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ANTIOXIDANT AND CYTOTOXIC EFFECTS OF CRUDE EXTRACT FROM A DIATOM, *Chaetoceros calcitrans* AND GREEN ALGA, *Nannochloropsis oculata*

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By

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Faculty: Institute of Bioscience

There is a growing trend in research focusing on marine microorganisms such as sponges, seaweeds and micro-algae as a potential source of bioactive compounds. In Malaysia, microalgae are widely used as live feed in shrimp and fish hatcheries. The nutrient-rich source of marine microalgae gives a promising potential to explore its new biomedical applications. This study was carried out to investigate the antioxidant capacity and cytotoxic effects of different polarities of crude solvent extracts namely hexane, dichloromethane, ethyl acetate and methanol from a diatom, *Chaetoceros calcitrans* and a green alga, *Nannochloropsis oculata*. The antioxidant properties were determined by 1, 1-diphenyl-2-picrylhrazyl (DPPH) radical-scavenging, ferric-reducing antioxidant power (FRAP), ferrous-ion chelating and folin assays. Results revealed that crude ethyl acetate (EA) extract of *C.*

calcitrans contained the highest total phenolic content (3775.67 ± 0.08 mg gallic acid equivalent/g dried extract) and DPPH radical scavenging power (3722.93 ± 2.98 mg Trolox equivalent/g dried extract). The antioxidant activities of extracts from *N. oculata* were lower compared to the extract from *C. calcitrans,* except for EA extract of *C. calcitrans* showed the highest chelating power compared to others. MTT assay was used to determine the cytotoxic properties of crude solvent extracts from *C. calcitrans* and *N. oculata* towards various cancer cell lines. Results showed that EA extract of *C. calcitrans* significantly inhibited the growth of MDA-MB-231 cells among the cancer cell lines tested, with IC_{50} 60 µg/mL only after 72 hours treatment period. Thus, EA extract of *C. calcitrans* was used in further assay to determine the mode of cell death with three different concentrations : 30 (½ IC_{50}), 60 (IC_{50}) and 120 (2x IC_{50}) µg/mL for 72 hours treatment. In the cell cycle with flow cytometry analysis, EA extract of *C. calcitrans* arrested the cell cycle of MDA-MB-231 cells at G2/M phase when treated with 60 µg/mL. However, when cells were treated with low concentration (30 µg/mL) of EA extract, significant growth arrest was occurred at G_1 phase. Apoptosis was induced when the concentration was increased to 120 µg/mL. The mode of cell death was mainly apoptosis, which was proven by acridine orange/propidium iodide (AO/PI) dual staining method, Annexin V-FITC and DNA fragmentation (TUNEL) assays. Morphology of the treated cells which was observed through AO/PI staining method showed the presence of blebbing cells, chromatin condensation and DNA fragmentation as well as intake of some PI stain, proving that

apoptosis has occurred. Early apoptosis was analysed by Annexin V-FITC apoptosis test and DNA fragmentation test showed that DNA was cleaved into fragments. These results indicated the presence of apoptotic cells increased with increasing concentration of the extract. The changes of expression level of apoptotic, and proliferative-related genes caused by EA extracts of *C. calcitrans* were profiled using multiplex gene expression profiler (GeXP). Cells treated with EA extract for 6, 12 and 24 hours did not show significant changes in the expression levels of most of the pro-apoptotic genes. The expression of pro-apoptotic genes of the cells treated for 48 hours with low concentration (30 μg/mL) of the extract was highly up-regulated. However, the expression level was down-regulated when treated with high concentration of EA extract (120 μg/mL). At 72 hours of treatment period, most of the pro-apoptotic genes especially caspases related genes such as caspases-3, -4, -9, BAK1 and p21 were found to be up-regulated. Conversely, genes that involved in p53 network especially Bax and ING3 as well as anti-apoptotic (Bcl-2) were found to be downregulated. These findings provided some mechanisms of EA extract of *C. calcitrans*-induced apoptosis in the human breast cancer cells via the caspases induction pathway. In conclusion, *C. calcitrans* is more cytotoxic compared to *N. oculata* (solvent extracts). EA extract of *C. calcitrans* showed the best antioxidant activities and cytotoxicity compared to others, and induced apoptosis in MDA-MB-231 cells. The induction of apoptosis involved important pro-apoptotic genes : p21, casp-3, -4, -9 and Bak1.

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

ANTIOKSIDAN DAN KESAN SITOTOKSIK EKSTRAK MENTAH DARIPADA DIATOM, *Chaetoceros calcitrans* DAN ALGA HIJAU, *Nannochloropsis oculata*

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Terdapat perkembangan dalam bidang penyelidikan berfokus kepada mikrorganisma marin seperti span, rumpai laut dan mikroalga, sebagai sumber sebatian bioaktif. Di Malaysia, mikroalga digunakan secara meluas sebagai makanan di pusat penetasan ikan dan udang. Sumber nutrisi mikroalga yang tinggi ini menjanjikan potensi untuk dieksploitasi bagi kegunaan baru dalam bidang bioperubatan. Kajian ini dilaksanakan untuk menyiasat kapasiti antioksidan serta kesan sitotoksik ekstrak yang berpolariti berbeza iaitu heksana, diklorometana, etil asetat dan metanol daripada diatom, *Chaetoceros calcitrans* dan alga hijau, *Nannochloropsis oculata***.** Ujian antioksidan yang dijalankan adalah 1, 1-diphenyl-2-picrylhrazyl (DPPH), daya pengurangan ferum (FRAP), ‘chelate’ ion ferus (FIC) dan kajian folin. Hasil kajian menunjukkan bahawa ekstrak mentah etil asetat (EA) daripada *C. calcitrans* mengandungi kandungan fenolik (3775.67 ± 0.08 mg setara dengan asid galik/g ekstrak kering) dan

aktiviti penggesatan radikal DPPH (3722.93 ± 2.98 mg setara dengan Trolox/g ekstrak kering) yang paling tinggi. Aktiviti antioksidan bagi ekstrak *N. oculata* adalah rendah berbanding dengan ekstrak daripada *C. calcitran*, kecuali ekstrak EA daripada *N. oculata* menunjukkan keupayaan pengilatan yang paling tinggi berbanding dengan yang lain. Ujian MTT digunakan untuk menentukan sifat ketoksikan ekstrak daripada kedua-dua *C. calcitrans* dan *N. oculata* terhadap pelbagai jujukan sel kanser. Keputusan menunjukkan ekstrak EA daripada *C. calcitrans* merencat pertumbuhan sel MDA-MB-231 secara signifikan di kalangan sel kanser lain yang dikaji, dengan IC_{50} 60 μg/mL selepas 72 jam rawatan. Oleh itu, EA ekstrak daripada *C. calcitrans* digunakan selanjutnya untuk menentukan cara kematian sel dengan tiga kepekatan yang berlainan: 30 (½ IC_{50}), 60 (IC_{50}) and 120 (2x IC_{50}) μg/mL selama 72 jam. Dalam kitaran sel dengan analisis sitometri aliran, ekstrak EA tersebut menyekat kitaran sel MDA-MB-231 pada fasa G2/M apabila dirawat dengan kepekatan 60 μg/mL. Namun, ketika sel dirawat dengan kepekatan ekstrak EA yang rendah (30 μg/mL), penyekatan pertumbuhan secara signifikan berlaku pada fasa G1. Apoptosis diaruh apabila kepekatan ditingkatkan kepada 120 μg/mL. Mod utama kematian sel ialah apoptosis yang dibuktikan melalui kaedah pewarnaan berganda ‘akridina jingga/propidium iodida’ (AO/PI), ujian ‘Annexin V-FITC’ dan fragmentasi DNA (TUNEL). Morfologi sel yang dirawat yang diperhatikan melalui kaedah pewarnaan AO/PI menunjukkan ‘blebbing’, kondensasi kromatin dan fragmentasi DNA serta pengambilan masuk pewarna PI membuktikan

berlakunya apoptosis. Kehadiran apoptosis awal dianalisis dengan ujian Annexin V-FITC dan ujian fragmentasi DNA menunjukkan bahawa DNA telah difragmentasi kepada serpihan. Keputusan ini menunjukkan bahawa bilangan sel apoptosis bertambah dengan peningkatan kepekatan ekstrak. Perubahan dalam tahap penzahiran gen yang berkaitan dengan apoptosis dan perebakan yang disebabkan oleh ekstrak EA daripada *C. calcitrans* diprofilkan dengan "multipleks ekspresi gen" (GeXP). Sel yang dirawat dengan ekstrak EA selama 6, 12 dan 24 jam tidak menunjukkan perubahan yang signifikan pada tahap penzahiran kebanyakan gen pro-apoptotik. Penzahiran gen pro-apoptotik sel yang dirawat selama 48 jam pada kepekatan rendah (30 μg/mL) ekstrak telah ditingkatkan. Walau bagaimanapun, tahap penzahiran telah diturunkan apabila dirawat pada kepekatan yang tinggi ekstrak EA (120 μg/mL). Pada 72 jam rawatan, penzahiran kebanyakan gen pro-apoptotik terutamanya gen yang berkaitan dengan caspase seperti casp-3, -4, -9, BAK1 dan P21 didapati ditingkatkan. Sebaliknya, penzahiran gen yang terlibat di dalam rangkaian p53, terutamanya Bax dan ING3 serta gen anti-apoptosis (Bcl-2) didapati diturunkan. Penemuan ini membekalkan beberapa mekanisma apoptosis yang diaruh oleh ekstrak EA daripada *C. calcitrans*. Kesimpulannya, *C. calcitrans* lebih sitotoksik berbanding dengan *N. oculata* (ekstrak pelarut). Ekstrak EA daripada *C. calcitrans* menunjukkan aktiviti antioksidan dan sitotoksik yang terbaik berbanding yang lain, dan mengaruh apoptosis dalam sel MDA-MB-231. Aruhan apoptosis tersebut melibatkan gen pro-apoptotik yang penting: p21, casp-3, -4, -9 and Bak1.

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I certify that a Thesis Examination Committee has met on **19 September 2011** to conduct the final examination of Goh Su Hua on her thesis entitled "**Antioxidant and cytotoxic effects of crude extract from a diatom, *Chaetoceros calcitrans* and green algae, *Nannochloropsis oculata***" 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 at Universiti Putra Malaysia or at any other institution.

GOH SU HUA

Date: 19 September 2011

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