UNIVERSITI PUTRA MALAYSIA

CYTOTOXIC EFFECTS OF DAMNACANTHAL AND DAMNACANTHAL-DOXORUBICIN COMBINATION ON HUMAN BREAST CANCER CELL LINE (MCF-7)

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Chairman : Associate Professor Noorjahan Banu Binti Mohamed Alitheen,PhD

Faculty : Biotechnology and Biomolecular Sciences

Damnacanthal is a biologically active antraquinone derivative isolated from roots of *Morinda* sp., belonging to the *Rubiaceae* family. This plant has a long history of use as a food in tropical regions throughout the world. The purpose of this study was to examine the *in vitro* cytotoxicity effect of damnacanthal and combination of damnacanthal with doxorubicin on human breast carcinoma (MCF-7). MTT assay was used to determine the cytotoxic properties of damnacanthal towards various cancer cell lines, normal cell lines and immune cells. Results showed that damnacanthal displayed strong killing effect towards the cancerous cell lines and moderate effect towards normal cells lines. Among the cancer cell lines, damnacanthal showed strong cytotoxic towards MCF-7 cells with CD_{50} 8.2 ± 0.7 µg/mL after 72 hours treatment period. In contrast, no cytotoxicity was detected in primary mouse splenocytes, mouse thymocytes and mouse bone marrow. In

particular, damnacanthal showed cytotoxicity in a dose- and time-dependent manner in the MTT assay. Thus, MCF-7 cells were used to determine the mode of cell death with damnacanthal alone and combination with doxorubicin. In the cell viability assay, the percentage viability of MCF-7 cells is lower when the cells are treated with combination of damnacanthal and doxorubicin than doxorubicin alone. In the cell cycle with flow cytometry analysis, damnacanthal arrested the cell cycle of MCF-7 cells at G1 phase and doxorubicin arrested at G2/M phase. The percentage population of MCF-7 cells enter sub G0 phase are increased when the cells were treated with combination of damnacanthal and doxorubicin. The mode of cell death was mainly apoptosis by acridine orange/propidium iodide (AO/PI) dual staining method and Annexin V-FITC/PI assays. Gross morphology of treated cells observed through AO/PI showed the presence of blebbings cells, chromatin condensation and DNA fragmentation, indicating apoptosis. Annexin V-FITC/PI staining showed that substantial early apoptotic cells were detected in MCF-7 cells treated with damncanthal alone and in combination with doxorubicin. The changes of percentage intracellular proteins were analyzed using flow cytometer. The percentage of Bcl-2 protein is higher than p53 protein in the control group. While in the treatment group, the percentage of Bcl-2 protein decreased significantly and p53 protein increased significantly. The changes of expression level of apoptotic related genes caused by damnacanthal and doxorubicin were profiled using multiplex gene expression profiler (GeXP). The expression levels of genes that give significant signal were not much different at 12 and 24 hours. Out of 15 genes that were analyzed, four genes showed detection in expression level; BAX, p21, caspase-3 and caspase-7. The expression of these pro-apoptotic genes is up-regulated in the treated group compared to control. Among the treated group, the combination treatment of

damnacanthal and doxorubicin showed higher expression level of these pro-apoptotic genes. The findings in the intracellular protein detection and changes in the expression level of apoptotic related genes reflect that the mechanism of damnacanthal induced apoptosis in the human breast cancer cells was by the intrinsic and caspases induction pathway. In conclusion, damnacanthal is more toxic towards cancer cell lines rather than normal cell lines and primary cells and showed the potential use as a promising agent for apoptosis induction alone and/or with combination with doxorubicin. The induction of apoptosis involved important pro-apoptotic genes which are BAX, p53, p51 caspase-3 and caspase-7.

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

KESAN SITOTOSIK OLEH DAMNACANTHAL DAN KOMBINASI BERSAMA DOXORUBICIN TERHADAP SEL KANSER PAYUDARA MANUSIA (MCF-7)

Oleh

MUHAMMAD YUSRAN BIN ABDUL AZIZ

Januari 2013

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Damnacanthal ialah molekul aktif terbitan antraquinone secara biologi yang telah dipencilkan daripada akar spesis *Morinda* dari Famili *Rubiaceae.* Tumbuhan ini mempunyai sejarah penggunaan yang panjang sebagai makanan di kawasan tropika di seluruh dunia. Tujuan kajian ini adalah untuk mengkaji secara *in vitro* kesan sitotoksik damnacanthal dan gabungan damnacanthal dengan doxorubicin pada sel karsinoma payudara manusia (MCF-7). Ujian MTT digunakan untuk menentukan sifat-sifat sitotoksik damnacanthal terhadap pelbagai jujukan sel-sel kanser, sel-sel normal dan sel-sel imun. Keputusan menunjukkan bahawa damnacanthal mempunyai kesan pembunuhan yang mantap terhadap sel-sel kanser dan kesan yang sederhana terhadap sel-sel normal. Di antara sel-sel kanser, damnacanthal menunjukkan kesan sitotoksik yang kuat ke atas sel MCF-7 dengan CD_{50} 8.2 ± 0.7 μg / mL selepas 72 jam tempoh rawatan. Sebaliknya, sitotoksisiti tidak dikesan di sel limpa mencit, sel

timus mencit dan sel sumsum tulang mencit. Secara khususnya, damnacanthal mempamerkan kesan sitotoksik berkadar secara langsung dengan dos dan masa yang digunakan. Oleh itu, sel MCF-7 digunakan selanjutnya untuk menentukan cara kematian sel dengan damnacanthal sahaja dan kombinasi dengan doxorubicin. Dalam ujian viabiliti sel, peratusan viabiliti sel MCF-7 adalah lebih rendah apabila sel-sel dirawat dengan gabungan damnacanthal dan doxorubicin berbanding doxorubicin sahaja. Dalam kitaran sel dengan analisis sitometri aliran, damnacanthal menyekat kitaran sel MCF-7 pada fasa G1 dan doxorubicin menyekat kitaran sel pada fasa G2/M. Peratusan sel MCF-7 memasuki fasa sub G0 meningkat apabila sel-sel dirawat dengan gabungan damnacanthal dan doxorubicin. Mod utama kematian sel ialah apoptosis yang dibuktikan melalui kaedah perwarnaan berganda 'akridina jingga/propidium iodida' (AO/PI) dan ujian Annexin V-FITC/PI. Morfologi sel yang dirawat diperhatikan melalui kaedah pewarnaan AO/PI menunjukkan kehadiran 'blebbing', kondensasi kromatin dan fragmentasi DNA, membuktikan berlakunya apoptosis. Dalam sel MCF-7 yang dirawat dengan damnacanthal sahaja dan kombinasi dengan doxorubicin, sel apoptotik awal yang ketara dikesan dalam ujian perwarnaan Annexin V-FITC/PI. Perubahan peratusan protein intrasel dianalisis menggunakan sitometri aliran. Peratusan protein Bcl-2 adalah lebih tinggi daripada protein p53 dalam kumpulan kawalan. Manakala dalam kumpulan rawatan, peratusan protein Bcl-2 menurun dengan ketara dan protein p53 meningkat dengan ketara. Perubahan dalam tahap penzahiran gen yang berkaitan dengan apoptosis yang disebabkan oleh damnacanthal dan doxorubicin diprofilkan dengan "multipleks ekspresi gen" (GeXP). Tahap penzahiran gen yang memberikan isyarat yang penting tidak banyak berbeza pada 12 dan 24 jam rawatan. Daripada 15 gen yang dianalisis, hanya 4 gen menunjukkan pengesanan dalam rawatan; BAX, p21, caspase-3 dan

caspase-7. Penzahiran untuk 4 gen pro-apotosis ini didapati meningkat dalam kumpulan rawatan berbanding dengan kumpulan kawalan. Di antara kumpulan rawatan, rawatan gabungan damnacanthal dan doxorubicin menunjukkan tahap penzahiran 4 gen pro-apoptotik lebih tinggi. Penemuan dalam pengesanan peratusan protein dihasilkan dalam sel dan perubahan dalam tahap penzahiran gen yang berkaitan dengan apoptosis membekalkan mekanisme apoptosis teraruh oleh damnacanthal dalam kanser payudara sel-sel manusia melalui laluan induksi intrinsik dan caspases. Kesimpulannya, damnacanthal adalah lebih toksik terhadap sel-sel kanser daripada sel-sel normal dan sel-sel asas dan menunjukkan potensi penggunaan sebagai aruhan apoptosis sama ada sendirian dan/atau dengan kombinasi dengan doxorubicin. Aruhan apoptosis tersebut melibatkan gen pro-apoptotik yang penting iaitu BAX, p53, p51 caspase-3 dan caspase-7.

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I certify that a Thesis Examination Committee has met on 15 January 2013 to conduct the final examination of Muhammad Yusran Bin Abdul Aziz on his thesis entitled "Cytotoxic Effect of Damnacanthal and Its Combination With Doxorubicin on Human Breast Cancer Cells (MCF-7)" 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.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were 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 in Universiti Putra Malaysia or at any other institution.

MUHAMMAD YUSRAN ABDUL AZIZ

Date: 15 January 2013

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