells. Data are reported as means ± standard error (SE). Significant differences (*$P < 0.05$ or **$P < 0.01$) between CMARE-treated and untreated control cells.

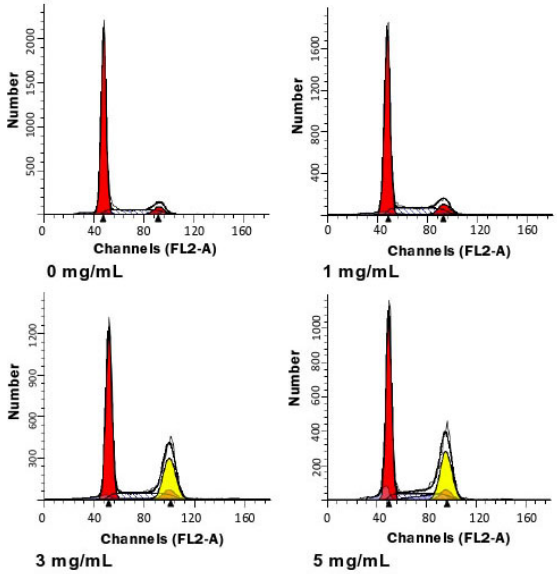

Figure 6. Flow cytometry graphs depicting cell cycle distribution and presence of sub-dipliod G_0/G_1 peaks in BPH-1 cells treated with *Croton membranaceus* aqueous root extract (CMARE) for 48 h compared to those in the untreated control cells.

Effect of CMARE on Bcl-2 family proteins

To understand the mechanism of apoptosis induction by CMARE in BPH-1 cells, we examined the effect of the CMARE extract on the mRNA and protein levels of Bax and Bcl-2 using real-time PCR and Western blot analysis. CMARE induced a significant dose-dependent increase in the expression levels of Bax mRNA. However, increasing doses of CMARE did not show a significant increase in the mRNA expression level of Bcl-2 (Figure 7).

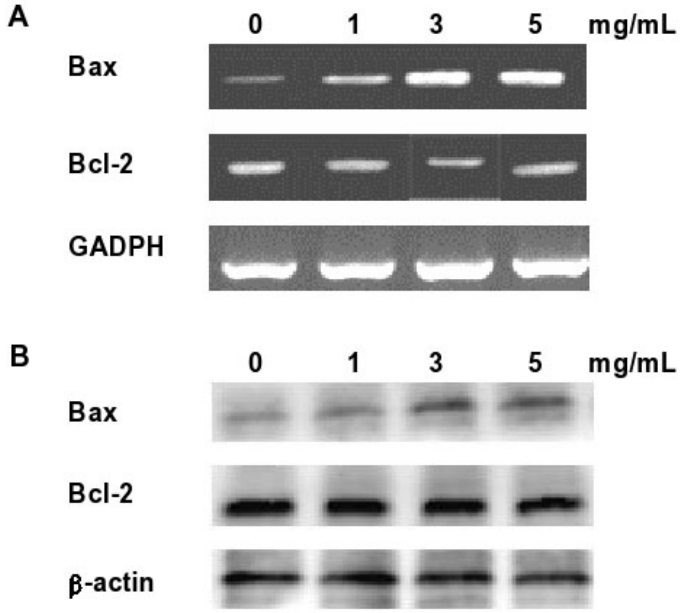

Figure 7. A. Effect of 48-h treatment with *Croton membranaceus* aqueous root extract (CMARE) on the mRNA expression levels of Bax and Bcl-2 in BPH-1 cells determined using reverse transcription polymerase chain reaction (RT-PCR). GADPH was used as an internal control. **B.** Effect of 48-h treatment with *Croton membranaceus* aqueous root extract (CMARE) on the levels of Bax and Bcl-2 proteinsin BPH-1 cells determined using Western blot analysis.

DISCUSSION

Although the exact pathogenesis of BPH and prostate cancer is still not clearly understood, several studies show that reduction of apoptosis is associated with the development of BPH (Siegfried et al., 1993; Kyprianou et al., 1996). Hence, induction of apoptosis has emerged as a potential target for optimizing the medical treatment for BPH and prostate cancer. Prostate epithelial cells derived from normal humans and BPH patients that have undergone prostatectomy are useful *in vitro* models for examining the cause of BPH and determining the mechanism of action of potential novel therapies (Kaseb et al., 2007). Medicinal plant extracts often contain diverse biologically active compounds with different mechanisms of action. Thus, whether apoptosis is one of the mechanisms of action of the medicinal plants used in improving symptoms associated with BPH and prostate cancers remains to be established.

A previous study has shown the cytotoxic activity of the methanolic root extracts of *C. membranaceus* against human normal and cancerous prostate cells *in vitro* (Bayor, 2008). However, to the best of our knowledge, this is the first *in vitro* study in which the effect of CMARE on BPH-1 cells has been examined. Our study showed that CMARE treatment induced a significant dose- and time-dependent decrease in the viability of BPH-1 cells. Our results are consistent with the results of significant antiproliferative activity of CMARE observed in the epithelial and stromal cells of Sprague-Dawley rats reported previously (Afriyie at al., 2013b). Furthermore, the significant decrease in the viability of BPH-1 cells observed in our study was similar to that observed in previous *in vitro* studies performed using extracts from plants such as *Olea europaea*, *Serenoae repentis* fructus, and *Commiphora mukul* used for the management of BPH and/or prostate cancer (Acquaviva et al., 2012; Hostanska et al., 2007). Additionally, our results showed that significant inhibition of BPH-1 cells was observed only after 48 h of treatment with any of the doses administered. This observation suggests that CMARE may have a slow onset of action and/or long duration of action.

Disruption of the molecular mechanisms that regulate cell proliferation and apoptosis in stromal and epithelial cells has been implicated in both the development of BPH, and progression of prostate cancer (Kyprianou et al., 2000). Apoptosis is characterized by features such as cell shrinkage, membrane blebbing, chromatin condensation, and DNA ladder formation with multiple fragments, which are eventually engulfed by macrophages (Majno and Joris, 1995; Collins et al., 1997). These cellular and morphological alterations are established hallmarks for the induction of apoptosis and are associated with a significant increase in the apoptotic index (Bayne et al., 2000). Because apoptosis occurs through a complex signaling cascade, which is tightly regulated at multiple points by activators, effectors, and regulators, more than a single assay is required to detect and count apoptotic cells, and to determine a distinct cellular feature. Hoescht 33258 staining showed that treatment of BPH-1 cells with CMARE induced a significant dose-dependent induction of apoptosis associated with characteristic brightly stained blue cells with nuclear condensation.

Fragmentation of DNA during which is characterized by the presence of a sub-diploid peak during the G_0/G_1 phase, is also a hallmark of apoptosis (Darzynkiewicz et al., 2001; Huguenin et al., 2004). Treatment with the different doses of CMARE resulted in obvious dose-dependent sub-diploid peaks during the G_0/G_1 phase, similar to findings of a related study where the administration of guggulsterone, an extract of *C. mukul* induced apoptosis with sub-diploid cells and significant DNA fragmentation in PC-3 human prostate cancer cells (Singh et al., 2005). The dose-dependent presence of BAD in BPH-1 cells treated with CMARE suggests the occurrence of DNA fragmentation, a characteristic hallmark of apoptosis (Cohen, 1993).

Maintenance of the mitochondrial membrane potential is fundamental for the performance and survival of cells (Mathur et al., 2000). Loss of mitochondrial potential, a key indicator of apoptosis in cells, results in insertion of proapoptotic proteins into the cell membrane, possible oligomerization of Bad, bax, Bid, or bid, and the release of cytochrome c into the cytoplasm because of the development of pores in the mitochondrial membrane (Bouchier-Hayes et al., 2005; Armstrong, 2006). The JC-1 dye has been used in several apoptosis studies to detect the loss of mitochondrial membrane potential (Castedo et al., 2002). Our findings showed that CMARE induced a significant dose-dependent loss of mitochondrial membrane potential in the BPH-1 cells. Further studies are required to establish the role of caspase-3 and caspase-9 to completely elucidate the apoptotic mechanism of CMARE.

The Bcl-2 family of proteins, namely, Bax and Bcl-2, play a key role in maintaining the mitochondrial permeability by controlling and regulating the apoptotic mitochondrial events (Cory and Adams, 2002). The differential interaction among the pro-apoptotic proteins (i.e., Bax and Bak) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) and other cellular proteins regulates apoptosis (Reed, 1997; Chao and Korsmeyer, 1998). The Bax/Bcl-2 ratio is useful because Bax expression is upregulated during apoptosis, Bax translocates into the mitochondria, and thus causes the release of cytochrome c and a decrease in the mitochondrial membrane potential (Liu et al., 2003). Our results showed that the administration of CMARE for 48 h induced a significant dose-dependent upregulation of mRNA and protein levels of Bax in the BPH-1 cells. However, no significant change was observed in the mRNA and protein levels of the anti-apoptotic Bcl-2 when BPH-1 cells were treated with CMARE for 48 h. Thus, the molecular expressions of Bax and Bcl-2 observed in our study suggest an increase in the Bax/Bcl-2 ratio in BPH-1 cells treated with CMARE and confirm the ability of CMARE to induce apoptosis at the molecular level similar to that observed at the cellular level.

Previous studies have shown that extracts from several *Croton* species such *C. zambesicus*, *C. argyratus*, and *C. pierrei* induce apoptosis, and these extracts are used in the management of tumors (Sandoval et al., 2002; Block et al., 2005; Morales et al., 2005). Several studies have proven the efficacy of beta-sitosterol and its glycosides in inhibiting the growth and improving the symptoms of BPH and prostate cancer (Wilt et al., 2000; Awad et al., 2005). Beta-sitosterols exhibit dose-dependent morphological and characteristic features of apoptosis such as an increase in the presence of sub G_0/G_1 population, upregulation of mRNA and protein levels of Bax, and an increase in Bax/Bcl_2 ratio in BPH, prostate cancer, and other cell lines (Duan, 2005; Park et al., 2007). Our results of the induction of apoptotic activity of CMARE are similar to those observed using extracts from other *Croton* species. Phytochemical investigations of the ethyl acetate fraction of the root extracts of *C. membranaceus* showed the presence of beta-sitosterol and beta-sitosterol-3-D-glucoside among other isolates (Bayor et al., 2007). Thus, apoptosis induced by the CMARE in BPH-1 cells may be attributed to the presence of beta-sitosterol and beta-sitosterol-3-D-glucoside similar to that observed in previous studies. The presence of these phytosterols may explain in part the mechanism of action underlying the cytotoxic effect of CMARE on prostate cancer cells *in vitro* (Bayor et al., 2008). Our findings support the ethnomedicinal use of CMARE in the management of prostatic tumors because of the ability of CMARE to induce apoptosis in BPH-1 cells. Further *in vitro* and *in vivo* studies are required to confirm the use of CMARE in prostate cancer cells.

In conclusion, to the best of our knowledge, our study showed for the first time that CMARE suppresses the proliferation of BPH-1 cells; and apoptosis is one of the mechanisms underlying the antiproliferative effect of CMARE. The effect of CMARE was characterized by significant nuclear condensation, DNA fragmentation, appearance of subdipliod cells, loss of membrane potential, and significant upregulation of Bax proteins. These observations provide the rationale for further preclinical and clinical studies to evaluate the efficacy of CMARE in the management of BPH.

Conflicts of interest

The authors declare no conflict of interest.

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