UNIVERSITI PUTRA MALAYSIA

CYTOTOXIC EFFECTS OF ETHANOL EXTRACT OF MALAYSIAN INDIGENOUS MARINE MICROALGA, *CHAETOCEROS CALCITRANS* ON HUMAN BREAST CANCER CELL LINE MCF-7

SIYAMAK EBRAHIM NIGJEH

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By

SIYAMAK EBRAHIM NIGJEH

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

August 2011

DEDICATION

I dedicate this work especially to my Parents for their continuous prayer, encouragement and patience.

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

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Chairman: Professor Abdul Rahman bin Omar, PhD

Institute: Institute of Bioscience

Algae are being used by millions of humans and animals around the world as nutritional or pharmaceutical ingredients due to their vast diversity, diverse chemical compositions and biological activities. In the current study, cytotoxic effects of Malaysian a marine microalga, *Chaetoceros calcitrans* were evaluated. Briefly, *Chaetoceros calcitrans* was identified and cultured. The *Chaetoceros calcitrans* ethanol extract (EEC) was screened using MTT assay for cytotoxicity on various human cancer cell lines, K562 (leukemia), HT29 (colorectal), HeLa (cervical), MCF-7 (breast) and also on normal cells, MCF10A (human normal breast cell) and PBMC (peripheral blood monolayer cell). Tamoxifen was used as a positive control.

The mode of cell death induced by EEC was determined by Annexin V/PI followed by flow cytometry cell cycle analysis. The effects of EEC treatment on MCF-7 cells in

expressions of apoptotic related genes, Bax, Bcl-2, BCL2L1, TNF, P53, Fas, Caspase3, Caspase7 and Caspase9 and cell cycle genes, P21Cip1, CyclinA2, CDK2 and MDM2 were determined using GeXP system, a multiplex quantitative gene expression technology.

Overall, the EEC showed different IC_{50} values on the tested cell lines at different time points (24, 48 and 72 hours). The lowest IC_{50} value for MCF-7 (3.00±0.65 µg/ml) compared to that for MCF-10A cells (12.00±0.59 µg/ml) was obtained at 24 hour after treatment. Besides that the IC_{50} value of EEC on MCF-7 (3.00±0.65 µg/ml) was lower than the IC_{50} value of Tamoxifen (12.00±0.52). EEC was not cytotoxic towards PBMC even at its highest concentration. Hence, the EEC can be considered having anti cancer properties. Based on Annexin V/PI and cell cycle flow cytometry analysis, it was found that inhibition of cell growth by EEC on MCF-7 cells is through the induction of apoptosis without cell cycle arrest. The apoptotic cells at subG0/G1 phase in treated MCF-7 cells at 48 and 72 hours showed 34 and 16 folds increase compared to treated MCF-10A cells which showed only 6 and 7 folds increase at the same time points, respectively. The results from this study also showed that EEC induced apoptosis of MCF-7 cells without cell cycle arrest that is associated with the modulation of expression of selected cell cycle and apoptosis related genes such as MDM2, p21Cip1 as well as increasing the expression of pro-apoptotic protein Bax and decreasing the expression of the antiapoptotic protein Bcl-2. The EEC treatment on MCF-7 cells caused the fold changes of MDM2 to decrease from 1.8 at 6 hours to 1.4 at 24 hours; the fold changes of CyclinA2 also decreased from 3.5 at 6 hours to 1.5 at 24 hour whilst,the

fold changes of p21Cip1 also increased from 0.8 at 6 hours to 1.9 at 24 hour. The EEC treated cells also modulate the fold changes of the key caspase molecules where the fold changes of caspase3 and caspase7 decreased from 1.5 and 2.1 at 6 hour to 1.3 and 2.0 at 24 hours, respectively.

The Bax activation might have been involved in the release of cytochrome c from the mitochondria and clustered with APAF-1, a apoptotic protease activating factor 1 and resulted in activation of caspase9 (although it was not determined in the experiment) which then cleaved the downstream caspease3, 6 and 7 that lead to apoptosis. Hence, EEC induced apoptosis of MCF-7 cells associated with expression of several major regulators of the cell cycle such as CDK2, MDM2 and Cyclin A2 and apoptotic related genes by increasing in Bax/Bcl-2 ratio that in turn activate the caspase-dependent pathway. This study also showed that EEC could induce apoptosis through a caspase-dependent pathway by activating caspase3 and 7 in MCF-7 cells. Based on the results obtained from this study, it appears likely that EEC has the potential cytotoxic effect on breast cancer. However, more studies were required which include isolation, characterization and synthesis of the active compound (s) found in EEC that was associated with the strong apoptotic effects against breast cancer cells.

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

KESAN SITOTOSIK EKSTRAK ETANOL MICROALGA MARIN TEMPATAN MALAYSIA, CHAETOCEROS CALCITRANS KE ATAS SEL KANSER PAYU DARA MANUSIA

Oleh

SIYAMAK EBRAHIM NIGJEH

Ogos 2011

Pengerusi: Profesor Abdul Rahman bin Omar, PhD

Institut: Institut Biosains

Alga digunakan oleh manusia dan haiwan di seluruh dunia sebagai ramuan pemakanan atau farmaseutikal. Ini di sebabkan oleh terdapat nya pelbagai jenis alga yang mempunyai pelbagai komposisi kimia dan juga aktiviti biologi. Dalam kajian ini, ciri ciri antikanser marin mikroalga Malaysia *Chaetoceros calcitrans* telah dikaji. Secara ringkas, *Chaetoceros calcitrans* telah dikenalpasti, dikultur dan ditulenkan. *Chaetoceros calcitrans* ethanol extract (EEC) yang tulen telah disaring dengan menggunakan asai MTT sebagi salah satu kajian antikanser terhadap pelbagai jenis kanser sel K562 (Leukemia), HT29 (koloretal), HeLa (Servikal), MCF-7 (Payudara) dan juga terhadap sel normal, MCF10A (sel normal payudara manusia) dan PBMC (sel mononuklear darah periferi) untuk menentukan IC_{50}% bagi setiap jenis sel. Tamoksifen telah digunakan

sebagai kawalan positif. Aktiviti kematian sel yang dirargsangkan oleh EEC telah diukur oleh Annexin V / PI ditentukan oleh analisa kitaran sel sitometri aliran. Kesan rawatan EEC pada sel MCF-7 terhadap ekspresi gen apoptosis seperti Bax, Bcl-2, BCL2L1, TNF, P53, Fas, Caspase3, Caspase7 and Caspase9 dan gen kitaran sel, P21Cip1, CyclinA2, CDK2 dan MDM2 telah ditentukan mengguna sistem GeXP, iaitu teknologi ekspresi gen kuantitatif multipleks.

Pada keseluruhan, nya EEC menunjukkan nilai-nilai IC_{50} berbeza pada sel yang telah diuji pada masa berlainan (24, 48 dan 72 jam). Nilai IC_{50} yang terendah diperoleh untuk MCF-7 (3.00±0.65 µg / ml) berbanding dengan sel MCF-10A (12.00±0.59 µg / ml) pada 24 jam selepas rawatan. Selain nilai IC_{50} EEC pada MCF-7 (3.00±0.65 µg / ml) adalah lebih rendah daripada nilai IC_{50} Tamoxifen (12.00±0.52), EEC tidak mempunyai kesan sitotoksik pada PBMC walaupun dalam kepekatan yang berbeza. Maka, EEC mengandungi agen antikanser khusus.

Berdasarkan analisis Annexin V / PI dan sitometri aliran kitar sel, didapati bahawa perencatan pertumbuhan sel oleh EEC pada sel MCF-7 ialah melalui apoptosis tanpa rencatan kitaran sel. Walaupun, sel MCF-10A yang telah dirawat dengan EEC mengalami apoptosis, peratusan sel apoptotik tersebut adalah lebih rendah berbanding dengan sel MCF-7. Sel apoptotik pada fasa subG0/G1 dalam sel MCF-7 yang telah dirawat pada 48 dan 72 jam telah menunjukkan 34 dan 16 kali ganda lebih tinggi berbanding dengan MCF-10A yang telah dirawat yang hanya menunjukkan 6 dan 7 kali ganda pada titik masa sama.

Keputusan dari kajian ini juga menunjukkan yang apoptosis sel MCF-7 yang telah dirawat dengan EEC tanpa rencatan kitaran sel berkait rapat dengan modulasi ekspresi gen kitaran sel dan gen apoptosis seperti MDM2, p21Cip1 serta pertambahan ekspresi protein pro-apoptotic Bax dan juga penurunan ekspresi protein anti-apoptotic Bcl-2. Rawatan EEC pada sel MCF-7 telah menyebabkan perubahan gandaan MDM2 berkurang dari 1.8 pada 6 jam kepada 1.4 pada 24 jam,perubahan gandaan CyclinA2 berkurang dari 3.5 pada 6 jam kepada 1.5 pada 24 jam. Manakala, perubahan gandaan p21Cip1 telah meningkat dari 0.8 pada 6 jam kepada 1.9 pada 24 jam. Sel yang dirawat oleh EEC juga mempengaruhi perubahan gandaan molekul caspase yang utama di mana perubahan fold caspase3 dan caspase7 masing-masing berkurang dari 1.5 dan 2.1 pada 6 jam kepada 1.3 dan 2.0 pada 24 jam.

Pengaktifan Bax mungkin telah menyebabkan pembebasan sitokrom c dari mitokondria dan berkumpul dengan APAF-1, (apoptotic protease activating factor 1) dan menyebabkan pengaktifan caspase9 (walaupun ia tidak ditentukan dalam eksperimen) yang kemudiannya telah memisahkan caspase3, 6 dan 7 yang menyebabkan apoptosis. Maka, EEC mengaruh apoptosis sel MCF-7 yang berkait dengan ekspresi beberapa pengatur utama kitaran sel seperti CDK2, MDM2 dan Cyclin A2 dan gen berkaitan apoptotik dengan penambahan pada nisbah Bax / Bcl-2 yang seterusnya menyebabkan pengaktifan laluan bersandarkan-caspase. Kajian ini juga menunjukkan EEC boleh mengaktifkan apoptosis melalui laluan bersandarkan caspase-dengan mengaktifkan caspase 3 dan 7 dalam sel MCF-7.

Berdasarkan keputusan yang diperoleh daripada eksperimen ini, EEC berpotensi untuk dijadikan sebagai rawatan kanser payudara. Namun, lebih banyak kajian seperti pengasingan, pencirian dan sintesis bahan aktif dalam EEC yang berkaitan dengan kesan antiproliferasi dan apoptosis terhadap sel kanser payudara masih diperlukan.

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I certify that a Thesis Examination Committee has met on 8-8-2011 to conduct the final examination of Siyamak Ebrahimi Nigjeh on his thesis entitled " Cytotoxic Effect Of Ethanol Extract Of Malaysian Indigenous Marine Microalga, Chaetoceros Calcitrans, On Human Breast Cancer Cell Line, 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.

Members of the Examination Committee are as follows:

Sabariah Abdul Rahman, PhD
Professor
Institute of Bioscince
Universiti Putra Malaysia
(Chairman)

Ahmad Bustamam Hj Abdul, PhD
Professor
Institute of Bioscince
Universiti Putra Malaysia
(Internal Examiner)

Latifah Saiful Yazan, PhD
Associate Professor
Faculty of Medicine
Universiti Putra Malaysia
(Internal Examiner)

Kamaruddin Mohd Yusoff, PhD
Professor
Faculty of Medicine
Universiti Malaysia
(External Examiner)

SEOW HENG FONG, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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:

Abdul Rahman bin Omar, PhD
Professor
Institute of Bioscience
Universiti Putra Malaysia
(Chairman)

Fatimah Md Yusoff, PhD
Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

Noorjahan Banu Mohamed Alitheen, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia
Date:

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 other institutions.

SIYAMAK EBRAHIMI NIGJEH
Date:

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