UNIVERSITI PUTRA MALAYSIA

SUPPRESSION EFFECT OF EURYCOMANONE ON THE GROWTH OF HUMAN HEPATOMA CELLS (HEPG2) BY INDUCING p53-MEDIATED APOPTOTIC PATHWAY

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By

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Chairman: Professor Dr Asmah Rahmat, PhD

Faculty: Institute of Bioscience

Eurycomanone is a compound found in *Eurycoma longifolia* Jack and has been reported that it had a cytotoxic effect against various cancer cell lines. The aim of this study was to isolate eurycomanone from the roots of *E. longifolia*, investigate the cytotoxicity against human hepatoma cell line, HepG2, and determine the mode of action. *In vivo* study using nude mice as an animal model was also carried out to further confirm the ability of eurycomanone in liver cancer suppression. Eurycomanone was extracted from the roots of *E. longifolia*. The methanol extract was partitioned with diethylether, saturated with water. The aqueous soluble portion was further partitioned with butanol (BuOH) and water. The BuOH-soluble portion was subjected to silica gel column chromatography, TLC and finally HPLC to afford eurycomanone.

The anti-proliferation assay was carried out using the MTT Cell Proliferation Assay. The cells were treated with crude extract of *E. longifolia* (CE) and eurycomanone at increasing concentrations for 72 hours. The findings showed that CE inhibited cell proliferation towards human malignant melanoma cell (HM3KO), human cervical cancer cell (Hela), human liver cancer cell (HepG2) and human ovarian carcinoma cell ($CaOV_3$) with an IC_{50} of 60±0.25 µg/ml, 60±0.25 µg/ml, 45±0.15 µg/ml and 79±0.16 µg/ml respectively. The extracts did not inhibit the cell proliferation for both normal cell lines used, human normal skin cell (CCD11114sk), and human normal liver cells, Chang's liver. The activity of eurycomanone towards HepG2 gave an IC_{50} of 3.8±0.12 µg/ml and significantly increased apoptosis in HepG2 cells. Eurycomanone also showed less toxicity towards both normal liver cells, Chang's liver (17±0.15 µg/ml) and WLR-68 (20±0.22 µg/ml) as compared to tamoxifen (1.4±0.31 µg/ml) and vinblastine sulfate (4.2±0.37 µg/ml).

In vivo study confirmed the effect of eurycomanone in the inhibition of tumor growth. Nude mice were inoculated with HepG2 cells, subcutaneously in the right flank. When the tumor volume reached 100 mm^3, eurycomanone (6 mg/kg and 17 mg/kg) was applied intraperitoneally once a day, for 30 days. CE was also administered to the mice bearing tumor to compare the effectiveness between both of them. Data showed that tumor size in mice treated with eurycomanone was significantly reduced at concentration of 17 mg/kg compared to control and CE. Relative tumor growth ratio (TC) was calculated with percentage value of 39.9% and relative tumor volume (RTV)

of 1.5±0.09 was recorded. Growth reduction was associated with significantly reduced mitotic index. Hoechst 33258 staining was carried out *in vitro*, to prove the presence of apoptosis in HepG2 cells treated with eurycomanone (5 µg/ml). The characteristics of apoptosis including chromatin condensation, DNA fragmentation and apoptotic bodies were found following eurycomanone treatment. Further investigation on the cell cycle progression in HepG2 cells under eurycomanone treatment using a flow cytometry approach with PI staining was done. The cell cycle distribution was examined at various times and indicated doses. Vinblastine sulfate and genistein were used as a positive control. Eurycomanone appeared to affect processes that could inhibit the cell proliferation by inducing G2/M arrest in a time-dependent manner in HepG2 cells, with 39.9% of cells accumulated in G2/M phase. Flow cytometry with annexin-V/propidium iodide double staining was carried out to further confirm that eurycomanone induced apoptosis in HepG2 cells. Eurycomanone was shown to induce apoptosis in HepG2 cells in a time-dependent manner. After 72 hrs of exposure, only 5.6% cells were alive indicating that almost all of the cells underwent apoptosis. In the quadrant of annexin V^+/PI^-, 74.1% of the cells were detected. Increased cell population was observed at late apoptotic quadrant with a percentage of 15.3%. The protein expression of Bcl-2, Bax, p53 and cytochrome C were studied via flow cytometry in order to find the mechanism of action of eurycomanone. This study found that the apoptotic process triggered by eurycomanone involves the up-regulation of p53 tumor suppressor protein. The increased of p53 was followed by an increase of pro-apoptotic Bax and decrease of anti-

apoptotic Bcl-2. Active Bax and inactive Bcl-2 induced the level of cytochrome C which leads to apoptosis.

In conclusion, this present study indicated that eurycomanone has cytotoxic effect towards HepG2 cells. *In vivo* study suggested that eurycomanone has a high potential in inhibiting solid tumor growth in mice. These findings also concluded that the anticancer effect of eurycomanone against HepG2 cells was via inducing apoptosis through the up-regulation of p53 and Bax, and down-regulation of Bcl-2 which increased the levels of cytochrome C.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN PERENCATAN EURIKOMANON KE ATAS PERTUMBUHAN SEL HEPATOMA MANUSIA (HEPG2) DENGAN MENGARUH TAPAKJALAN APOPTOTIK DIPERANTARA p53

Oleh

Yusmazura Zakaria

September 2009

Pengerusi: Profesor Dr Asmah Rahmat

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Eurikomanon merupakan sebatian yang terkandung di dalam *E. longifolia* Jack di mana kajian terdahulu menunjukkan bahawa ia mempunyai kesan ketoksikan terhadap beberapa jenis titisan sel kanser. Kajian ini bertujuan untuk memencilkan eurikomanon daripada *E. longifolia*, mengkaji kesan ketoksikan terhadap sel kanser hepar manusia, HepG2 dan menentukan mekanisme tindakannya. Kajian secara *in vivo* menggunakan tikus 'nude' sebagai model haiwan juga dilakukan untuk mengesahkan lagi keupayaan eurikomanon dalam merencat pertumbuhan sel kanser hepar. Eurikomanon diekstrak daripada akar *E. Longifolia*. Ekstrak metanol dipartisikan dengan dietileter dan air. Fraksi larut air kemudian dipartisikan dengan butanol dan air. Fraksi larut butanol seterusnya dilakukan kromatografi silika gel, TLC dan HPLC bagi mendapatkan sebatian aktif eurikomanon.

Asai antiproliferatif dijalankan menggunakan kaedah MTT. Sel didedahkan dengan ekstrak kasar *E. Longifolia* (EK) dan eurikomanon pada kepekatan yang semakin meningkat selama 72 jam. Hasil kajian menunjukkan ekstrak kasar *E. longifolia* merencat proliferasi sel kanser kulit manusia (HM_3KO), sel kanser serviks manusia (Hela), sel kanser hati manusia (HepG2) dan sel kanser ovari manusia (CaOV3) dengan IC_{50} masing-masing 60±0.25 µg/ml, 60±0.25 µg/ml, 45±0.15 µg/ml dan 79±0.16 µg/ml. Ekstrak kasar tidak menunjukkan kesan perencatan pada kedua-dua sel normal yang digunakan iaitu sel normal kulit manusia (CCD11114sk) dan sel normal hepar manusia, Chang's liver. Eurikomanon juga memberikan aktiviti antikanser yang tinggi terhadap sel HepG2. Eurikomanon secara signifikan merencat pertumbuhan sel HepG2 dengan IC_{50} 3.8±0.12 µg/ml dan mengaruh apoptosis. Eurikomanon juga menunjukkan kesan ketoksikan yang lebih rendah terhdap kedua-dua sel normal hepar manusia, Chang's liver (17±0.15 µg/ml) dan WLR-68 (20±0.22 µg/ml) jika dibandingkan dengan tamoxifen (1.4±0.31 µg/ml) dan vinblastin sulfat (4.2±0.15 µg/ml).

Kajian *in vivo* mengesahkan kesan eurikomanon dalam merencat pertumbuhan kanser. Tikus 'nude' diinokulat dengan sel HepG2 secara suntikan bawah kulit pada bahagian rusuk kanan. Apabila isipadu tumor mencapai 100 mm^3, eurikomanon (6 mg/kg dan 17 mg/kg) diberikan secara suntikan bawah perut, sekali sehari selama 30 hari. EK juga diberikan kepada tikus yang telah ditumbuhi tumor untuk membandingkan keberkesanan antara keduanya. Data menunjukkan saiz tumor yang dirawat eurikomanon menurun secara signifikan pada kepekatan 17 mg/kg

dibandingkan dengan kawalan dan EK. 39.9% nilai TC (nisbah pertumbuhan relatif tumor) dicatat dengan RTV (isipadu relatif tumor) 1.5±0.09. Penurunan kadar pertumbuhan selari dengan penurunan indeks mitotik.

Pewarnaan Hoechst 33258 dilakukan untuk mengesahkan bahawa apoptosis berlaku dalam sel yang dirawat eurikomanon. Ciri-ciri apoptosis termasuk kondensasi kromatin, fragmentasi DNA dan kehadiran badan apoptotik dikesan berikutan rawatan eurikomanon. Kajian selanjutnya dijalankan untuk melihat kesan eurikomanon dalam merencat kitar sel menggunakan sitometer aliran. Vinblastin sulfat dan genistein diguna sebagai kawalan positif. Eurikomanon didapati memberi kesan terhadap sel HepG2 dengan mengaruh penahanan sel pada fasa G2/M bergantung kepada masa pendedahan dengan 39.9% sel dicatatkan. Sitometer aliran menggunakan annexin-V/PI dilakukan untuk mengesahkan lagi bahawa eurikomanon mengaruh apoptosis pada sel HepG2. Selepas 72 jam diberikan eurikomanon, hanya 5.6% sel yang masih hidup, menunjukkan sebahagian besarnya telah mengalami apoptosis. 7.1% daripada sel dikesan dalam kuadrant V^+/PI^-, tetapi peningkatan populasi sel dicatat pada kuadrant fasa akhir apoptosis dengan nilai 15.3%. Kajian terhadap pengekspresan protein Bcl-2, Bax, p53 dan sitokrom C dilakukan untuk mengenalpasti mekanisme tindakan eurikomanon. Antibodi spesifik terhadap protein-protein tersebut digunakan dalam analisis menggunakan sitometer aliran. Hasil mendapati proses apoptosis yang dicetuskan oleh eurikomanon melibatkan peningkatan protein p53. Peningkatan p53 diikuti oleh peningkatan Bax dan penurunan

Bcl-2 yang seterusnya membawa kepada peningkatan sitokrom C yang akan mengaktifkan proses apoptosis.

Kesimpulannya, hasil kajian mencadangkan eurikomanon mempunyai potensi yang tinggi dalam merencat pertumbuhan tumor *in vivo*. Berdasarkan kajian ini juga, dapat disimpulkan bahawa eurikomanon adalah toksik terhadap sel HepG2 dengan mengaruh apoptosis melalui peningkatan p53 dan Bax, dan menurunkan aras Bcl-2 yang akan mengaruh peningkatkan aras sitokrom C.

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Yusmazura

2009

I certify that a Thesis Examination Committee has met on **15 September 2009** to conduct the final examination of **Yusmazura Zakaria** on or her thesis entitled **"Eurycomanone Suppressed the Growth of Human Hepatoma Cell (HepG2) Via p53-Mediated Apoptotic Pathway"** 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 **Doctor of Philosophy**.

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This thesis submitted to the Senat of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been fully acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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YUSMAZURA ZAKARIA

Date: 10 December 2009

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

γ-GT	gamma-glutamyl transpeptidase
ACN	Acetonitrile
ACUC	Animal Care Unit Commitee
AFB1	Aflatoxin B1
AFP	Alfa-fetoprotein
AIDS	Acquired immune deficiency syndrome
AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease activating factor-1
ASEAN	Association of Southeast Asian Nations
ATCC	American Type Culture Collection
BH	Bcl-2 homology domains
BuOH	Butanol
BW	Body weight
CaOV3	Human ovarian carcinoma cell
CCD11114sk	Human normal skin cell
CDK	Cyclin-dependent kinases
CE	Crude extract of *E. longifolia*
CKI	Cyclin-dependent kinase inhibitor
DCP	des-γ-carboxy prothrombin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethyline diamine tetra acetic acid
EGCG	Epigallocathecin gallate
ELISA	Enzyme-Linked Immunosorbent Assay