Bioactivity of bizzy nut extract in prostate cancer cells

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Cola acuminate is one of the three edible forms of bizzy nut that has been used for medicinal and therapeutic purposes. However, the precise compound responsible for its biological effects has not been completely identified. Using sequential solid-liquid extraction of bizzy nut coupled with bioactive screening, ether extract was obtained (Biz-2) and it possesses tumor inhibitory activity specific for prostate cancer cells. Enrichment of this tumor inhibitory activity (Biz-2Fr.3) resulted in the elimination of caffeine and tannin, suggesting that Biz-2-Fr.3 activity is due to a unique set of compounds. Biz-2Fr.3 contains three to four unique compounds with a molecular mass ranging from 120 to 440 amu as evident by high performance liquid chromatography (HPLC), UV-Vis spectroscopy and LC/MS. Biz-2Fr.3 was found to exhibit growth inhibition and cytotoxicity against the hormone-independent (DU-145) and hormone-dependent (LNCaP) prostate cancer cell lines via microculture tetrazolium (MTT) assay. In the DU145 cell, Biz-2Fr.3 induces a growth-inhibition with a GI₅₀ of 120 ppm with no apparent toxicity in normal transformed prostate cells. The inhibition of DU145 cell proliferation by Biz-2Fr.3 was as a result of retardation of the cell cycle occurring mainly in the G1 phases of the cycle. This cell cycle arrest was associated with the decrease in cyclin D protein levels following Biz-2Fr.3 treatment. It was observed that Biz-2Fr.3 did not elicit any toxicity as evidenced by biochemical markers of liver injury which caused decrease in body weight or serum protein profiles. These results suggest that C. acuminate possesses an anti-cancer activity that is distant from its previously reported biological effects.

Key words: Cola acuminate, bizzy nut, prostate, toxicity.

INTRODUCTION

Prostate cancer (PCa) is a major public health problem worldwide. Epidemiological studies have illustrated a wide variation in PCa incidence among different ethnic populations. The incidence of PCa is lower in Asia and China as compared to Western countries such as Europe and the Americas (American Cancer Society, 2013, 2014; Torre et al., 2016). It is estimated that, in the United States, 241,740 men will be diagnosed, and 28,170 men will die of cancer of the prostate in 2016 (Howlader et al., 2017). At the same time, the incidence rate of the diseases in African American men is one-half to three times higher as compared to other ethnic groups (American Cancer Society, 2016). Interestingly, a thirty-year retrospective study on PCa in Black men...
men from the Caribbean island of Jamaica indicated a lower PCa incidence as compared to black men from the USA and the United Kingdom (Gibson et al., 2011). This disparity between prostate cancer among blacks is presumed due to the difference in life-style and diet.

Studies over the past ten years have provided convincing evidence that natural products can suppress cancer cell growth in cultures and in some animal models. Although the mechanisms of cancer inhibition by natural products vary, they all appear to exert their effect at one or more stages in the cell cycle. For example, Berberine, a naturally occurring isoquinoline alkaloid present in the roots, rhizome, mediates its potential effectiveness in prostate cancer cells through interference with cell cycle progression and induction of apoptosis (Martena et al., 2006). Andrographolide (AGP), a natural compound isolated from Andrographis paniculata, displayed selective inhibitory properties in androgen resistant prostate cancer cells through induction of the G2/M phase cell cycle arrest leading to apoptosis (Varma et al., 2011; Yan et al., 2012; Wong et al., 2011). CKBM, a product contained in the water extracts of wu wei zi (Schisandra chinensis), ginseng (Panax ginseng), hawthorn (Fructus Crataegi), jujube (Ziziphus jujube), soybean (Glycine Max) and baker’s yeast (Saccharomyces cerevisiae) inhibits cell proliferation through the induction of the G2/M cell cycle arrest and dose dependent apoptotic effect (Luk et al., 2005).

Chemopreventive agents, isolated from natural products including silibinin, selenium, inositol hexaphosphate, decursin, apigenin, acacetin, curcumin, and epigallocatechin-3 gallate have been identified in laboratory studies as potential useful agents in managing PCa (Singh and Agarwal, 2006; Deep et al., 2016; Tilley et al., 2016). In a clinical trial, lycopene was found to be associated with a decreased risk of PCa development and to inhibit the growth of PCa cells (Dahan et al., 2008; Kumar et al., 2008; Maru et al., 2016). Epigallocatechin-3 gallate (EGCG), a compound found in green tea has been shown to inhibit both androgen dependent and androgen-independent prostate cancer growth (Kalifatidis et al., 2016; Kumar et al., 2016).

EGCG appears to target prostate cell proliferation by inhibiting the production of androgens thus inhibiting 5-alpha reductase and depleting the level of polyamines, both of which are involved in prostate cell proliferation. Other sources of dietary-based natural products that have been examined as a possible treatment and prevention regime for prostate cancer are garlic, grape seed extract, green tea, bitter almonds, and tomato based products. Although several of the natural product remedies are being used clinically and as over the counter medication for treatment of prostate disease but none of them have emerged as a gold standard for prevention or treatment of the disease.

In the laboratory, interest is placed on natural products that contain phytoestrogens, phyto-androgens, or compounds that are anti-androgenic in nature. Recently, it was reported that the common Jamaican herbal medicine, (Bizzy), contains a putative non-steroidal compound with bioactivity in both breast and prostate cancer cells (Fontenot et al., 2007; Solipuram et al., 2009). C. acuminata, also known as obi or bizzy nut to the Ettu people of Jamaica, is a “cure-all” herbal medicine. It reportedly affects many biological processes, which are directly, or indirectly, modulated by hormones. Available ethnobotanical information suggests that C. acuminata may contain bioactive chemicals that possess estrogenic and androgenic properties (Kamatenesi-Mugish and Orlyem-Origa, 2005; Endrini et al., 2011; Osterburg et al., 2009; Lowe et al., 2014).

To begin to elucidate the biology of bizzy nut, several of its hormonally bioactive extracts that were capable of inhibiting the growth of different cancer cell lines were identified (Fontenot et al., 2007). The putative androgenic effects of a Biz-2Fr.3 extract (acetone extract of bizzy Nut) on pathways mediated by an androgen receptor (AR) in LNCaP cells was demonstrated. This bioactive fraction of Bizzy nut is able to induce apoptosis in a prostate cell and modulate AR-dependent gene expression (Solipuram et al., 2009). Furthermore, it was demonstrated that Biz-2Fr.3 induces apoptotic cell death and the cytostatic effect was twice as potent in AR positive LNCaP cells line as compared to the AR negative DU145 cells. However, the biochemical mechanism by which Biz-2Fr.3 induces toxicity in prostate cancer and its effects on prostate cell function is undefined. According to recent studies, many chemopreventive and/or chemotherapeutic agents can cause cell death via the induction of apoptosis. Therefore, the induction of apoptotic cell death is an important mechanism in the anticancer properties of many drugs. In this study, the anti-cancer effects of Biz-2Fr.3 in the two main stages of prostate cancer, namely, androgen-sensitive and androgen-insensitive tumors is reported. It is shown that a HPLC enriched fraction of bizzy nut is able to inhibit prostate cancer cell growth via induction of the G1--cell cycle blockage. Under the conditions used in this study, bizzy nut did not affect the viability of non-neoplastic human prostate epithelial cells and showed no signs of toxicity in mice. Taken together, a potent anticancer bioactive fraction from bizzy nut which is apparently safe in mice has been identified.

**MATERIALS AND METHODS**

**Cell culture**

All cell lines used in this study were obtained from the American type culture collection (ATCC; Rockville, MD, USA). The human prostate cancer cell lines DU145, LNCaP, and PC-3 were cultured in RPMI-1640 (Invitrogen, Grand Island, NY, USA) with 4 mM L-glutamine, and no phenol red and adjusted to contain 10% fetal bovine serum and 100 U/mL penicillin-streptomycin. The human
breast cancer cell lines MCF-7 and MCF-10A were cultured in DMEM-F12 with 4 mM L-glutamine and adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.005 mg/ml/mL bovine insulin and 10 nM dehydroisoandrosterone, 10%; fetal bovine serum (BD Biosciences, San Jose, CA, USA). The non-neoplastic immortalized adult human prostate epithelial cell line RWPE-1 was cultured in K-SFM supplemented with recombinant human epidermal growth factor (rhEGF) and bovine pituitary extract (BPE). The cells were incubated at 37°C in 95% air, 5% CO₂ atmosphere until they approached 80% confluence.

Cell viability analysis

For experiments involving cell growth and gene induction studies, cells were grown for 24 h in appropriate medium containing 5% fasting blood sugar (FBS) that was stripped three times with dextran-coated charcoal or in corning™ serum free medium, with L-glutamine and without phenol red. Cells were plated in 96-well plates (2 x 10³ cells/well) and allowed to attach overnight. Bizzy nut extract was added at five to 10-fold dilutions of a 1E4 ppm stock Biz-2Fr.3 solution in 0.5% dimethyl sulfoxide (DMSO) to a 96-well plate. As a control and reference, 10-8 M DHT and 100 ng/ml TNF-a were added to separate wells of each plate and each treatment and time point had four replicates. In each treatment, the final concentration of vehicle solvent (DMSO) did not exceed 0.1% v/v in the medium. After 24 h exposure to the test compounds, the effect on cell viability and gene expression was determined. Cytotoxicity was determined by the CellTitre 96® aqueous one solution cell proliferation assay (Promega, Madison, WI) or sulforhodamine B assay according to the manufacturer’s instructions. After incubation with 3-(4,5-dimethyl-2-yli)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS), absorbance at 490 nm was measured using a ELX800UV universal microplate reader (Bio-Tek, Inc.). The absorbance data from the sulforhodamine B assay and the CellTitre 96® aqueous one assay was analyzed using Prism 5.

Analysis of cell cycle progression

Cells were seeded in a 75 cm² flask at a density of 5 x 10⁶ cells/flask. Cells were treated with Biz-2Fr.3 (five to 10-fold dilutions of 1E4 ppm stock), and incubated for 0, 6, 12 or 24 h. Cells were trypsinized, harvested, and fixed in 1 ml of 80% cold ethanol in test tubes and incubated at 4°C for 15 min. After incubation, cells were centrifuged at 1,500 rpm for 5 min and the cell pellets were resuspended in 500-μl propidium iodide (10 μg/ml) containing 500 μg/ml RNase (Sigma, MO, USA). Then, the cells were incubated on ice for 30 min and filtered with 53 μm nylon mesh. The cell cycle distribution was determined from 20,000 cells using the Beckman Coulter Cytotox flow cytometer. The stained cells were analyzed by ModFit software for cell cycle distribution, including sub-G1, G0/G1, S, and G2/M phases.

Western blot analysis

Immunobloting was performed as previously described (Stahl et al., 1998; Washington et al., 2001). Cells at 80% confluence were treated with Biz-2Fr.3 (5 to10-fold dilution of 1 E4 ppm) for 24 h. Treated cells were trypsinized, washed in PBS, and then pelleted by centrifugation at 100 g for 5 min. The cell pellet was then suspended in lysis buffer [20 mM Tris-HCl pH 7.4, 2 mM EDTA pH 7.4, 2 mM EGTA pH 7.4, 6 mM β-mercaptoethanol, 10 mg/ml of leupeptin, 2 mg/ml of aprotinin and 1% Nonidet (NP-40)] and sonicated (Soniprep 150, MSE, USA) at 26 amplitude microns on ice. The cell lysate was centrifuged at 140,000 g for 15 min at 4°C and the supernatant was collected and stored at 70°C. The concentration of protein was determined using a BCA protein assay reagent, according to the manufacturer's instructions. An equal amount of protein (25 μg) was separated by 10% SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane, blocked overnight with 1% skimmed milk in TBS at 4°C then reacted with the following antibodies: anti-BC12 0.5 μg/ml and anti-Bax 0.5 μg/ml (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-AR 1.5 μg/ml, anti-PARP 0.5 μg/ml and anti-GDP1G 0.5 μg/ml (Cell Signaling, Danvers, MA), mouse monoclonal antibody against CDK1, CDK4 or Actin (Ab-1) (Santa Cruz Biotechnology). After reaction with horseradish peroxidase-conjugated goat anti-mouse antibody, the immune complexes were visualized by using the ECL-detection reagents following the manufacturer’s procedure. The immunoblot signal was captured using an Alphalnnotec Fluorochrom HD 9900 (Alpha Innotech, San Leandro, CA) equipped with a CDD camera and curves and graphs were fitted with GraphPad Prism 5.0 software (GraphPad, San Diego CA).

Immunofluorescence staining

Cells were grown on microscope slides and induced with Biz-2Fr.3 for 24 h. Cells were fixed with 100% methanol (-20°C for 10 min) and cross linked with 4% paraformaldehyde at room temperature for 10 min. The slides were blocked with 1% rabbit serum solution at room temperature for 1 h. Slides were probed with anti-AR at a 1:100 dilution for 1 h at room temperature, washed and then incubated with fluorescence-labeled anti-rabbit IgG (1:5000 dilution) for an additional hour at room temperature. For dual antibody staining, slides were washed with TBS-T and blocked in 1% sheep serum for 1 h and probed with anti-——tubulin (1:200) for 1 h. The tubulin signal was developed by addition of Cy3-labeled anti-mouse IgG for 1 h. Slides were washed with TBS and stained with prolong gold anti-fade reagent containing DAPI (4,6-diamidino-2-phenylindole). Slides were visualized using a Nikon Optiphot fluorescent microscope scope with green fluorescent (525 nm) and red fluorescent (620 nm) filters.

Solid-liquid extraction of bizzy nut

C. acuminata, commonly known as bizzy nut is from the west and central African genus of the family Sterculiaceae. Ripened bizzy nut was obtained from Lambe River, Jamaica in August and the dark brown nut was blended to a fine powder before use. A 1.2 kg sample of finely ground nut was sequentially extracted in a Soxhlet apparatus (120 cm X 500 cm) using 100% hexane, ether, acetone, and methanol, and water to produce five independent extracts with compounds of unique polarity. The extraction mixture was refluxed for seven days at temperatures corresponding to the boiling point of the respective solvent and the extraction monitored by HPLC chromatography. Following extraction, particulate matter was removed by filtering the samples through a 0.45 μm glass-fritted filter, and the extracts evaporated to dryness using a combination of simple distillation and rotary evaporation. All extracts were dissolved in 50% DMSO and represent the starting point for characterizing the bioactivity of bizzy nut.

HPLC with diode array detection (HPLC-DAD) analysis

HPLC purification of Biz-2Fr.3 was performed on an Agilent 1200 Series system (Agilent, USA), equipped with diode array UV/VIS detector, a quaternary LC- pump, a degassing unit auto injector column oven, a fraction collector and chem station data system.
Chromatographic separation was performed on a 10 micron, 250 mm x 4.6 mm. i.d. semi preparative Phenomenex C18 reversed phase column, (Agilent, USA) at 25°C with a guard column (4.6Å~12.5 mm, 5 μm, Zorbax eclipse plus). The mobile phase consists of water (Solvent A was water/formic acid 100:0.1(v/v)) and acetonitrile/isopropanol (solvent B, acetonitrile/isopropanol 70:30 (v/v)) which was used for gradient elution. The gradient starts linear at 20% B up to 70% B at 15 min, 50% B at 30 min, 60% B at 35 min and then down to 6% B at 41 min. Sample volume was 1200 uL, at a flow rate of 5 mL min-1 and peak detection at 250 nm and 280 nm.

HPLC with electrospray ionization mass spectrometry (HPLC-ESI-MS)

LC-MS identification of Biz-2Fr.3 analytes were performed on the Agilent Technologies Agilent 6210 Quadrupole LC-MS equipped with a mass analyzer and an electrospray ionization interface. An octadecyl silica (C18) column, 10 cm x 2.1 mm i.d., 100 Å pore C18 Ace® analytical column and guard column, from Mac Mod Analytical, Inc (Chadds Ford, PA) was used for analysis. The MA analysis parameters were as follows: Q1 MS; polarity positive, ion source, turbo spray (ESI); declustering potential (DP), 80 V; entrance potential (EP), 10 V; curtain gas, 20 psi; ion spray voltage (IS), 5500 V; temperature, 550°C; ion source gas 150 psi; ion source gas 250 psi; interface. The LC mobile phase consisted of gradient water; methanol: ammonium acetate (v/v/w). The mobile phase started with 100% A for the first min, followed by a linear increase to 100% B from 1 to 16 min. This was followed by 100% B from 16 to 31 min, then a linear decrease to 100% A from 31 to 40 min. The injection volume was 20 μl and the eluent flow rate was 0.25 ml/min.

NMR and FTIR Analysis of Biz-2Fr.3

1H NMR and 1H-decoupled 13C-NMR spectra of Biz-2Fr.3 in DMSO-d6 analysis were obtained using a Bruker ARX 500 MHz NMR. A 30° pulse width was used for the 1H NMR, with a 1 s pulse delay. A 30° pulse width was used for the 13C-NMR spectra, with a 2 s pulse delay. The hydrogen and carbon chemical shifts were referenced to the DMSO peaks, which were set to 2.50 ppm for hydrogen and 39.50 ppm for carbon, respectively. The Attached Proton Test (APT) was used to distinguish between two groups of signals, namely, methyl/methene and methylene/quaternary.

Animal study

C57BL/6 male mice of age 4 to 6 weeks were used for the subacute toxicity profiling. They were fed ad libitum with standard feed, and had free access to water. They were also maintained under standard conditions of humidity, temperature, and 12 h light/dark cycle. The animals were acclimatized for a week before the commencement of the study. A standard protocol was drawn up in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experiments in conscious animals.

Dosing

C57BL6 mice of average weight between 22 and 24 g were selected by stratified randomization and then divided into four groups of five mice. Group II, Group III, and Group IV were given 10, 100 and 200 mg/kg body weight, respectively, of Biz-2 or Biz-2Fr.3 fraction orally every 24 h for 21 days. Biz-2, is a crude ether extract of the C acuminate nut and Biz-2Fr.3 is a partial HPLC purified fraction of Biz-2 containing both active and inactive ingredients. Group I served as the control group and received DMSO in PBS. The body weight of each mouse was expressed using a sensitive balance during the acclimatization period, once before commencement of dosing, once daily during the experimental period and on the day of sacrifice. The first day of dosing was taken as Day 0 and blood was collected on Day 21 and used for biochemical analysis.

Determination of biochemical parameters

Blood was collected by the cardiac puncture technique. The clear serum supernatant was prepared and stored in a clean sample bottle for the biochemical tests. A mini blood chemistry panel was performed which included measurement for blood glucose, total serum protein, serum albumin, serum cholesterol, serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), serum urea, and serum creatinine. All hematological parameters were determined at room temperature following standard laboratory procedures.

Organ weight

The liver, kidney, brain, prostate, and stomach of mice in the various groups were excised on the Day 21 immediately after blood collection. Following excision, the organs were trimmed of extraneous tissues, placed on a saline soaked gauze pad to retard desiccation and were immediately weighed (paired organs were weighed together) to one decimal place and calculated for organ weight ratio.

Statistical analysis

All numerical data were expressed as mean ±SEM. In each assay, three or four measurements were made. Means for the treatment groups were compared using analysis of variance and Duncans’s multiple range test (P< 0.05). To analyze the absorbance density from Western blot data, a two-tailed t test (P< 0.05) was used to compare the mean (n=4) for each treatment group with the mean for the untreated control group. The GraphPad Prism 5.0 software program (GraphPad, San Diego CA) was used for the statistical analysis.

RESULTS

Enriching the anti-tumor bioactivity in bizzy nut

Previously we reported that a crude ether extract of bizzy Nut (Biz-2) contains bioactive compounds that elicited an anti-proliferative effect in both hormone---responsive (LNCaP cell) and hormone-resistant (DU145 cell) prostate cancer cells suggesting that Biz-2 may be a potential agent for managing PCa. To generate an enriched fraction of the bioactive compounds present in Biz-2, a reverse phase HPLC chromatographic separation method was developed using a 2.6 μm C18 250b x 4.6 ID phenomenex column and the Agilent 1200 Series HPLC equipped with diode array detector, a quaternary pump, and a standard fraction collector. The analytes present in Biz-2 were separated based on retention time and a peak threshold corresponding to 5 nng of analytes at 250 nm absorbance. Optimizations of
the Biz-2 enrichment conditions were performed on an analytical size column and include chromatography column selection, mobile phase buffer optimization and detection wavelength. In several HPLC runs, a mobile phase of 70%/acetonitrile/30% isopropanol was able to identify five peaks having an absorbance response greater than 5 ng using our separation conditions. The HPLC conditions that were developed resulted in excellent separation with each peak having a resolution factor (K') of 2.1 to 2.4. Fractionation of the Biz-2 extract by HPLC revealed the presence of five distinct peaks (Fr.1-5), all eluting within the first 25 min of the HPLC run (Figure 1A). The resulting peak areas of the five-distinct fraction were used as a marker for evaluation of Biz-2 extraction efficiency. There were no significant differences in the peak areas of the five fractions among different batches of extractions and HPLC separation. Therefore, the extraction method for identifying the bioactivity in bizzy nut was selected as extraction with 100% hexane 70°C for 24 h, followed by 100% diethyl ether at 50°C for 48 h.

**Identification of bioactivity in HPLC Fraction**

To determine which of the HPLC fraction contains the observed bizzy nut bioactivity, each HPLC peak was concentrated to remove the HPLC solvent, dissolved in 10% DMSO, and were subjected to growth-inhibitory screens in LNCaP prostate cancer cells using the MTS viability assay (Figure 1B). The percent growth-Inhibition was determined and the resulting inhibition data analyzed using Sigmoidal Dose-response with a variable slope was used to determine which fraction contained the bioactivity. Analysis of the GI50 in DU145 cells indicated that most of the activity eluted in peak 3. The GI50 associated with peak 3 was between 150 to 210 ppm as compared to the other HPLC fractions which has a GI50 greater than 500 ppm. The strongest correlation between the inhibitory activity of the HPLC fractions was observed in peak 3 of the HPLC chromatograph. The GI50 for peak 3 (referred to as Biz-2Fr.3) in DU145 cells was 100±1.3 ppm; 2.5 times lower than that of the crude extract (Figure 1B)

**Chemical characterization of Biz-2Fr.3**

Next, we examined the purity and determined the number of compounds present in peak 3 (Biz-2Fr.3Fr.3) using U-visible and LC-MS spectroscopy. A spectrum scan of peak 3 from 210 to 700 nm detected the presence of at
least two to three individual compounds with maximum absorbance at 278 nm and 284 nm (Figure 2A). To provide clues as to the chemical identity of Biz-2Fr.3, LC-MS spectroscopy analysis was subjected peak 3. LC-MS because of its sensitivity, reproducibility, speed and versatility was applied. An Agilent 6210 Quadrupole LC-MS with an Agilent Ultra-2 fused silica capillary MS column both positive and in the negative ion mode was used to analyzed Biz-2Fr.3 because it provides more information about chemical structure. LC-MS chromatography analysis resulted in the identification of various compounds present in Biz-2Fr.3. The LC-MS resulted in three major peaks with retention times of 1.1, 5.8 and 7.5 min (Figure 2B). Characterization of the molecular ions at 1.1 min revealed a range of analytes with m/z ranging from 120 to 445 amu. The MS peaks at 5.8 and 7.5 min contained a single molecular ion with amu of 181 (Figure 2B.)

**In vitro growth Inhibitory properties of Biz-2Fr.3 in normal and prostate cancer cells**

To determine if there were any selectivity of anti-proliferative effects of Biz-2Fr.3 in towards prostate cancer, a panel of cell lines representing functional disease states was tested. The panel contains four androgen receptor-selected lines representing hormone-responsive (LNCaP cell), hormone-resistant (DU145), hormone refractory (PC-3) cell and normal transformed (RWPE-1) prostate cells. Cells (1E4) were grown in 96-well plates, induced with varying concentration of Biz-2Fr.3 in for 24 h and the degree of the cytotoxicity measured using the MTT assay. Biz-2Fr.3 was found to inhibit the proliferation of all tested cell lines in a concentration-dependent and time-dependent manner (Figure 3). The growth inhibitory concentration (GI50) of Biz-2Fr.3, calculated by the four-parameter logistic method, shows higher values in normal transformed prostate cells than in prostate cancer cell lines (p>0.001) implying Biz-2 possesses relatively selective cytotoxicity towards cancer cells (Figure 3). Biz-2Fr.3 was found to be 5 times more potent towards the androgen insensitive cell DU145 line as compared to the normal transform RWP-1 (GI50 of 126 ppm versus 650 ppm). The androgen responsive cell line showed a marginal sensitivity (260 ppm LNCaP verses 126 ppm DU145), whereas the hormone refractive PC-3 was unaffected by Biz-2Fr.3. To corroborate the cytotoxicity of Biz-2Fr.3, toward prostate cancer cells, its growth inhibitory activity in MCF-7 was examined which possesses a functional AR receptor. Growth of MCF-7 cells in the presence of Biz-2Fr.3, resulted in little to no toxicity. The GI50 after 24 and 48 h was 600ppm, similar to that observed in the normal untransformed prostate cells. Thus, Biz-2Fr.3 produces a significant increase in its cell inhibitory activity in DU145 prostate cells implicating that Biz-2Fr.3 possesses relatively selective cytotoxicity towards cancer cells.

**Inhibition of cell cycle progression by Biz-2Fr.3**

Observing that Biz-2Fr.3 elicited a greater anti-proliferative effect in DU-145, it was used as a model to examine the mechanism of Biz-2Fr.3 anti-proliferative activity. PI staining coupled to flow cytometry was used to determine where in the cell-cycle Biz-2Fr.3 induced proliferation blockage. Analysis of untreated, synchronized DU-145 cells by flow cytometry showed that 40% of the cells were in G1/S, and 17% in the G2/M phase of the cell cycle. Upon treatment with Biz-2Fr.3, there was a significant increase in G1 cells, which was accompanied by a decrease of cells in the S phase after 24 and 36 h. (Figure 4). As summarized in Figure 4, treatment of DU145 cells with Biz-2Fr.3 for 48 h resulted in a significantly higher number of cells in the G1 phase at the concentrations used, 250 ppm (72%), compared with non-Biz-2Fr.3 treated control (41%). These experiments suggested that Biz-2Fr.3 induces G1-phase cell cycle arrest in DU145 cells. When these experiments were performed in LNCaP, we observed an accumulation of cells in the G2/M phase of the cell cycle (data not shown). Next, Biz-2Fr.3 inhibition of cell cycle regulatory proteins in prostate cancer cells was examined. Considering the essential role of cycling D/p27 in the G1/S transition, it was investigated whether the Biz-2Fr.3 induced accumulation of cells in G1 phase in the DU-145 was a direct modulation of cyclin D expression. To test this hypothesis, the protein expression levels of cycling D1 in DU145 prostate cancer cell lines following exposure to 250 ppm Biz-2Fr.3 for 36 h was measured. As observed low levels of cyclin D1 in DU145, was unaffected by Biz-2Fr.3 treatment. There was an increase in cycling D1 levels in LNCaP and a decrease in its level in PC-3 cells following Biz-2Fr.3 treatment (Figure 5). Since the AR functions as a driver of G1 progression through cross-communication with the cell cycle machinery and regulation of transcription of genes that control the G1-S transition the effect of Biz-2Fr.3 on AR levels was examined. Analysis of AR levels suggested that Biz-2Fr.3 regulation of cycling D1 levels might be dependent on AR levels since there was an apparent increase in its protein levels following Biz-2Fr.3 exposure (Figure 5). In DU-145 cells (which contains a hormone insensitive AR) the levels of AR was inhibited in the presence of Biz-2Fr.3. Taken together, these results suggest that AR is involved in Biz-2Fr.3 inhibition of prostate cancer cells.

**Body weight changes in mice fed Biz-2Fr.3 for 21-days**

Given that Biz-2Fr.3 has the potential to inhibit prostate
cancer cell growth, the pharmacological potential of the extract and its safety in C57BL/6 mice was evaluated. The effect of Biz-2Fr.3 on the hematology, kidney and liver profile of C57BL/6 mice was investigated. Acute
toxicity study (LD50) of Biz-2Fr.3 was performed in 8 week old B57BL/6 mice using doses as high as 500 mg/kg. Animals were gavaged daily with 0, 10, 100 or 200 mg/kg body weight and changes in body weight, signs of toxicity, mortality, and general behaviors were observed. As observed no signs of toxicity or mortality up to the dose of 500 mg/kg was seen. However, there were partial decreases in activity and increase in respiratory rates in mice receiving 200 and 500 mg/kg of Biz-2Fr.3. The changes in body weight of mice over 21 days following oral gavage of Biz-2Fr.3 are shown in Figure 6. There was a significant increase in body weight in the treatment groups as compared to the control (Figure 6 (control, 22.22±0.117 g versus 200 ppm Biz-2Fr.3, 24.24±0.20 g; p=0.0001). The increase in overall body weight was more pronounced in the low dose group. The effect of Biz-2Fr.3 on organ reproductive and detoxification tissues were examined in mice after a 21-day treatment as shown in Table 1. We observed no significant (P>0.05) difference in the weight of the liver, kidney or brain of mice after Day 21. However, there were a significant (P<0.05) increase in wet weight of urogenital sinusus (control, 135.11 ug ± 5.12 g versus Biz-2Fr.3, 176.09 ± 25.20 g ug) after correcting for changes in body weight in the treatment group.

Clinical biochemistry of mice exposed to Biz-2Fr.3 for 21-days

There were no consistent significant differences in serum profiles between treated and control animals following Biz-2Fr.3 exposure. Table 2 shows the effect of Biz-2Fr.3 on hematological parameters in mice. After Day 21, there was a significant (P<0.01) effect on the total WBC in the treated group compared to the control group. There was a two-fold increase in the WBC (21.1±0.4 control versus 6.20±2.10) at the high doses of Biz-2Fr.3 throughout the study. There was also no significant (P>0.05) effect on mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), or mean corpuscular haemoglobin concentration (MCHC) in the treated groups as compared with the control group. However, there was a significance decrease in the platelet levels between the control group and treatment group. Administration of 100 mg/kg Biz-2Fr.3 for 21 days resulted in a 29% decrease in platelets (Control 1354 103 uL⁻¹ versus 953 103 uL⁻¹ in treatment group).
Figure 4. Time dependent effect of Biz-2fr.3 on cell cycle progression of du-145 human prostate cancer cells. Cells were cultured in complete medium and treated either with vehicle (0.5% DMSO in medium) or 250 ppm of Biz-2Fr.3. After the indicated time of treatment, cells were harvested, washed with cold PBS buffer, and digested with RNase. Cellular DNA was stained with propidium iodide and flow cytometric analysis was done to determine the cell cycle distribution as described in the materials and methods.

Biochemical profile of kidney and liver of mice exposed to Biz-2Fr.3

The effects of Biz-2Fr.3 on kidney and liver functions are illustrated in Figure 7 after day 21 of daily gavage of Biz-2Fr.3 liver AST was significantly increased (P<0.05) in the treatment group as compared to the control (Group IV, 101.00±16.00 versus control, 47.33±1.86). There were also a significant (P<0.05) increase in ALT in Group II and IV with no significant change in the middle dose group (Group III). Alkaline phosphatase was markedly decreased in the entire treated group but not significantly (P>0.05) different from the control. There was no statistical significant (P>0.05) difference in glucose, urea, or creatinine levels.

DISCUSSION

Identification of prostate specific bioactivity

Bizzy Nut is generally recognized for its enriched caffeine
Figure 5. Regulation of G1 cell cycle regulatory proteins by Biz-2Fr.3 in DU145 Cells. The DU145 cells were cultured in complete medium and treated either with vehicle or 250 ppm Biz-2Fr.3 for 24 h, then subjected to SDS-PAGE followed by Western blot analysis, as described in materials and methods. Blot were probed with antibodies for cyclin D1, anti-AR and β-Actin.

constituents which are attributed to its reported bioactivity and therapeutic effects. In this study, solid-liquid extraction, using solvents of increasing polarity to identify and characterize medicinally relevant, putative anti-prostatic or anti–tumor compounds present in bizzy nut using a cancer cell inhibition assay screen was performed. The bizzy nut was sequentially extracted with 100% hexane, Biz-1; ether, Biz-2; acetone, Biz-3; methane, Biz-4 or water, Biz-5 using solid-liquid extraction. Our bioactivity drive isolation scheme used to isolate the tumor inhibitory activity (ether extract, Biz-2) resulted in the elimination of the major constituents of bizzy nut such as caffeine and tannin suggesting our tumor inhibitory activity is due to a unique compound. To characterize this new bioactivity, a liquid chromatography (LC) method was developed for fingerprinting and quantifying the anti-prostatic activity existing in Biz-2. Using this method, the chemical fingerprint of Biz-2Fr.3 was established, in which the separation of more than five analytes was accomplished in about 30 min, and 6 to 8 distinctive peaks were identified by LC/MS analysis. Characterization of the molecular ions re-showed the presence of the analyte, with m/z ranging from 120 to 225 m/z. Peaks two and three, on the other hand, contained a
single molecular ion with m/z of 181. The LC-MS data suggested that we have three groups of compounds in the Biz-2Fr.3.

**Mechanism of anticancer activity**

In order to systematically evaluate the toxicity of the enriched fraction of Biz-2, (Biz-2Fr.3) towards target prostate cancer cells, a cell line panel representative of different stages of prostate disease was compiled. The data presented here suggests that the mechanism of toxicity induced by Biz-2Fr.3 is dependent on the cell phenotype. Also, it was observed that prostate cancer cells were more sensitive to Biz-2Fr.3 induced toxicity as compared to breast or neuronal cells lines (data not shown). The order of sensitivity of prostate cells toward Biz-2Fr.3 was DU-145, LNCaP, PC-3 and normal prostate cells. This differential sensitivity of prostate cancer cell lines to Biz-2Fr.3 could be explained in part due to the difference in molecular characteristics of the three-different prostate cancer cell lines tested. LNCaP cells with hormone-sensitive and p53-wild type confer sensitivity to Biz-2Fr.3. DU145 is resistance to Biz-2Fr.3 and is hormone-insensitive and has a p53-mutant. It will be investigated whether these phenotypes and other factors contribute to the mechanism of action of Biz-2Fr.3 in prostate cancer cells. Prostate cancer development is dependent on androgens, and majority of patients respond to androgen ablation. However, virtually every patient will develop hormone-resistance prostate cancer and can no longer respond to androgen deprivation therapy (ADT). Therefore, the need to identify chemo preventative compounds that does not result in androgen resistance is urgently needed. In this study, it was demonstrated that our Biz-2Fr.3 bioactive extract is more potent to androgen insensitive DU145, suggesting that this extract may be developed as a treatment hormone-resistant prostate cancer. To begin to understand the mechanism of Biz-2Fr.3 anti-proliferative activity in DU-145 cell, flow cytometry was used to determine where in the cell-cycle Biz-2Fr.3 induces proliferation blockage. Biz-2Fr.3 was able to induce a G1 blockage in a dose and time dependent manner. Control of cell cycle progression in PCa and other cancer cells is considered to be a potentially effective strategy for the control of tumor growth (Endrini et al., 2011; Osterburg et al., 2009). Molecular analysis of human cancers has revealed that cell cycle regulators are frequently mutated in most common malignancies and natural product
Figure 7. Biochemical profile of mice exposed to chronic consumption of Biz-2Fr.3. Mice were dosed orally with 0, 10, 200 ppm Biz-2Fr.3 daily for 21 days. At the end of the treatment period, blood was collected by cardiac puncture and used to prepare serum for the biochemical tests. The values of blood glucose (GLU), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), serum urea and serum creatinine were determined following standard laboratory procedures. * Significantly different at p<0.05 compared to the control.

...tended to target the cell cycle pathway (Gioti and Tenta, 2015; Gunn et al., 2011; Rafi et al., 2002). The evaluation of ancient herbal medicines such as bizzy nut may indicate novel strategies for the treatment of prostate cancer, which remains the leading cause of cancer--related deaths in American men. In our present investigation, it shown that a naturally occurring specific bioactive fraction of bizzy nut significantly inhibits the proliferation and reduces the viability of DU145 as well as LNCaP cells (Figure 1), which suggests that Biz-2Fr.3 may be an effective chemotherapeutic agent against both androgen-sensitive and androgen-insensitive prostate cancer cells. There is significant global exposure of humans to bizzy nut (usually an ethanolic extract of the nut) in the form of a flavoring ingredient and food coloring (Burdock et al., 2009; Agency’s, 2011). In addition, oral exposure can be dated back to the late 19th century with no documented adverse side effects. Studies in animals on the effects of chronic (28 days) consumption of Bizzy nut and its active major constituent caffeine have been investigated in mice. These studies report that the chronic consumption of Bizzy nut and caffeine diets caused an apparent toxicity as side effects from a decrease in food intake and body weight and the observed effects are largely due to the high caffeine content (Umoren et al., 2009; Salahdeen et al., 2015; Moradi et al., 2016). Humans have consumed plant phytochemicals for an extensive period and so they are perceived to be reasonably safe. However, our phytochemical enrichment scheme may have concentrated unwanted chemical that may pose safety issues. Furthermore, Bizzy has been consumed in Jamaica for century without any reported side effect. Given that bizzy nut may have multi--targeting properties along with relatively lower systemic toxicity, the compounds in Biz-2Fr.3 can offer significant therapeutic advantages for prevention and treatment of PCa in Jamaica (Lowe et al., 2014; Jamaica, 2006; Mitchell and Ahmad, 2006). Since that Biz-2Fr.3 has the potential to inhibit prostate cancer cell growth, the pharmacological potential of the extract and evaluate its safety in mice was evaluated. To examine whether Biz-2Fr.3 administration induces toxicity, for 5-week C56B mice with Biz-2Fr.3 for 21 days was gavage-fed. Biz-2Fr.3 administration (at 10 or 200 mg/kg body weight) produces a slight increase in body weight (Figure 6). At the time of sacrifice (at 8 weeks of age), there was no considerable difference in organ (liver, lung, kidney, and spleen) weights between Biz-2Fr.3 and control groups (Table 1). Additionally, the levels of creatinine ALP and alanine aminotransferase (ALT) in the sera were not increased by Biz-2Fr.3 administration (Figure 7). However, the activity of aspartate aminotransferase (AST) was significantly increased relative to the control.
Table 1. Relative organ weight of mice in the presence of Biz-2Fr.3 over a 21 day period.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Group I (0.5% DMSO)</th>
<th>Group II (Biz-2Fr.3 10 mg/kg)</th>
<th>Group III (Biz-2Fr.3 100 mg/kg)</th>
<th>Group IV (Biz-2Fr.3 200 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>378.33±13.31</td>
<td>372.75±51.142</td>
<td>450.25±34.55</td>
<td>344.25±57.55</td>
</tr>
<tr>
<td>Liver</td>
<td>1185.75±74.90</td>
<td>1364.14±100.94</td>
<td>1181.25±93.75</td>
<td>1312.16±72.01</td>
</tr>
<tr>
<td>Kidney</td>
<td>275.11±9.64</td>
<td>321.25±36.62</td>
<td>304.75±29.15</td>
<td>305.28±26.52</td>
</tr>
<tr>
<td>Prostate</td>
<td>135.33±5.50</td>
<td>204.25±55.37a</td>
<td>199.75±7.32a</td>
<td>176.09±25.62a</td>
</tr>
<tr>
<td>Stomach</td>
<td>366.51±43.13</td>
<td>294.25±25.78</td>
<td>316.33±70.46</td>
<td>335.52±14.15</td>
</tr>
</tbody>
</table>

Values are represented as mean ±SD of quintuple expressed in mg. Superscript letters differ significantly (aP<0.05) from the control.

Table 2. Hematological analysis of mice exposure to Biz-2Fr.3.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Control, 0.2% DMSO)</th>
<th>Group II (Biz-2Fr.3 10 mg/kg)</th>
<th>Group III (Biz-2Fr.3 100 mg/kg)</th>
<th>Group IV (Biz-2Fr.3 200 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (103/μL)</td>
<td>2.1±0.4</td>
<td>5.4</td>
<td>5.06±0.71</td>
<td>6.20±2.10</td>
</tr>
<tr>
<td>RBC (106/μL)</td>
<td>7.54±0.29</td>
<td>8.51±0.48</td>
<td>8.36±0.41</td>
<td>6.77±0.93</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>11.10±0.50</td>
<td>12.66±0.73</td>
<td>9.2±3.8</td>
<td>10.15±4.30</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>38.80±1.30</td>
<td>42.13±2.20</td>
<td>41.76±1.58</td>
<td>35.30±0.85</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>51.40±0.30</td>
<td>49.50±0.25</td>
<td>49.96±0.61</td>
<td>52.25±0.30</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>14.75±0.05</td>
<td>14.86±0.08</td>
<td>15.00±0.15</td>
<td>15.00±0.30</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>28.65±0.35</td>
<td>30.00±0.20</td>
<td>30.06±0.27</td>
<td>28.75±0.05</td>
</tr>
<tr>
<td>CHCM (g/dL)</td>
<td>26.05±0.25</td>
<td>27.33±0.08</td>
<td>27.10±0.27</td>
<td>25.95±0.35</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>13.25±0.35</td>
<td>12.70±0.15</td>
<td>14.00±0.23</td>
<td>14.00±0.20</td>
</tr>
<tr>
<td>PLT (103/μL)</td>
<td>1354.00±211</td>
<td>1145.00±10.69a</td>
<td>953.00±68.87b</td>
<td>1125.50±203a</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>6.00±0.20</td>
<td>6.10±0.25</td>
<td>5.86±0.12</td>
<td>7.45±1.35</td>
</tr>
</tbody>
</table>

Values are represented as mean ±SEM of triplicates. Values on the same row followed by superscript letters differ significantly (a, P<0.01; b, P<0.001) from the control. WBC, differential leukocyte count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume, MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, Red cell distribution width; PLT, Platelets; MPV, Mean platelet volume.

As AST is not a liver specific enzyme a high level of this enzyme can also be released from skeletal and cardiac muscle or red blood cells (Etuk and Muhammad, 2010). These results indicate that the chronic administration of Biz-2Fr.3 (10 or 200 mg/kg/day) was not toxic to the kidney or liver in mice. In the present study, the inhibition of prostate cancer cell growth and cell cycle progression are observed at 120 ppm Biz-2Fr.3 concentrations. It is hard to predict whether such concentration of Biz-2Fr.3 was achievable in the in vivo studies in the absence of pharmacokinetic data. Nonetheless, the Biz-2 concentrations used in the present study are within the range employed in previous studies to document cellular effects of this natural product (Salahdeen et al., 2015). In summary, the present study identified a bioactive component of bizzy nut that suppresses the growth of androgen--responsive (LNCaP) as well as androgen-independent (DU145) human prostate cancer cells in association with G1 phase cell cycle arrest. The Biz-2Fr.3-mediated cell anticancer activity is not associated with any sign of toxicity in normal prostate cells or in mice. Collectively, these results suggest that the Biz-2Fr.3 fraction of bizzy nut should be seriously considered for further investigation to determine its possible chemopreventive and/or therapeutic efficacy against prostate cancer in humans. A sample of finely ground bizzy root was sequentially extracted with solvents of increasing polarity and the resulting ether extract (Biz-2) was fractionated on a reversed phase high performance liquid chromatography (RP-HPLC) (A). Growth-inhibitory activity (GI50) of each fraction were determined (B) and spectra of each peak was monitored from 190 to 400 nm to identify the number of putative bioactive compounds in each peak (C).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES


