Boesenbergin A, a chalcone from *Boesenbergia rotunda* induces apoptosis via mitochondrial dysregulation and cytochrome c release in A549 cells in vitro: Involvement of HSP70 and Bcl2/Bax signalling pathways

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**ABSTRACT**

The anti-cancer effect of Boesenbergin A (BA) isolated from *Boesenbergia rotunda*, via the induction of apoptosis resulting from mitochondrial dysfunction was assessed in human non-small cell lung cancer (A549) cells. The apoptotic mechanisms of BA induction on cancer cells were studied in the present study for the first time. Nuclear stain, measuring the accumulation of sub-G\(_1\) cell population and DNA ladder were done to determine the apoptosis. Further investigations into the depletion of mitochondrial membrane potential and release of cytochrome c determined that BA treatment induced apoptosis via the regulation of the expression of pro-survival and pro-apoptotic Bcl-2 family members. The involvement of both intrinsic and extrinsic caspases (caspase 3/7, 9 and 8) were significantly increased. Moreover the role of free radicals was significantly found to be elevated with concomitant decrease in HSP70. In conclusion the results from the current study indicated BA could be a promising agent for the treatment of lung cancer.

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1. Introduction

Cancer is the number one killer in the entire world (Aggarwal et al., 2006). According to the National Cancer Registry (Lim, Yahaya, & Lim, 2003), a number of 11815 males and 14274 females, which made up a total of 26089 cancer cases, were diagnosed among all residents in Peninsular Malaysia in the year 2002. Non-small cell lung cancer (NSCLC) accounts for...
~80–85% of all cases of lung cancer (Dresler et al., 2000). Lung cancer has been listed as the most typical malignancy in Malaysia by the Ministry of Health Malaysia based on discharge and mortality in government hospitals. The discharge rate in 1998 was 19 out of 100,000 populations. There were 38,836 cancer admissions to government hospitals in 1998 and lung cancer was the most common cause of cancer admission among adult males. Based on a research conducted over 20 years in Kuala Lumpur, lung cancer was found to be the leading cause of cancer fatality in both males and females, with a total of 24% and 14% of cancer deaths in men and women, respectively, attributed to lung cancer (Lim, 2002; Rajiv, Shamir, Swee, & Beena, 2012). Although chemotherapy and radiation therapy are available to treat NSCLC, they are largely ineffective and highly toxic with a low survival rate (Carteci et al., 1993). This toxicity and resistance to the current chemotherapy made researchers focus on new drug candidates from dietary bioactive agents, targeting apoptosis, a programmed cell death, as physiological process that provides an effective, non-inflammatory way to remove redundant or damaged cells from tissues thereby securing tissue homeostasis (Martin, 2006). A multitude of signals activated by variable triggers, such as growth factors, cell–cell interactions, changing nutrient conditions, hypoxic conditions, and cytotoxic damage affect the status of the apoptotic machinery (Hanahan & Weinberg, 2011; Syam et al., 2011). Therefore, it is an effective means of treating cancer, including NSCLC (Molina, Yang, Cassivi, Schild, & Adjei, 2008).

Zingiberaceae is a family of perennial herbs. They are terrestrial, rarely epiphytic, aromatic, with fleshy tuberous or non-tuberous rhizomes, usually with tuber-bearing roots. In the plant kingdom, Zingiberaceae is known as one of the biggest families. They are widely distributed throughout the tropics, especially in Southeast Asia. In Peninsular Malaysia, the Zingiberaceae are a component of the herbaceous ground flora of the rainforest. There are approximately 150 species of ginger (Zingiberaceae) belonging to 23 genera found in Peninsular Malaysia (Hasbawi et al., 2000; Ibrahim et al., 2010). Zingiberaceae are essential natural resources that are widely used for providing many useful products such as food, spices, medicines, dyes, perfume and aesthetics to human (Srirugsra, 1999). Recent studies have shown that Zingiberaceae contains active compounds, which possess pharmacological properties such as antioxidant, anti-inflammatory and anti-cancer activities (Abdelwahab et al., 2010; Taha et al., 2010).

Boesenbergia rotunda (L.) Mansf. Kulturpfl., previously known as Boesenbergia pandurata or Kaempferia pandurata is a perennial herb of the family Zingiberaceae (Seidemann, 2005; Tuchinda et al., 2002). It is used in Thai cuisine, by the name krachai, and is also commonly called Chinese ginger. It is used as flavouring agent and eaten as a vegetable, as well as having a variety of medicinal uses including cancer (Chotcbohngchatchai, Saralamp, Jenjittikul, Pornsiripongse, & Prathanturarun, 2012; Murakami, Ohigashi, & Koshimizu, 1994). Identification of several bioactive compounds from the rhizome extract of B. rotunda has been done previously (Fahey & Stephenson, 2002; Jing, Mohamed, Rahmat, & Bakar, 2010; Tuchinda et al., 2002). The bioactive compounds consist of boesenbergin, cardamonin, pinoestrobin, pinocembrin, panduratin A and 4-hydroxypanduratin A. These compounds have been found to show several bioactivities such as antioxidant, antibacterial, antifungal, anti-inflammatory, antitumour and anti-tuberculosis activities (Kiat et al., 2006). Panduratin A, a cyclohexenylchalcone derivative, has been found to inhibit the growth and induced apoptosis of HT-29 colon cancer cells (Yun, Kwon, Mukhtar, & Hwang, 2005). This compound also showed apoptotic effect and cell cycle arrest in A549 non-small cell lung cancer; PC3 and DU145 prostate cancer cells and MCF-7 breast cancer cells (Yun, Kweon, Hwang, & Mukhtar, 2006). Previously we have found that Boesenbergin A induced a significant cytotoxicity against A549 cells (Isa et al., 2012). However, much is needed to understand further in the use of functional foods and herbal preparation in treating lung cancer, particularly the benefits and risks in using such edible plant therapy for treatment. According to NIH, A 549 cells has considered as the best research tool to analyze the efficacy of potential anti-cancer agents to devise better cancer treatments for malignancies. More over this cell line has been considered as standard research tool for cell senescence, cytokine induction, protein expression, apoptosis, and receptor-ligand interactions (Giard et al., 1973). There is no literature available on the mechanism of BA as anti-cancer agent. Moreover, apoptosis is the most frequently observed mechanism of chalcone-mediated cell death (Orlikova, Tasdemir, Golais, Dicato, & Diederich, 2011). As such the present study was undertaken to investigate the apoptosis inducing property of BA and the involvement of mitochondria in the death process.

2. Materials and methods

2.1. Plant materials

The rhizomes of B. rotunda were purchased from Puchong Market, Selangor DarulEhsan, Malaysia. The material was identified by a botanist at the Faculty of Science, University Putra Malaysia, where a voucher specimen was deposited (BR-R11-01). The isolation and identification of BA (Fig. 1) was reported by us in detail previously (Isa et al., 2012).

2.2. Cell viability assay

Cells were obtained from American Type Cell Collection (ATCC) and were maintained in 37 °C incubator with 5% CO2.
saturation. Cells were maintained in RPMI-1640 medium that is supplemented with 10% Fetal Bovine Serum (FBS). Viability assay was done using MTT assay as previously described (Chung et al., 2012). Briefly, cells (5 x 10^4 cells/ml) were treated with BA at different concentration in 96-well plate and incubated for 24 h. The colorimetric assay is measured and recorded at absorbance of 570 nm. Results were expressed as percentage of control giving percentage cell viability after 24 h exposure to test agent. The potency of cell growth inhibition for test agent was expressed as IC_{50} value.

2.3. Multiparametric high content screening assay (HCS)

A multiparametric cytotoxicity assay was performed using multiparametric cytotoxicity reagent Kit™ (Cellomics Inc., Pittsburgh, PA, USA) as described previously (Mohan et al., 2012). Multiparametric cytotoxicity Kit™ contains four fluorescent dyes, consisted of blue fluorescent Hoechst 33342; Alexa Fluor® 488 Phalloidin stain; MitoTracker® Red CMXRos; and cytochrome c primary antibody together with DyLight™ 549 Conjugated Goat Anti-Mouse IgG. These reagents detect changes in nuclear condensation, membrane permeability, mitochondrial membrane potential and the release of cytochrome c respectively. Briefly, A549 cells were seeded overnight at density of 8000 cells/well into black flat-bottomed 96-well plates (Perkin–Elmer Inc., Wellesley, MA, USA). Following treatment with different concentrations of BA (10, 20 and 50 μg/ml), fixation and staining for imaging analysis of the A549 cells were performed according to the manufacturer’s instructions. Cells treated with 0.2% DMSO and 5 μM paclitaxel were used as negative and positive controls, respectively. Plates were analyzed using Thermo Scientific ArrayScan™ VTI HCS Reader (CellomicsInc., Pittsburgh, PA, USA). This is a computerized automated fluorescence imaging microscope that automatically identifies stained cells and measures the intensity and distribution of fluorescence in individual cells. Images for each fluoroprobe were acquired at different channels using suitable filters with 20× objective at fixed exposure time. The Cell Health Profiling BioApplication software was used for image acquisitions and analysis. For each well, at least 25 fields, corresponding to at least 500 cells were automatically acquired and analyzed. All experiments were performed in triplicates. Cell average intensity (Mean) under the modified object mask within selected range in each channel was used as assay indicator, and reported as average fluorescence intensity.

2.4. Caspase assay

The assay of caspase-8, -9 and -3/7 was performed using commercial kit (Caspase-Glo® 8 assay, Caspase-Glo® 9 assay and Caspase-Glo® 3/7 assay: Promega Corporation, Madison, WI, USA). Ten thousand cells/well of A549 cells were seeded over-night in a white-walled 96 well plate. The cells were then treated with 10, 20 and 50 μg/ml of BA and incubated for 24 h while untreated cells acted as control. The 96-well plate containing cells was taken out from the incubator and allowed to equilibrate at room temperature, Fifty microliters of Caspase-Glo® Reagent (-8, -9 and -3/7) were added to each well of a white-walled 96-well plate containing 50 μl of blank, negative control cells and treated cells in culture medium. The contents of the plate gently mixed using a plate shaker at 300–500 rpm for 30 s. It was then incubated at room temperature for 30 min in the dark. The reading was taken at every 10 min for 3 h by using the luminescence microplate reader (Infinite M200 PRO, Tecan, Männedorf, Switzerland).

2.5. DNA laddering

The Apoptotic DNA Ladder Detection Kit (Chemicon International Inc., Palo Alto, CA, USA) was used for DNA extraction from cells. Briefly, cells were treated with different concentrations of BA and were collected at 24 h post-treatment. The cells were washed with PBS and centrifuged for 5 min at 500g. After removal of the supernatant, the cells were lysed by the addition of 40 μl of TE (Tris and EDTA) lysis buffer, followed by the addition of 5 μl of Enzyme A (RNase A) and incubated at 37 °C for 10 min. Five microliters of Enzyme B (Proteinase K) were added and the lysate was further incubated at 50 °C for 30 min. Five microliters of ammonium acetate solution and 50 μl of isopropanol were added and mixed well and kept at 20 °C for 10 min. The samples were then centrifuged for 10 min at 16,000g to precipitate the DNA. After washing, the DNA pellet was added with 70% ice cold ethanol, air dried, and later dissolved in 30 μl of DNA suspension buffer. The extracted DNA samples were run on a 1.5% agarose gel in Tris–acetic acid–EDTA buffer. After electrophoresis, the gel was stained with ethidium bromide (Gibco BRL, Dac-kopatts, Scotland) and band obtained was visualized with a UV light transilluminator.

2.6. Measurement of reactive oxygen species generation

The production of intracellular ROS was measured using 2',7'-dichlorofluorescin diacetate (DCFH-DA). Briefly, 10 mM DCFH-DA stock solution (in methanol) was diluted 500-fold in Hank’s Balanced Salt Solution (HBSS) without serum or other additives to yield a 20 μM working solution. After 24 h of exposure to BA the cells in the 96-well black plate was washed twice with HBSS and then incubated in 100 μl working solution of DCFH-DA at 37 °C for 30 min. Fluorescence was then determined at 485-nm excitation and 520-nm emission using a fluorescence microplate reader (Tecan Infinite M 200 PRO, Männedorf, Switzerland) (Mohan et al., 2012).

2.7. Cell cycle analysis

A549 cells at concentration of 5 x 10^4 cells/ml were cultured in RPMI 1640 (PAA, Coelbe, Germany) medium containing 10% FBS and 1% penicillin/streptomycin seeded into 25 ml culture flask (TPP Brand) and treated with BA at different concentrations (10, 20 and 50 μg/ml) for 24 h. After the incubation, the cells were spun down at 1800 rpm for 5 min. The supernatant was discarded and the pellet was washed with PBS (phosphate buffer saline) twice to remove any remaining media. To restore the integrity, fixation of cell population for flow cytometry analysis was performed. Briefly, cell pellets were fixed by mixing 700 μl of 90% cold ethanol and kept at 4 °C overnight. The cells were then spun down at 200 g for 5 min and the ethanol was decanted. After washing once with
PBS, cells were resuspended in 600 µl of PBS. Twenty five micro liters of RNaseA (10 mg/ml) and 50 µl of propidium iodide (PI) (1 mg/ml) were added to the fixed cells for 1 h at 37 °C. PI has the ability to bind to RNA molecule and hence, RNase enzyme was added in order to allow PI to bind directly to DNA. The DNA content of cells was then analyzed by flow cytometer (BD FACSCanto™ II). The fluorescence intensity of sub-G1 cell fraction represents apoptotic cell population.

2.8. Protein detection by Western blotting

2.8.1. Extraction of whole protein from the cell

A549 cells at concentration of 5 × 10⁴ cells/ml were cultured in RPMI 1640 (PAA, Coelbe, Germany) medium containing 10% FBS was seeded into 75 mm culture flask (TPP Brand) and treated with 20 µg/ml BA at different incubation period (3, 6, 12 and 24 h). After the incubation the cells were spun down at 1000 rpm for 10 min. The supernatant was discarded and the pellet was washed twice with PBS (Phosphate Buffer Saline) to remove any remaining media. Estimation of the packed cell pellet volume was done and added 20 volume of mammalian cell lysis reagent (Proteo JET, Fermentas Life sciences, Ontario, Canada) to 1 volume of packed cells. The cells were then incubated for 10 min at room temperature on a shaker (900–1200 rpm) and centrifugation was done at 16,000–20,000 g for 15 min to clarify the lysate. The resultant lysate then transferred to a new tube and stored at –70 °C until analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.8.2. Western blotting analysis

After the protein extraction, the concentration of protein in the supernatant was determined with BCA protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (20 µg protein) were separated by SDS–PAGE system. The Gel used in this study was 10% Fluorescent SPRINT NEXT GEL™ SDS–PAGE (Amresco, Solon, OH, USA) and followed the protocol suggested by the manufacturer. Briefly, 10 ml of Fluorescent Sprint NEXT GEL™ were poured in a conical tube. To this 90 µl of freshly prepared 10% ammonium persulphate and 12 µl of TEMED were added. Tightly cap the tube and gently inverted without shaking. Immediately poured the solution between the glass plates and inserted the comb. The gel was allowed to polymerize completely about 15 min and assembled in the chamber. 1·NEXT GEL™ running buffer was prepared from 20·running buffer and used to fill the chamber. Five micro litters of protein samples were dissolved in equal volume of loading buffer and loaded into the gel prior to running with a constant voltage of 275 V until tracking gel dye reaches the bottom of gel. The electrophoresis apparatus (Bio-Rad, Hercules, CA, USA) were disassembled and allowed gel to cool briefly before removing from the plates. The

| Table 1 – Cytotoxicity of BA on different cancer and normal cells in vitro. |
|-----------------------------|-----------------------------|-----------------------------|
| **Cell line**               | **Origin of cells**         | **IC<sub>50</sub>**          |
| MCF-7                       | Oestrogen receptor-positive breast adenocarcinoma cells | 42.40 ± 2.23                |
| MDA-MB-231                  | Oestrogen receptor-negative breast adenocarcinoma cells | 28.00 ± 3.60                |
| A 549                       | Adenocarcinomic human alveolar basal epithelial cells | 20.22 ± 3.15                |
| HepG2                       | Liver hepatocellular cells  | 20.31 ± 1.34                |
| HT 29                       | Human colon adenocarcinoma cells | 94.10 ± 1.19                |
| B16-F10                     | Murine melanoma cells       | 25.22 ± 2.04                |
| CCD841                      | Normal colon epithelial cell line | 61.0 ± 4.91                |

IC<sub>50</sub> values were obtained from MTT assay. Data are reported as means ± SD for measurements in triplicate.

Fig. 2 – Representative images of A549 cells treated with control (0.2% DMSO) and 20 µg/ml of BA, and stained with Hoechst for nuclear, cell permeability dye, mitochondrial membrane potential dye and cytochrome c. The images from each row are obtained from the same field of the same treatment sample. A549 cells produced a marked reduction in mitochondrial membrane potential, and marked increases in membrane permeability and cytochrome c (magnification 20×).
proteins were then transferred to an Immobilon-P blotting membrane (Millipore, Bedford, MA, USA) using SEMI-PHOR Transfer machine (Hoefer scientific instruments, San Francisco, CA, USA). Spectra™ Multicolor Broad range Protein Ladder (Fermentas Life sciences, Ontario, Canada) was used for monitoring the protein migration during SDS–PAGE and to check the protein transfer efficiency. Detection of protein in western blot was done by using SNAP id protein detection system (Millipore, Bedford, MA, USA) as described by the manufacturer protocol. The membrane was firstly blocked with blocking buffer (DBT Buffer with 0.1% v/v Tween 20; Amresco Western max™, Solon, OH, USA), and then incubated with primary antibody for 10 min and emptied completely with vacuum. Then the membrane was washed three times with washing buffer (DBT Buffer with 0.1% (v/v) Tween 20; Amresco Western max™, Solon, OH, USA) with vacuum on. With vacuum off, the secondary antibody (Horseradish Peroxidase conjugated goat anti-IgG, Amresco Western max™, Solon, OH, USA) was added to the membrane and incubated for 10 min, and emptied completely with vacuum. Finally, the membrane washing step was repeated 3 times with washing buffer. The immunoreacted proteins were detected using a chemiluminescene system (ECL Westernblot substrate, Abcam, England). Antibodies to Bax, Bcl2, Hsp70 and B-actin were purchased from Santa Cruz Biotechnology, USA.

2.9. Statistical analysis

Results were reported as mean ± SEM for at least three analyses for each sample. Normality and homogeneity of variance assumptions were checked. Statistical analysis was performed according to the SPSS-16.0 package and GraphPad prism 3.0. Analyses of variance were performed using the ANOVA procedure.

Fig. 3 – Changes in total nuclear condensation (A), membrane permeability (B), mitochondrial membrane potential (C) and cytochrome c localization (D) were measured simultaneously in A549 cells. Following treatment with BA, statistically significant nuclear condensation, increased cell permeability, decrease of mitochondrial membrane potential and cytochrome c release from mitochondria with good p values were observed. Cells treated with 0.2% DMSO and 5 µM paclitaxel were used as negative and positive controls, respectively. Data is representative of three independent experiments. *P < 0.05 and **P < 0.01, versus vehicle control.
3. Results

3.1. Antiproliferative activity

Cell viability was analyzed using the MTT assay, which measuring the metabolic activity of cell. The IC50 of BA on various cells lines were given in Table 1. In the A549 cells treated with BA, metabolic activity decreased followed by 24 h treatment with an IC50 of 20.22 ± 3.15, meanwhile in the control plate, cell viability and metabolism was not affected.

3.2. Boesenbergin A-induced apoptosis in A549 cells

To confirm the presence of apoptosis, we examined nuclear morphological changes of A549 cells by determining nuclear condensation and fragmentation hallmark for apoptosis (Fig. 2). Hoechst 33342 staining showed that a part of the cells displayed nuclear condensation at 24 h after BA treatment. The nuclear intensity which is directly corresponding to apoptotic chromatin changes: blebbing, fragmentation and condensation where quantitated in fig. 3A. Meanwhile, concurrent increase in the cell permeability also was observed (Fig. 3B).

3.3. Boesenbergin A-induced MMP disruption and release of cytochrome c

MMP was significantly reduced on cells treated with BA (30% at 50 μg/ml p < 0.01) (Fig. 3C). Changes of mitochondrial membrane potential in A549 cells treated with BA at 20 and 50 μg/ml for 24 h showed a significant reduction of fluorescence intensity (Fig. 2), which reflected the collapse of MMP. Meanwhile, BA triggered the A549 cells to translocate the cytochrome c from mitochondria into cytosol during apoptosis significantly (Fig. 2). At 50 μg/ml BA triggered the cytochrome c release by 2-fold (p < 0.01) (Fig. 3D).

3.4. Caspase-8, -9 and -3/7 Assay

As shown in Fig. 4A, BA significantly stimulated both caspase-8, -9 and -3/7. There was more than one fold increase in all caspase activities in all the three concentration treatment compared to the control. Induction of the apoptosis enzymes was confirmed to be higher in treated A549 cells when compared to non-treated cells and hence, concludes that BA induces apoptosis through both intrinsic and extrinsic pathways.

3.5. Boesenbergin A induced cell death includes increased ROS formation

The generation of ROS is always connected with MMP disruption and apoptosis (Castedo et al., 1996). Thus we examined the levels of ROS in A549 cells treated with BA. ROS was monitored by the oxidation-sensitive fluorescent dye DCFH-DA. A concentration depended increase in DCF fluorescence was detected in treated cells (Fig. 4B).

3.6. Cell cycle analysis and DNA content

Flow cytometric analysis of cell cycle and DNA content were performed to determine the ability of BA to induce cell cycle arrest and apoptosis. There were no significant changes of G0/G1, G2/M phase in dose-dependent treatment of BA on A549 cells. The S phase cells were increased upon treatment but were not statistically significant. However, sub-G1 phase, which is also known as apoptotic cells, showed significant increase due to concentration dependent manner (Fig. 5A–D). BA increased the apoptotic sub-G1 peak of A549 cells to 3%, 6% and 22% at concentrations of 10, 20 and 50 μg/ml, respectively (Fig. 5E). This apoptotic property of BA was further confirmed as fragmented DNA upon exposure to BA. The fragmented DNA was clearly observed in the treated cells, whereas untreated control cells did not provide ladders (Fig. 6).

3.7. Western blot analysis

Whole cell lysate of treated and untreated A549 cells were probed with the appropriate antibody to compare Bax, Bcl-2 and Hsp70 protein levels in these samples by western blot. Fig. 7 showed Bax gradually increased as the concentration of treatment increases. The Bcl-2 protein level was also found to be decreased after treatment for 24 h. It was also observed that Hsp70 protein level was downregulated in a concentration-dependent manner. β-Actin was used as loading control, which showed equal intensity bands, confirming equal protein concentration in all samples.

4. Discussion

The consumption of natural and healthy foods is currently the major interest of consumers. It attracts much more...
attention if it has also been used as functional food (Manchali, Chidambaram Murthy, & Patil, 2011). But in many instances the usage of such foods has been left as traditional or custom practice rather than scientifically validated. *B. rotunda* is such kind of food practiced for its major pharmacological activities including cancer therapy. Our previous research has shown that the main constituent in this plant is BA has the potential to act as an anti-cancer agent; as such this time we have evaluated BA for its potential to kill the lung cancer cells in vitro via apoptosis and the role of mitochondria in the death process.

The cytotoxicity analysis conducted in this research has revealed that BA inhibits the growth of A549 cells with an IC$_{50}$ of 20 μg/ml. Though the inhibitory concentration of BA is bit high, it has been noted that the chalcones normally exert their anti-cancer activities in the middle to low micromolar range (Yadav, Prasad, Sung, & Aggarwal, 2011). For instance the IC$_{50}$ at 24 h for some chalcones such as Butein in HL60 (18 μg/ml), Xanthohumol in HA22T-VGH (35 μg/ml), in Hep 3B cells (50 μg/ml), and Flavakavib in squamous carcinoma KB cells (20 μg/ml) are near to the IC$_{50}$ of BA (Ho, Liu, Chen, Duan, & Lin, 2008; Kim et al., 2001). This lead us to further study the cell death mechanism of BA in the present study. As such, first the A549 cells treated with BA were tested using the multiparametric high content screening (HCS) assay. Based on this assay, results showed four important signs of apoptosis, which included nuclear chromatin condensation, increase of membrane permeability, reduction of mitochondrial membrane potential and release of cytochrome c. These findings confirmed that BA is capable of inducing apoptosis in A549 cells in concentration-dependent manner. These characteristics also differentiate apoptosis from necrosis. Nuclear chromatin condensation and polymerization of F-actin are signs of early apoptosis (Bursch et al., 2000). F-Actin gathered in cell areas, where apoptotic bodies were formed. Reorganization of F-actin network is fundamental for the proper formation of apoptotic bodies (Grzanka et al., 2010). This is confirmed by Takesono et al., which reported that F-actin accumulates in membrane blebs as they start to retract (Takesono, Heasman, Wojciak-Stothard, Garg, & Ridley, 2010).

Mitochondria play as a central checkpoint of apoptosis control. It assimilates various signals including endogenous factors (Ca$^{2+}$, Mg$^{2+}$, K$^+$, and Na$^+$, ATP, ADP, and NADP) as well as exogenous factors. At the level of mitochondrial membrane, these mitochondria organelles collect the sum of death-inducing and life-preserving signals, thus predomination of lethal signals over the vitals will cause the mitochondria to initiate mitochondrial membrane potential (MMP) (Kroemer, Galluzzi, & Brenner, 2007). As a result, the release of cytochrome c, which is an early indication of mitochondrial changes, triggered the activation of the caspase cascade. The opening of mitochondrial permeability transition pore has been linked to enhanced permeability and loss of mitochondrial membrane potential. So the role of mitochondria

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**Fig. 5** – DNA analysis of A549 cells. Rapidly proliferating A549 cells were exposed to BA for 24 h with 10 (B), 20 (C) and 50 (D) μg/ml were tested for DNA content and the population on each phase were quantified (E). *’ Indicates a significant difference ($p < 0.05$).
in A549 cell apoptosis was investigated by the detection of changes in MMP, as studies reported that the release of mitochondrial apoptotic factors, such as cytochrome c, is influenced by the opening of the permeability transition pore and its consequences (Koya et al., 2000). The results were in well agreement with the literature and suggest that BA may act on mitochondria, and lead to subsequent apoptosis.

The close relationship between extrinsic and intrinsic pathways had always showed that they do not function independently (Yan et al., 2010). It is well documented that cytochrome c release occurs in the apoptosis of intrinsic pathway, by targeting and activating caspase-9, thus leading to the activation of effector caspases (-3, -6 and -7). Studies have also reported that the extrinsic pathway, where binding of ligand-receptor activates caspase-8, influence the intrinsic pathway. This is because active caspase-8 can induce activation of Bid, a BH3 domain-containing pro-apoptotic Bcl2 family member (Li et al., 2002). The treatment with BA on A549 cells activated the caspases-3/7, -8 and -9. The activation of the caspase 9 and the significant release of cytochrome c detected earlier shows the substantial role of mitochondria in the BA mediated apoptosis. Moreover, the activation of caspase-8 further suggested that the extrinsic pathway merged with the intrinsic pathway that leads to the activation of effector caspase-3/7 (Shih, Yeh, & Yen, 2005). Since the Caspase cascade leads to apoptosis via fragmentation of DNA, we observed the endonuclease cleavage product via gel electrophoresis. Our control cells did not displayed any ladder due to the DNA of non-apoptotic cells which remains largely intact (Elmore, 2007). In this regard, a dose-related trend of increased DNA fragmentation implicated to apoptosis was observed in the present study.

Oxidative stress is measured to be a vital state to promote programmed cell death in response to a variety of signals and pathophysiological situations. Moreover the studies verified that the ROS is an inevitable factor in the cell death where mitochondria are significantly involved (Ham et al., 2012). To know this relationship we have measured the ROS level upon BA treatment. The results clearly underline this relation significantly (p < 0.05). In addition, the HSP 70 protein, which is known to protect cells from stress by preventing the protein aggregation during the oxidative stress, was found to be significantly down regulated with the BA treatment. Since elevated HSPs are well known for the promotion of tumour progression, the down regulation of HSP could accelerate the potency of free radicals to promote mitochondria mediated apoptosis via oxidative stress. Hsp70 also has been discovered to directly bind to block the recruitment of procaspase 9 to the apoptosome by binding itself to the apoptosis protease-activating factor-1 (Apaf-1) (Schmitt, Gehrmann, Brunet, Multhoff, & Garrido, 2007). Apart from the HSPs apoptosis is strictly controlled by antiapoptotic and proapoptotic proteins, including the Bcl-2 family protein, and can be mediated by several different pathways (Heo et al., 2010; Kang et al., 2012). Since we have observed many signs of mitochondria relation in the BA mediated apoptosis, immunoblotting assays were performed to check the role of Bcl2 and Bax. These results were constant with the previous findings that showed clear involvement of Bax and Bcl2 at protein level.

The mode of cell death induced by many natural compounds was closely associated and influenced by cell cycle progression (González-Sarrías, Li, & Seeram, 2012). Besides, cell cycle control has been proven to be a major event in ensuring precise cellular division (Mohan et al., 2010). Since the regulation of cell cycle progression is considered to be a potentially powerful strategy for tumour growth control, the flow cytometry cell cycle analysis was performed. Our results indicated that cell cycle distribution was not altered in A549 cells in the parameter used in the present study. Even though there was a slight increase in S phase, it was not statistically significant (p > 0.05). But the significantly increased sub-G1 (p < 0.05) phase accumulation was very clear, which further confirms the apoptosis findings in our previous assays.

Chalcones represent an important group of the polyphenolic family, widely present in many edible plants (Kaisoon, Siriamornpun, Weerapreeyakul, & Meeso, 2011). Even though in the current research we have not done any SAR study, from the literature it can be seen that the presence of methoxy groups in the A ring of BA could be aid this compound to exert anti proliferative effect. Moreover, the presence of α, β unsaturation present in the basic skeleton, which generally helps the chalcones for the biological effects (Boumenjdel et al., 2008).In conclusion, this study is, to the best of our knowledge, the first to demonstrate the biological mechanisms underlying the anti-cancer effects of the BA in A549 cells. Our results also indicate that the apoptotic process induced in A549 cells is associated with the dysregulation of
mitochondria. This was very clearly evident while observing the level of MMP, cytochrome c, ROS, HSP70, and Bcl-2/Bax levels. BA is thus worth in further investigation to determine corresponding signalling pathway involved in apoptosis. This could provide new avenues to therapeutic targeting and subsequently, new alternatives in treatment of lung cancer from functional food.

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