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By

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Chairman: Ahmad Bustamam Abdul, PhD

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Boesenbergin A (BA) is a chalcone isolated from *Boesenbergia rotunda*, containing methoxyl and hydroxyl groups in its structure. These groups have been reported to exert anti-proliferative, antioxidant and anti-inflammation activities. However, there has been no literature reported on the biological activities of BA and its effects on lung cancer. The current *in vitro* study was designed to investigate the apoptotic induction of BA in A549 cells, as well as its antioxidant and anti-inflammatory activities. Human hepatocellular carcinoma cell (HepG2), human prostate cancer cell (PC3), colon adenocarcinoma cell (HT-29), non-small cell lung cancer cell (A549) and normal hepatic cells (WRL-68) were used to evaluate the cytotoxicity of BA using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

assay. The antioxidant activity of BA was assessed by the Oxygen Radical Absorbance Capacity (ORAC) assay and compared to quercetin as a standard reference antioxidant. The anti-inflammatory assay was performed by inducing murine monocytic macrophage cell line (RAW 264.7) using 100 U/mL of interferon-γ (IFN-γ) and 5 µg/mL of lipopolysaccharide (LPS) with or without the presence of BA, and compared concurrently to L-Nitro-Arginine Methyl Ester (L-NAME) as positive control. The production of nitric oxide (NO) after inducing the inflammation was then assessed using Griess reaction. Multiparametric High Content Screening (HCS) assay was conducted to detect changes in total nuclear intensity (chromatin condensation and fragmentation), cell permeability, mitochondrial membrane potential and release of cytochrome c. Caspase assay for caspase-8, -9 and -3/7 were analyzed using luminescence reader, whereas DNA fragmentation was detected through gel electrophoresis. To measure the production of intracellular ROS, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was used. Cell cycle analysis was performed using Flow Cytometer. Expression of Bax, Bcl2 and Hsp70 protein were detected by using the western blot analysis.

Toxic effect of BA on different cell types, reported as IC_{50}, yielded 20.22 ± 3.15, 10.69 ± 2.64, 20.31 ± 1.34, 94.10 ± 1.19, and 9.324 ± 0.24 µg/mL for A549, PC3, HepG2, HT-29 and WRL-68, respectively. ORAC results are reported as the equivalent concentration of trolox that produces the same level of antioxidant activity as the sample~~s~~ tested at 20µg/mL. BA displayed low antioxidant activity, when the results of ORAC assay were reported as

trolox equivalents, where BA (20 µg/mL) and quercetin (5 µg/mL) were equivalent to a trolox concentration of 11.91 ± 0.23 and 160.97 ± 0.02 µM, respectively. For the anti-inflammatory assay, the results clearly showed a concentration-dependent decrease in NO production in the induced RAW 264.7 cells, with a significantly low level being evident even at 25 and 50 µM of BA concentrations, with a level of 29.69 ± 2.5 and 25.69 ± 2.0 µM of NO production respectively. The anti-inflammatory activity of BA was significant between concentration of 25 µM to 50 µM but with no significant cytotoxicity towards RAW 264.7 cells at 50 µM. The anti cancer activities of BA was investigated using A549 cell line, as BA was found to be cytotoxic to A549 cells (IC_{50}< 30 µg/mL). The HCS results showed the BA-induced A549 cells exhibited a concentration-dependent increase in total nuclear intensity, cell permeability and cytochrome c release, whereas the cells mitochondrial membrane potential was decreasing ($p<0.05$), ($p<0.01$). Luminescence assay for caspase-8, -9 and -3/7 revealed an elevation of all three caspases in concentration-dependent manner ($p<0.05$). A549 cells treated with BA showed DNA fragmentation at the concentration of 20 and 50 µg/mL of BA. Production of ROS was also elevated in a concentration-dependent manner, indicated by the increase of DCF fluorescence intensity in treated cells ($p<0.05$). Protein analysis by western blot showed an increase of Bax, whilst Bcl2 and Hsp70 protein were subsequently decreased due to time-dependent treatment ($p<0.05$). Cell cycle analysis by flow cytometer showed a significant concentration-dependent increase in the sub G1 phase ($p<0.05$). A slight increase of cell arrest was observed in S phase but it was not statistically significant ($p>0.05$), which confirmed cell cycle arrest was not

induced in treated A549 cells. Collectively, results presented in this study demonstrate that BA inhibited the proliferation of non-small lung cancer *in vitro*, leading to a possible induction of apoptosis, which was later confirmed to be induced through the extrinsic and intrinsic pathways. Moreover the role of free radicals was significantly found to be elevated with concomitant decrease in Hsp70. This compound also showed low antioxidant capacity and significant anti-inflammatory activity. BA can be a potential drug to treat lung cancer and inflammation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGARUHAN APOPTOSIS DI DALAM SEL A549 *IN VITRO* OLEH BOESENBERGIN A YANG DIASINGKAN DARIPADA *BOESENBERGIA ROTUNDA*

Oleh

NORBAITI BINTI MOHD ISA

Julai 2013

Pengerusi: Ahmad Bustamam Abdul, PhD

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Boesenbergin A (BA) ialah sejenis calkon daripada *Boesenbergia rotunda* yang mengandungi kumpulan methoxyl dan hydroxyl di dalam strukturnya. Kumpulan ini pernah dilaporkan mengandungi aktiviti anti-proliferatif, antioksida dan anti-radang. Walaubagaimanapun, belum terdapat sorotan literatur yang melaporkan tentang aktiviti biologi BA dan kesannya terhadap kanser paru-paru. Kajian *in vitro* kini direka bertujuan untuk menyiasat pencetusan apoptosis oleh BA pada sel A549, juga aktiviti antioksida dan anti-radangnya. Sel kanser hati manusia (HepG2), sel kanser prostat manusia (PC3), sel kanser kolon (HT-29), sel kanser paru-paru bukan kecil (A549) dan sel hati normal (WRL-68) telah digunakan untuk menilai

sitotoksisiti BA dengan menggunakan asai MTT. Aktiviti antioksida BA dinilai melalui asai ORAC lalu dibandingkan dengan quercetin yang bertindak sebagai anti oksida rujukan standard. Asai anti-radang telah dilaksanakan dengan meransang sel murine monocytic macrophage (RAW 264.7) menggunakan 100 U/mL interferon-y (IFN-y) dan 5 µg/mL lipopolysaccharide (LPS) dengan atau tanpa kehadiran BA, dan dibandingkan dengan L-Nitro-Arginine Methyl Ester (L-NAME) yang bertindak sebagai kawalan positif. Penghasilan nitric oxide (NO) selepas meransang keradangan kemudiannya dinilai menggunakan reaksi Griess. Multiparametrik High Content Screening (HCS) dijalankan bagi mengesan perubahan dalam intensiti nuklear seluruh (kondensasi nuclear dan fragmentasi), ketembusan sel, potensi membran mitokondria dan pelepasan sitokrom c. Asai caspase untuk caspase-8, -9 dan -3/7 telah dianalisis menggunakan pembaca luminesen, sementara fragmentasi DNA dikesan melalui kaedah gel elektroforesis. Bagi mengukur penghasilan ROS intraselular, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) digunakan. Kitaran sel analisis pula dilaksanakan dengan menggunakan flow cytometer. Ekspresi protein Bax, Bcl2 dan Hsp70 dikesan menggunakan analisis western blot.

Kesan toksik BA terhadap jenis sel yang berlainan, dilaporkan sebagai IC_{50}, masing-masing bernilai 20.22 ± 3.15, 10.69 ± 2.64, 20.31 ± 1.34, 94.10 ± 1.19, dan 9.324 ± 0.24 µg/mL untuk A549, PC3, HepG2, HT-29 and WRL-68. Keputusan ORAC dilaporkan sebagai kepekatan setaraf trolox yang menghasilkan aktiviti anti oksida yang sama paras dengan sampel yang diuji

pada 20 µg/mL. BA menunjukkan aktiviti anti oksida yang rendah apabila keputusan ORAC dilaporkan sebagai setaraf trolox, di mana BA (20 µg/mL) dan quercetin (5 µg/mL) adalah setaraf kepada kepekatan trolox iaitu 11.91 ± 0.23 dan 160.97 ± 0.02 µM. Bagi asai anti-radang, keputusannya jelas menunjukkan peningkatan secara kebergantungan-kepekatan dalam penghasilan NO dalam sel RAW 264.7 yang diransang, dengan paras rendah BA secara siknifikan menjadi bukti walau pada kepekatan 25 dan 50 µM, dengan penghasilan NO masing-masing pada paras 29.69 ± 2.5 dan 25.69 ± 2.0 µM. Aktiviti anti-radang BA adalah siknifikan antara kepekatan 25 µM hingga 50 µM tetapi tanpa sitotoksisiti yang siknifikan terhadap sel RAW 264.7 pada 50 µM. Aktiviti anti-kanser BA yang selanjutnya disiasat dengan menggunakan sel A549 memandangkan BA didapati bersifat sitotoksik terhadap sel A549 (IC_{50}<30 µg/mL). Keputusan HCS menunjukkan bahawa sel A549 yang diransang oleh BA mempamerkan peningkatan secara kebergantungan-kepekatan dalam intensiti nuklear seluruh, ketembusan sel dan pelepasan sitokrom c, manakala potensi membran mitokondria pula menurun ($p<0.05$), ($p<0.01$). Asai luminesen untuk caspase-8,-9 dan -3/7 mendedahkan peningkatan bagi ketiga-tiga caspase secara kebergantungan-kepekatan ($p<0.05$). Sel A549 yang dirawat dengan BA menunjukkan fragmentasi DNA pada kepekatan 20 dan 50 µg/mL ($p<0.05$). Penghasilan ROS ditingkatkan juga secara kebergantungan-kepekatan, diindikasi oleh peningkatan intensiti fluoresen dalam sel yang dirawat ($p<0.05$). Analisis protein oleh western blot menunjukkan peningkatan Bax, sementara protein Bcl2 dan Hsp70 menurun berikutan rawatan secara kebergantungan-masa ($p<0.05$). Analisis kitaran sel oleh

flow cytometer menunjukkan peningkatan secara kebergantungan-kepekatan pada fasa sub G1 ($p<0.05$). Sedikit peningkatan pada penangkapan sel dapat dilihat pada fasa S, akan tetapi ia tidak siknifikan secara statistik ($p>0.05$), yang membuktikan bahawa penangkapan sel tidak dicetuskan oleh BA pada sel A549 yang dirawat. Secara keseluruhan, keputusan yang dipersembahkan dalam kajian ini menunjukkan bahawa BA menghalang proliferasi ke atas sel kanser paru-paru bukan-besar secara *in vitro*, membawa kepada pendorongon apoptosis, yang selanjutnya disahkan didorong melalui laluan ekstrinsik dan intrinsik. Tambahan pula, peranan radikal bebas didapati secara meningkat secara siknifikan dengan dituruti penurunan Hsp70. Kompaun ini juga menunjukkan kapasiti antioksida yang rendah dan aktiviti anti-radang yang siknifikan. BA berpotensi menjadi ubat untuk merawat kanser paru-paru dan keradangan.

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APPROVAL

I certify that a Thesis Examination Committee has met on 4th July 2013 to conduct the final examination of Norbaiti binti Mohd Isa on her Master of Science thesis entitled "INDUCTION OF APOPTOSIS IN A549 CELLS IN VITRO BY BOESENBERGIN A ISOLATED FROM BOESENBERGIA ROTUNDA" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of Thesis Examination Committee were as follows:

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This thesis submitted to the Senate of University Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

NORBAITI BINTI MOHD ISA

Date: 4th July 2013

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