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Boesenbergin A from *Beosenbergia rotunda* induces an oxidative stress dependent cell death via the mitochondrial pathway in HepG2 cells

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Abstract: Background: *Boesenbergia rotunda* is a local edible plant predominantly consumed in Asia. Its consumption is on the increase as it is widely believed to improve health and also heal kidney diseases and cancer among others. However, the detailed mechanism of action of the plant in the treatment of cancer is yet to be fully explored. **Objective**: The present study focused on the use of boesenbergin A from *B rotunda* in the management of liver cancer as well as possible mechanisms through which it induces apoptosis in liver cancers (HepG2 cells). **Method**: Cell viability, acridine orange/propidium iodide, annexin V assay and ROS assays were performed to evaluate the cytotoxic effects of Boesenbergin A in HepG2 cells. In addition, cell cycle assay was done to determine Boesenbergin A-induced cell cycle arrest while caspases 3 and 9 assays were done to investigate the involvement of the mitochondria in Boesenbergin A-induced apoptosis in HepG2 cells. **Results**: We observed that beosenbergin A induces a G0/G1 cell cycle arrest in HepG2 cells and also induced apoptosis directly proportional to the dose of boesenbergin A treatment. The fluorescent micrographs revealed that boesenbergin A-induced cell death in HepG2 cells occur via apoptosis. In addition, boesenbergin A treatment significantly increased the level of caspase 9 and ROS HepG2 cells which suggest mitochondrial involvement in the cell death. **Conclusion**: Boesenbergin A was used successfully to treat HepG2 cells which demonstrates its potential use for the chemotherapy of liver tumors using natural compounds. The study also justifies the increase consumption of *B. rotunda* in local Asian diets for therapeutic benefits.

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Keywords – Liver cancer, HepG2 cells, boesenbergin A, *B. rotunda*, apoptosis, oxidative stress

Abbreviations

RPMI – Rosewell Park Memorial Institute, HepG2 – liver hepatoblastoma cells, DNA - Deoxyribonucleic acid, DMSO – Dimethyl sulfoxide, JNK – c-Jun N-Terminal Kinases, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide, SAPK – stress activated protein kinase, Apaf-1 – Apoptotic protease activating factor-1

1.0 Introduction

Liver cancer is the third most common cause of cancer death in the world that occurs predominantly in sub Saharan Africa and Asia. Liver cancer appears commonly as hepatoblastoma, cholangiocarcinoma, hemangiosarcoma and hepatocellular carcinoma (HCC) (Jemal et al., 2009). Amongst all these forms, hepatocellular carcinoma is the commonest accounting for between 70 to 80% of liver cancer cases. Severe viral infections such as HBV and HCV as well as aflatoxin B consumption are major risk factors while binge alcohol consumption, hemochromatosis and obesity are minor risk factors associated with liver cancer with chronic HBV and HCV infections

accounting for over 80% of the diagnosed HCC cases (Ferlay et al., 2010; Nordenstedt et al., 2010). Globally, the burden of liver cancer is increasing at an alarming rate and this portends great danger because the current therapeutic options of liver cancer are limited and survival after diagnosis is poor (Bakiri and Wagner, 2013). Therefore, the use of natural plant products is seen as a way to complement the current treatment measures in a bid reduce the prevalence of liver cancer especially in developing countries where it is difficult to manage the disease. Basically, plants are cheap and readily available making them suitable alternatives to the conventional treatments in middle and low-income countries.

Zingiberaceae is a family of local herbs commonly used in Asian folk medicine. South-east Asians use the fresh rhizomes of *B. rotunda* as food spices or condiments (Chan et al., 2008). In Malaysia, *B. rotunda* is locally known as 'Temu kunci' while in Thailand, it is known as 'krachai'. *Boesenbergia rotunda*, is the most common member of the *Zingiberaceae* (Bhamarapravati et al., 2006; Ching et al., 2007) and it has been associated with numerous pharmacological activities. The plant has been used by traditional healers in the treatment of leukorrhea, inflammation, dry mouth, ulcer, cancers, and kidney diseases (Morikawa et al., 2008). In addition, the plant is used as an aphrodisiac (Bhamarapravati et al., 2006; Ching et al., 2007; Kiat et al., 2006; Sudwan et al., 2007; Tuchinda et al., 2002). Boesenbergin A, pinostrobin, chromene are the major bioactive compounds to have been isolated from the rhizomes of *B. rotunda*. Boesenbergin A is a chalcone with two aromatic rings containing an unsaturated chain (Figure 1) and has been used in several bioassays (Isa et al., 2012).

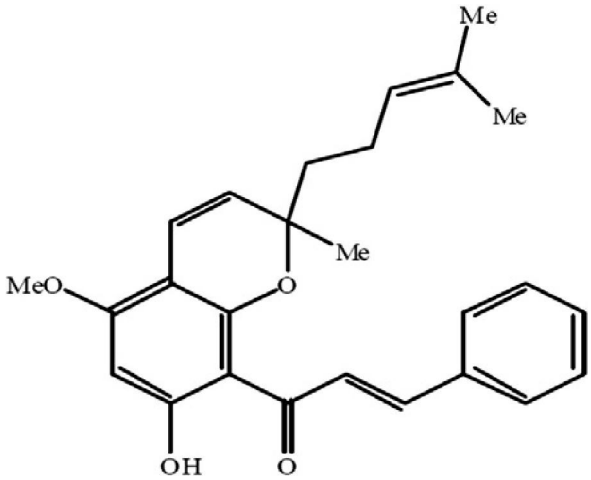

Figure 1: Structure of Boesenbergin A

For instance, a study conducted on lung cancer cells revealed the pure compound (boesenbergin A) to induce apoptosis via extrinsic and intrinsic pathways as well as decrease the levels of HSP70 (Isa et al., 2013). Similarly the anti-cancer, anti-inflammatory and antioxidant properties of boesenbergin A has been demonstrated on a panel of cancer cells (Isa et al., 2012). However, an in-depth understanding of the compound that includes its mechanism of action is a prerequisite to develop the pure boesenbergin A as an anticancer drug to be used for the treatment of liver cancer. Therefore, the current study focused on evaluating the cytotoxic properties of boesenbergin A. For the first time, we investigated the possible mechanisms through which boesenbegin A induced apoptosis in liver cancer (HepG2 cells).

2.0 Methods

2.1 Plant material collection.

The pure compound, boesenbergin A used in this study is a gift from Prof Aspollah M. Sukari of the department of Chemistry, University Putra, Malaysia (Isa et al., 2012).

2.2 Cell viability assay

Human hepatocellular carcinoma (HepG2 cells) was obtained from American Type Culture Collection (ATCC, Va, USA). The cells were maintained in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and grown in an incubator at 37^{o}C under 5% CO_2. About 5000 cells were seeded into each well of a 96 well plate for overnight incubation, followed by treatment with increasing concentrations of boesenbergin A and doxorubicin (positive control). After 72 hours, MTT (5mg/ml) was added for the viability assay as described previously by Syam (Syama et al., 2013). Results obtained were expressed as percentage cell viability of control after 72 hours exposure to test agents.

2.3 Morphological assessment of apoptotic cells by acridine orange (AO) propidium iodide (PI) double staining

Boesenbergin A induced cell death in HepG2 cells was monitored using acridine orange (AO) and propidium iodide (PI) double-staining according to standard procedures. Cells were seeded in a 6 well plate (10^5 cells/well) and incubated overnight. The cells were treated for 24 hours with 5, 15 and 30 µg/mL of boesenbergin A after which the cells were harvested, washed twice with PBS by centrifugation at 1000 x *g* for 5 min at 4^{o}C. The cell pellets were collected and 10 µL each of AO (10 mg/mL) and PI (10 mg/mL) were to the pellets in a 1.5 mL tube and mixed to form a suspension. Freshly stained cell suspension was dropped onto a glass slide and covered with a cover slip. Slides were observed under

fluorescent microscope within 30 min before the fluorescence fades.

2.4 Cell cycle analysis by flow cytometry

The HepG2 cells were seeded at a concentration of 10^6cells/T-25ml flask in RPMI media and incubated overnight. The cells were treated with 5, 15, 30 and 50 µg/mL of Boesenbergin A while the negative control cells were treated with 0.1% (v/v) DMSO both for 24 hours. After treatment, cells were harvested and washed with PBS. The cell cycle assay was performed using BD cell cycle reagent (CycleTest™ Plus DNA reagent kit, Becton Dickinson, Belgium) according to manufacturer's protocol and the result was analyzed on a flow cytometer.

2.5 Annexin V assay

HepG2 cells were seeded at a concentration of 2 x 10^5cells/T-25 flask in RPMI media and incubated overnight. The cells were treated with 5, 15, 30 and 40 µg/mL of Boesenbergin A while the negative control cells were treated with 0.1% (v/v) DMSO both for 24 hours. Cells were harvested after treatment and washed with PBS by centrifugation at 1000 x *g* for 5 min at 4°C. Annexin V assay was then carried out using FITC annexin V assay kit (BD Pharmingen, USA) following the manufacturer's protocol and the result was analyzed on a flow cytometer.

2.6 Measurement of reactive oxygen species

Total reactive oxygen assay was performed using Total ROS assay kit 520nm (ebioscience Inc, Affymetrix) following manufacturer's protocol. Cells were initially seeded in a 6 well plate at a density of 10^6 cells/well overnight. The cells were treated for 24 hours with 5, 15 and 30 µg/mL of boesenbergin A for 24 hours. Negative control cells were treated with 0.1% (v/v) DMSO for 24 hours. After the completion of reaction, the cells were analyzed on a flow cytometer.

2.7 Caspases 3 and 9 assays

Caspase 3 and 9 activities were determined using the colorimetric method (Genescript Colorimetric Assay kit, USA). Cells were initially seeded in a 6 well plate at a concentration of 10^6 cells/well overnight. The cells were treated for 24 hours with 5, 15, 30, 40 and 50 µg/mL of boesenbergin A while negative control cells were treated with 0.1% (v/v) DMSO for 24 hours. After the treatment period, Caspases 3 and 9 assays were performed separately according manufacturer's protocol. The absorbance of samples were read using a microplate reader at 405 nm and the results were analyzed.

2.8 Statistical analysis

Data obtained are presented as mean ± standard deviation and one-way analysis of variance (ANOVA) on SPSS software (SPSS Inc, USA) was used to determine the significant level means ($p<0.05$).

3.0 Results

3.1 Cell viability assay

The result in Figure 2 shows the percentage (%) cell viability of HepG2 cells after treatment with increasing concentrations of Boesenbergin A. The cell viability assay shows that like many other potent natural products, boesenbergin A has a very low IC_{50} (6.9 µg/mL) at 72 hours.

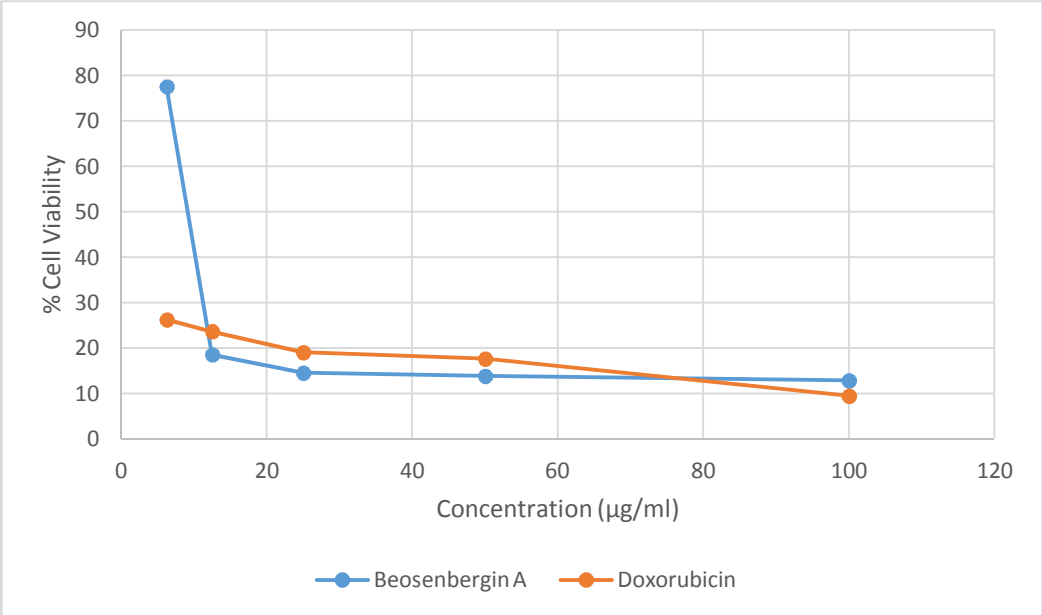

Figure 2: Percentage viability of Hepg2 cells after treatment with increasing concentrations of boesenbergin A for 72 hours. Analyses were performed in triplicates.

3.2 Fluorescence microscopy

AO and PI are intercalating nucleic acid-specific fluorochromes which emit green and orange fluorescence, respectively, when they are bound to DNA. Of the two, only AO can cross the plasma membrane of viable and early apoptotic cells. The criteria for identification are as follows: (i) viable cells appear to have green nucleus with intact structure; (ii) early apoptosis exhibit a bright- green nucleus showing condensation of chromatin in the nucleus; (Twelves et al.) dense orange areas of chromatin condensation shows late apoptosis and (iv) orange intact nucleus depicting secondary necrosis (Ciapetti et al., 2002). The untreated group (Fig 3A) contained more viable cells as shown by the blue arrows. Apoptosis and cell membrane blebbing was more evident in the treatments (Fig 3B, C and D) as depicted by the yellow and purple arrows respectively. Similarly secondary necrosis was evident in Figure 3D.

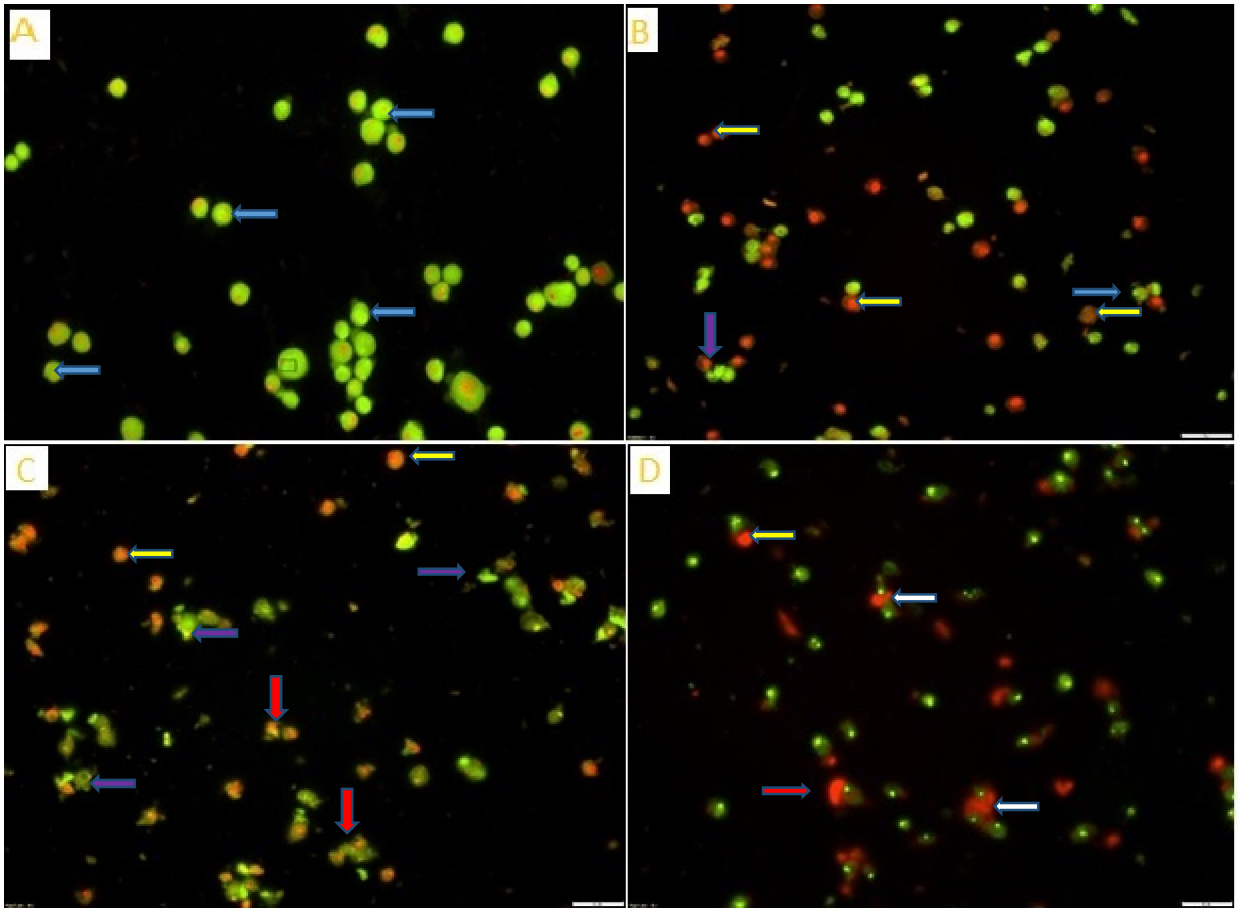

Figure 3: Fluorescent micrographs of HepG2 cells stained with acridine orange (AO) and propidium iodide (PI) after treatment with Boesenbergin A. (A – untreated cells, B, C and D are treatments with 5, 15 and 30 µg/mL of Boesenbergin respectively; blue arrows – viable cells, yellow arrows – dead cells, green arrows - chromatin condensation, red arrows – early apoptosis, white arrows – secondary necrosis, purple arrows - membrane blebbing). Results are representative of one of three independent experiments.

3.3 Annexin V assay

The annexin V assay histogram shows the distribution of cells in different quadrants (lower left quadrant shows the live cells, the lower right – early apoptotic cells, upper right – late apoptosis, upper left – necrosis) (Fig 4). The population of viable cells decreased significantly ($p<0.05$) in the treated cells as the treatment concentration increased. Conversely, there was a significant increase ($p<0.05$) in the percentage population of apoptotic cells (early and late apoptosis) after treatment with increasing dose of boesenbergin A.

3.4 Cell cycle assay

The DNA content histogram of HepG2 cell treated with boesenbergin A is shown in Figure 5. A significant decrease ($p<0.05$) lower than the control was observed in the population of *G0/G1* cells in the groups treated with 5, 15, 30 and 40 µg/mL of

boesenbergin A respectively. In the *S* phase however, there was a significant increase ($p<0.05$) higher than the control in the population of cells with increasing dose of boesenbergin A. Also in the *G2/M* phase, a significant increase was observed only in the group treated with 30µg/ml of boesenbergin A.

The *sub G0/G1* phase represents the fraction of cells with fragmented DNA. The number of treated cells with fragmented DNA is significantly higher ($p<0.05$) than the control cells (Fig 5).

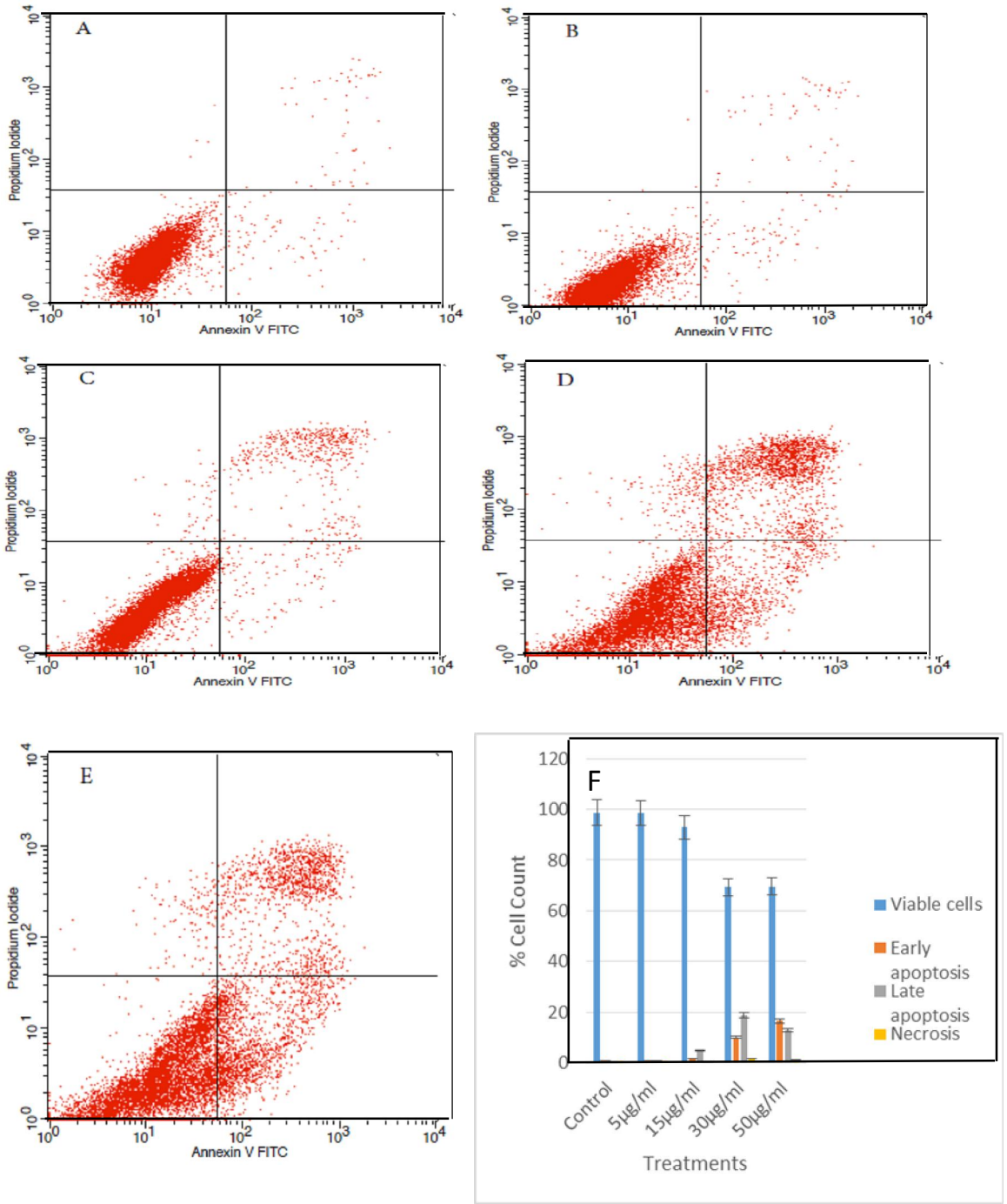

Figure 4: Annexin V assay result of boesenbergin treated HepG2 cells. A – untreated cells, B, C, D and E are 5, 15, 30 and 40 µg/mL treatments respectively. F – Percentage cell distribution in the various phases of cell death. Analyses were performed in triplicates

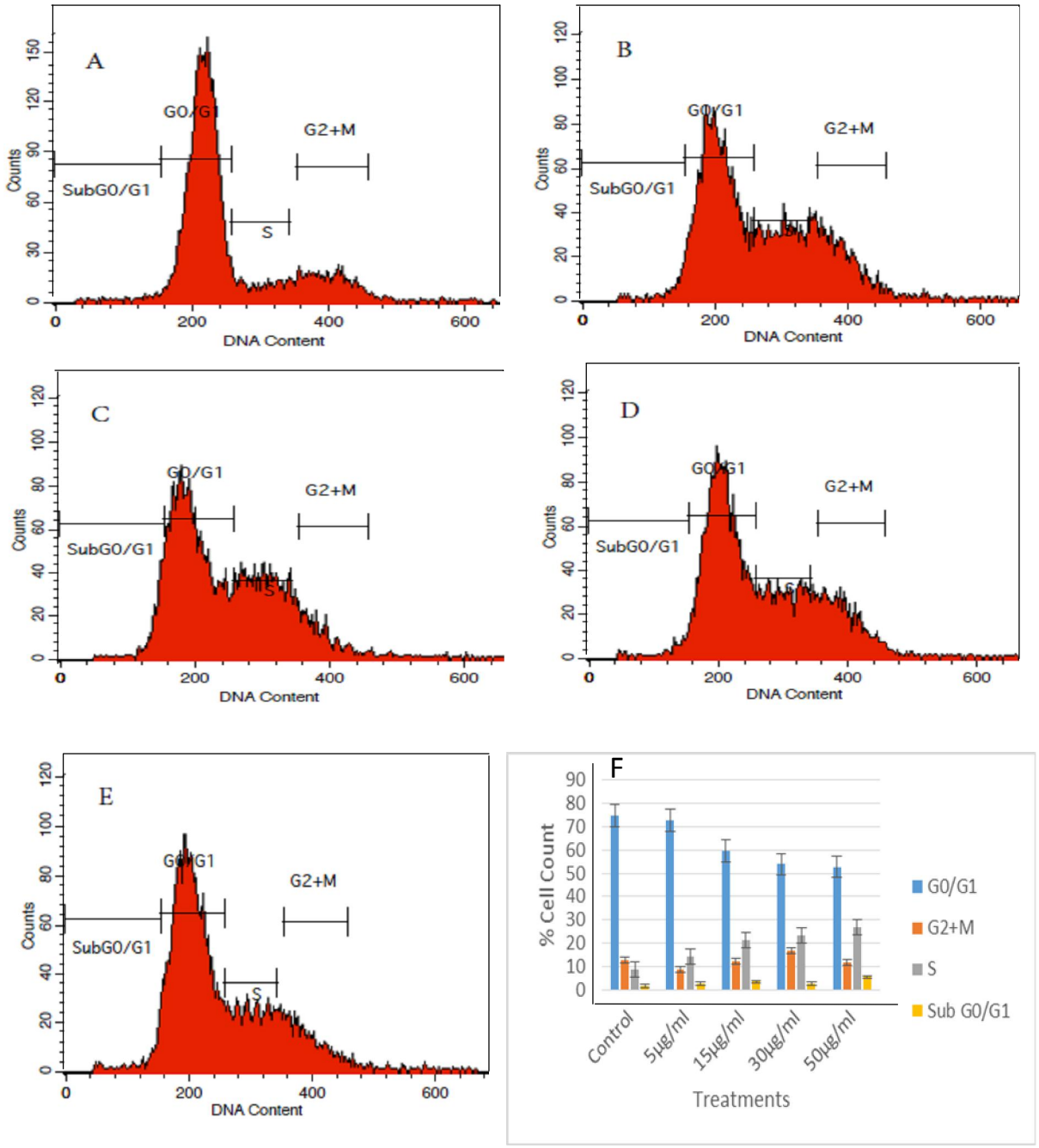

Figure 5: DNA content histogram of boesenbergin-treated HepG2 cells. A – untreated cells, B, C, D and E are 5, 15, 30 and 40 µg/mL treatments respectively. F – Percentage cell distribution in the cell cycle phases. Analyses were performed in replicates.

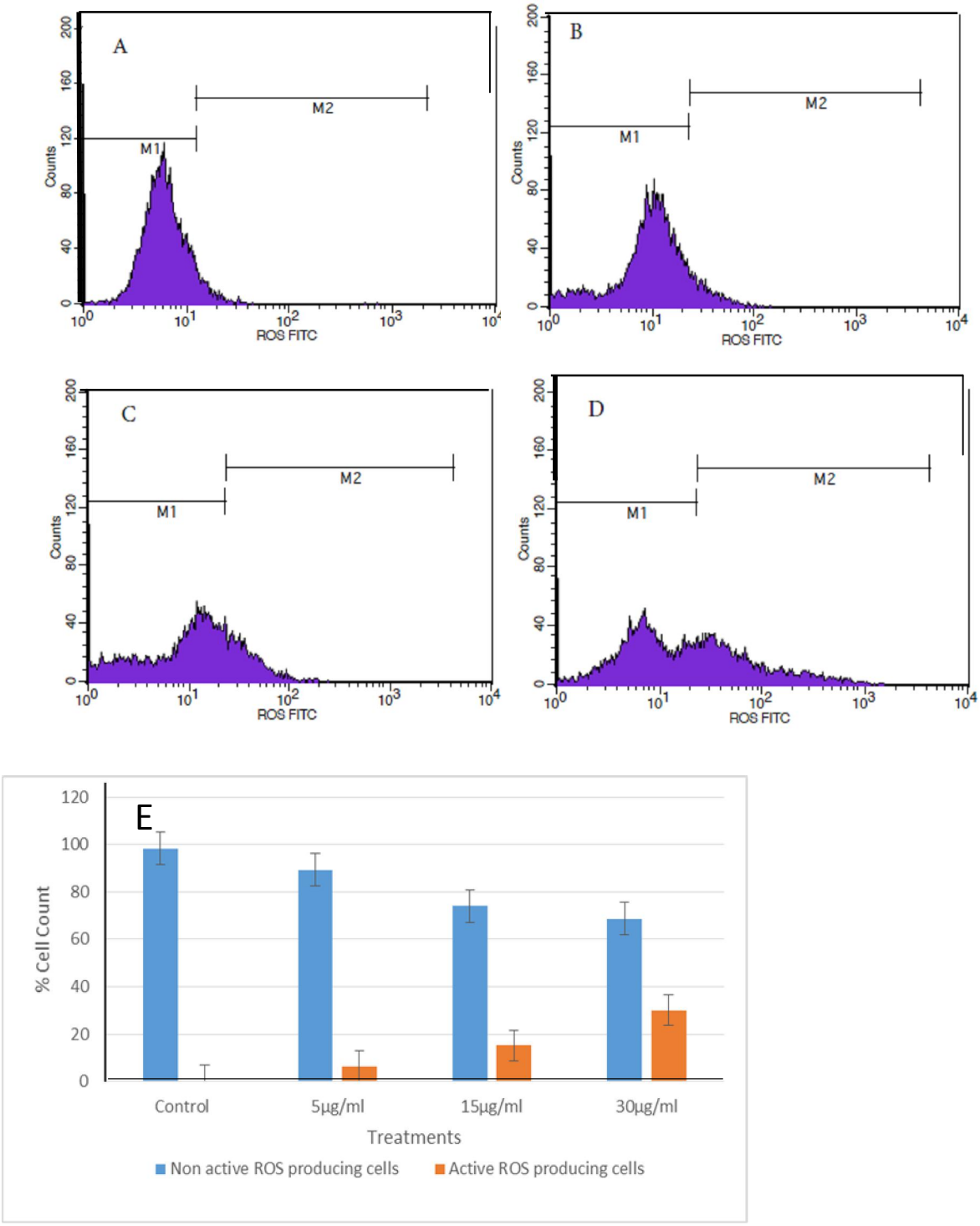

Figure 6: ROS assay result of boesenbergin-treated HepG2 cells. A – untreated (control) cells, B, C and D are 5, 15 and 30 µg/mL of boesenbergin A treatments respectively. E – Percentage cell distribution of active and non-active ROS producing HepG2 cells. Analyses were performed in triplicates

3.5 ROS Assay

The method of ROS assay used in this study only detected active and non-active ROS producing cells from the group of treated cells. The M1 column represents the non-active ROS producing cells while the M2 column represents the active ROS producing cells as shown in Figure 6. The percentage population of active ROS producing cells significantly increased ($p<0.05$) after treatment with 5, 15 and 30 µg/mL of boesenbergin A (Fig 6). Similarly the non-active ROS producing cells also decreased significantly ($p<0.05$) after treatment with 5, 15 and 30 µg/mL of boesenbergin A.

3.6 Caspases Assay

The treatment of HepG2 cells with boesenbergin A significantly increased ($p<0.05$) the activities of caspases 3 and 9 in the treated cells was observed. The activities of caspases 3 and 9 was significant at all the treatment points (Fig 7).

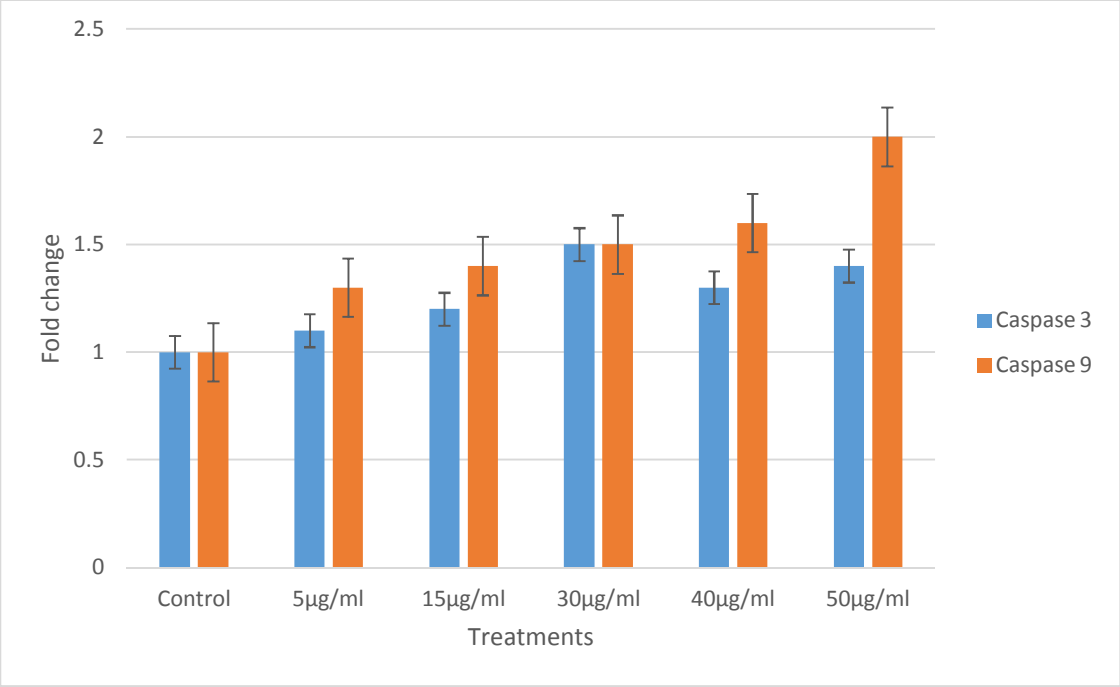

Figure 7: Caspase 3 and 9 activities after treatment with 5, 15, 30, 40 and 50 µg/mL of boesenbergin A.

4.0 Discussion

Plant secondary metabolites exert cytotoxic effects in cancer cells directly or by modulating the progression of tumors via numerous biochemical pathways (Kintzios and Barberaki, 2004; Singh et al., 2003). Boesenbergin A is a chalcone (Kaisoon et al., 2011) that belongs to the class of polyphenolic compounds with potent pharmacologic properties. We postulate that the high cytotoxicity (IC_{50} = 6.9µg/ml) of boesenbergin A may be due to the presence of the methoxy group because compounds with similar functional groups are known to be cytotoxic (Karthikeyan et al., 2015). Specifically, the α and β unsaturation found in compounds like boesenbergin A contribute to cytotoxic effects (Boumendjel et al., 2008).

Apoptosis is described as active programmed cell death. It is an integral physiological event that ends in cellular self-destruction, hence it is a novel strategy used to screen anticancer drugs (Fesik, 2005). Aside the biochemical changes that precedes apoptosis, cellular morphological anomalies like DNA fragmentation, blebbing of cell membrane, chromatin condensation, cell shrinkage and formation of apoptotic bodies are common features of apoptosis (Mohan et al., 2010). Fluorescent microscopy of boesenbergin A treated cells revealed some of these features thus confirming the occurrence of apoptosis in HepG2 cells. Another important finding is that cell death in HepG2 occurs via cell necrosis. The presence of these morphological anomalies lends credence to the virility of boesenbergin A in the induction of apoptosis in HepG2 cells. Similarly the annexin V

assays clearly distinguishes between early and late apoptotic cells. Similar mechanisms and apoptotic features had been observed in lung cancer cells treated with boesenbergin A (Syama et al., 2013). The dose-dependent treatment of HepG2 cells with boesenbergin A induced significant ($p<0.05$) apoptotic and necrotic cell death in HepG2 cells thus confirming its potency as a natural product that inhibit cancer cell growth. In a similar study conducted by Lust *et al*. (Lust et al., 2005), xanthohumol (chalcone) induced a dose dependent cell death in BCLL cells.

Inability to regulate the cell cycle leads to the development of cancer (Hartwell and Kastan, 1994). Cell cycle progression influences the mode of cell death triggered by many natural compounds (González-Sarrías et al., 2012). The regulation of cell cycle is an integral event that enables cell division (Mohan et al., 2010) and also growth of tumor cells. Boesenbergin A induced significant ($p<0.05$) *G0/G1* cell cycle arrest and cell death (*sub G0* cells) in HepG2 cells corresponding to the dose of treatment. However even with highest treatment dose in this study (50µg/ml of boesenbergin), more than 50% of the HepG2 cell population still remained in the *G0/G1* phase of cell cycle and this further affirmed *G0/G1* arrest. HTMC (2-hydroxy-2,3,4,6-tetramethoxychalcone), another chalcone from *C. pulcheriwa* also induced *G0/G1* phase cell cycle arrest in A549 lung adenocarcinoma cells(Rao et al., 2010). The induction of *G0/G1* cell cycle arrest in HepG2 cells by boesenbergin A demonstrates its ability to inhibit the growth of tumor cells.

From the result of the ROS assay, the population of non-active ROS producing cells decreased significantly ($p<0.05$) with increasing dose of boesenbergin A. However, the population of the active ROS producing cells increased significantly ($p<0.05$) with corresponding increase in dose of beosenbergin A. In other words, the dose of boesenbergin A used to treat the cells is directly proportional to the population of cells that will be induced to produce ROS. One of the ways through which intracellular ROS induces cell death is through membrane lipid peroxidation which causes the membrane to loose its symmetry(Halliwell and Gutteridge, 2007). HepG2 cells stained with AO/PI dyes after exposure to boesenbergin showed loss of membrane symmetry (Figure 3). More so, Skulache (Skulachev, 1996) and Simon (Simon et al., 2000) had reported of an association between mitochondrial derived ROS and the initiation of caspase cascades that leads to apoptosis. The mitochondria can produce ROS that stimulates apoptosis under physiologic and pathologic conditions (Jia et al., 2012). Thus, the increased production of ROS in the treated cells is presumed to have occurred via mitochondrial action.

Beosenbergin A treatment increased the activities of caspases 3 and 9 levels in the treated HepG2 cells. Caspase 9 is an upstream regulator caspase that activates the downstream caspase 3 for the execution of cell death. Mitochondrial assault leads to the release of cytochrome c that activates caspase 9, the main caspase of the intrinsic mitochondrial pathway. The significant increase in caspase 9 activity following boesenbergin treatment strongly implies that the death of HepG2 cells is via mitochondrial pathway apoptosis. Xanthohumol is a similar chalcone that induced caspases 9 and 3 dependent cell death in HCT 116 derived colon cancer cells. The compound is a potent initiator of the caspase cascade, hence its use as an anticancer agent (Pan, Becker, & Gerhäuser, 2005). Butein (another chalcone), was also found to induce apoptosis in HL60 leukemic cells via the activation of caspase 3 (Kim et al., 2001). Tumor cells are observed more frequently in the intrinsic pathway than in the extrinsic pathway because the intrinsic pathway is more sensitive (Mohan et al., 2010).

The findings of this study shows that boesenbergin A treatment of HepG2 cells triggered apoptosis via the mitochondrial pathway.

Conclusion

The cytotoxic effects of boesenbergin A in HepG2 cells was successfully evaluated in this particular study. Globally, there is more consumption of plant foods that are known to improve health. The efficacy of *Boesenbergia rotunda* was evaluated using one of its most active component, boesenbergin A in the current study which revealed the plant to be therapeutically useful for the induction of cell death in HepG2 cells. In addition, the study provides evidences which supports the involvement of the mitochondria in the cell death induced by boesenbergin A. So therefore, further in vivo and clinical studies on the potential use of boesenbergin A for the chemotherapy of liver cancer is recommended as this will enable the use of the compound as an anti-HepG2 drug.

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Conflict of interest

The authors declare that there is no conflict interest.

Supplementary data

All data are contained within the manuscript.

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