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THE MECHANISMS OF ACTION OF *ACANTHASTER PLANCI* ACTIVE FRACTION IN THE REGULATION OF *PCSK9* GENE EXPRESSION AND THE UPTAKE OF CHOLESTEROLS IN HUMAN HEPATOCELLULAR CARCINOMA CELL LINE, HepG2

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Atherosclerosis caused by an elevated level of cholesterols leads to numerous cardiovascular diseases (CVDs) which ranked as the leading cause of mortality globally. This pathophysiological condition leads to the impediment of blood flow to vital organs such as heart and brain. Unfortunately, the widely used drug, statin, to reduce cholesterols, produces adverse effects like muscle weakness and nausea and ineffective in high-risk patients consumed at high dosages. The discovery of proprotein convertase subtilisin-kexin type 9 (PCSK9) that plays a critical role in reducing the levels of LDL receptor (LDLR) and the uptake of cholesterols has paved a new way in providing a new therapeutic target in lowering the levels of cholesterols. Therefore, it is an urgent need to search for small molecule inhibitors that reduce the levels of *PCSK9*. Thus, the main aims of this study were to elucidate the mechanisms of action of natural products prepared from a marine organism, *Acanthaster planci*, in reducing the levels of *PCSK9* gene expression in human hepatocellular carcinoma cell line (HepG2), and its effect in increasing the levels of LDLR protein and the uptake of LDL-cholesterols (LDL-C). Cytotoxicity study using MTS assay showed methanolic extract and most fractions prepared from *A. planci* only significantly reduced the cell

growth at high concentration. The extract and fractions were then used to treat the cells that were transiently transfected with *PCSK9* promoter-luciferase constructs followed by luciferase assay. Fraction 2 of *A. planci* (EF2) showed the most potent activity in reducing the transcriptional activity of *PCSK9* promoter. This fraction exhibited the lowest level of *PCSK9* promoter to 30% of control at low concentration of 6.25 μg/mL. The effect of EF2 in downregulating the expression of *PCSK9* mRNA was determined by real time polymerase chain reaction (RT-PCR). The lowest expression levels of *PCSK9* mRNA at 41% of control were found when HepG2 cells were treated with 6.25 μg/mL of EF2. Seven 5'end deletion PCSK9 promoter fragments (D1-D7) were then used to determine the *cis*-acting elements responsible in mediating the inhibitory effects of EF2. Site-directed mutagenesis showed mutated peroxisome proliferator responsive elements (PPRE) attenuated the downregulatory effect of EF2 on *PCSK9* promoter activity indicating the interaction of PPRE and its corresponding transcription factor, PPARα played a major role in mediating the inhibitory action of EF2. Western blot analysis indicated that MEK-MAPK and PKCα components of signal transduction pathways were phosphorylated and activated by EF2 in reducing *PCSK9* expression. The involvement of MAPK and PKC pathways in the PCSK9 inhibitory action were further confirmed with the treatment of HepG2 cells with MEK inhibitor (PD98059) and PKCα inhibitor (HA-100 dihydrochloride) followed by real-time PCR. The results show that both inhibitors attenuated the inhibitory action of EF2 on the level of PCSK9 mRNA. EF2 was also used to determine its effect in regulating the protein levels of LDLR and LDL-C uptake by HepG2 cells by using immunocytochemistry. It was revealed that the levels of LDLR protein and the uptake of LDL-C were increased to the highest levels of 3.0 and 2.0-fold as compared to control, respectively in EF2 treated cells as compared to untreated control. The findings strongly indicate that *A. planci* EF2 produced a potent effect in reducing the gene expression levels of *PCSK9*, which in turn, increasing the protein levels of LDLR and more importantly, uptake of LDL-C. It is hoped that this study will pave the way for the natural products from *A. planci* to be developed as lipid lowering agent, and thus, as therapeutic intervention against the progression of atherosclerosis.

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MEKANISMA TINDAKAN FRAKSI AKTIF *ACANTHASTER PLANCI* DI DALAM PENGAWALATURAN PENGEKSPRESAN GEN *PCSK9* DAN PENGAMBILAN KOLESTEROL DALAM SEL KANSER HATI MANUSIA, HepG2

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Aterosklerosis yang disebabkan oleh peningkatan paras kolesterol yang tinggi membawa kepada pelbagai penyakit kardiovaskular (CVD) yang disenaraikan sebagai punca utama kematian di seluruh dunia. Keadaan patofisiologi ini menghalang pengaliran darah ke organ-organ penting seperti jantung dan otak. Malangnya, ubatan yang digunakan secara meluas, statin, untuk mengurangkan kolesterol telah menyebabkan kesan sampingan seperti melemahkan otot dan menyebabkan rasa loya dan kurang berkesan kepada pesakit berisiko tinggi walaupun statin diambil pada dos yang tinggi. Penemuan proprotein penukaran subtilisin-kexin jenis 9 (*PCSK9*) yang memainkan peranan penting dalam mengurangkan paras reseptor lipoprotein berketumpatan rendah (LDLR) dan pengambilan kolesterol telah membuka jalan baru dalam usaha menurunkan kadar kolesterol. Oleh itu, ianya menjadi satu keperluan yang mendesak untuk mencari molekul perencat kecil untuk mengurangkan *PCSK9*. Justeru, kajian ini dilaksanakan bertujuan untuk mengenalpasti mekanisme tindakan produk semulajadi daripada organisma laut, *Acanthaster planci*, di dalam mengurangkan tahap pengekspresan gen *PCSK9* dalam sel hati manusia, HepG2, dan potensinya dalam meningkatkan kadar protein LDLR dan pengambilan kolesterol (LDL-C). Kajian sitotoksisiti melalui asai MTS menunjukkan ekstrak metanol dan fraksi-fraksi yang disediakan daripada *A. planci* hanya mengurangkan pertumbuhan sel dengan ketara pada kepekatan tinggi. Ekstrak dan fraksi yang dihasilkan kemudiannya digunakan untuk menguji sel HepG2 yang ditransfeksikan dengan

konstruk promoter *PCSK9*-luciferase secara transien disusuli dengan asai luciferase. Fraksi 2 *A. planci* (EF2) menunjukkan potensi terbaik di dalam mengurangkan aktiviti transkripsi promoter *PCSK9*. Fraksi ini menghasilkan pengurangan aktiviti promoter *PCSK9* terendah kepada 30% berbanding sampel kawalan pada kepekatan rendah 6.25 μg/mL. Kesan EF2 di dalam menurunkan pengekspresan mRNA *PCSK9* ditentukan melalui reaksi berantai polimerase-masa nyata (RT-PCR). Tahap pengekspresan terendah mRNA *PCSK9* adalah 41% berbanding kawalan yang dihasilkan semasa sel HepG2 dirawat dengan 6.25 μg/mL EF2. Tujuh fragmen pemotongan penghujung 5' promoter *PCSK9* kemudian digunakan untuk mengenalpasti elemen tindakan *cis* yang bertanggungjawab dalam membantu kesan perencatan oleh EF2. Mutagenesis terarah-tapak menunjukkan elemen responsif proliferator peroksisom (PPRE) termutasi mengurangkan kesan pengurangan EF2 terhadap aktiviti promoter *PCSK9* yang menunjukkan interaksi PPRE dan faktor transkripsinya, PPARα memainkan peranan utama di dalam membantu tindakan perencatan EF2. Analisis Western Blot mendapati bahawa komponen MEK-MAPK dan PKCα dari jalan transduksi isyarat telah difosforilasi dan diaktifkan oleh EF2 di dalam mengurangkan pengekspresan *PCSK9*. Penglibatan jalan transduksi isyarat MAPK dan PKC dalam tindakan perencatan PCSK9 telah disahkan lagi dengan rawatan sel HepG2 dengan perencat MEK (PD98059) dan perencat PKCα (HA-100 dihydrochloride) diikuti oleh RT-PCR. Keputusan menunjukkan bahawa kedua-dua perencat melemahkan tindakan perencatan EF2 terhadap kadar mRNA PCSK9. EF2 juga digunakan untuk menentukan kesan di dalam mengawalatur kadar protein LDLR dan pengambilan LDL-C oleh sel HepG2 dengan menggunakan imunositokimia. Adalah didapati bahawa kadar protein LDLR dan pengambilan LDL-C meningkat ke tahap tertinggi iaitu masing-masing 3.0 dan 2.0 kali ganda berbanding dengan sampel kawalan. Dapatan ini menunjukkan bahawa EF2 *A. planci* menghasilkan kesan yang kuat di dalam mengurangkan kadar pengekspresan gen *PCSK9*, yang seterusnya meningkatkan kadar protein LDLR dan pengambilan LDL-C. Adalah diharapkan kajian ini akan membuka jalan agar produk semulajadi daripada *A. planci* dapat dibangunkan sebagai agen merendahkan kadar lipid, dan sebagai intervensi terapeutik terhadap perkembangan atherosklerosis.