



UNIVERSITI PUTRA MALAYSIA

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

YUSMAZURA ZAKARIA

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By

YUSMAZURA ZAKARIA

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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September 2009

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 (CaOV₃) with an IC₅₀ 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 IC₅₀ 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 mm³, 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 $\mu\text{g/ml}$). 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.



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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

Fakulti: Institut Biosains

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 (HM₃KO), sel kanser serviks manusia (Hela), sel kanser hati manusia (HepG2) dan sel kanser ovari manusia (CaOV3) dengan IC₅₀ 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 IC₅₀ 3.8±0.12 µg/ml dan mengaruh apoptosis. Eurikomanon juga menunjukkan kesan ketoksikan yang lebih rendah terhadap 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 mm³, 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**.

Members of the Thesis Examination Committee were as follows:

Sabariah Abd Rahman, PhD

Associate Professor
Institute of Bioscience
Universiti Putra Malaysia
(Chairman)

Rozita Rosli, PhD

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Chong Pei Pei, PhD

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Yasmin Anum Mohd Yusof, PhD

Professor
Faculty of Medicine and Health Sciences
Universiti Kebangsaan Malaysia
Cheras, Kuala Lumpur
Malaysia
(External Examiner)

BUJANG BIN KIM HUAT, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 15 December 2009



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:

Asmah Rahmat, PhD

Professor
Faculty Medicine and Health Science
Universiti Putra Malaysia
(Chairman)

Fauziah Othman, PhD

Professor
Faculty Medicine and Health Science
Universiti Putra Malaysia
(Member)

Abdah Md. Akim, PhD

Faculty Medicine and Health Science
Universiti Putra Malaysia
(Member)

Azimahtol Hawariah Lope Pihie, PhD

Professor
Faculty Science and Technology
Universiti Kebangsaan Malaysia
(Member)

Noor Rain Abdullah, PhD

Research Officer
Bioassay Unit
Institute for Medical Research
(Member)

HASANAH MOHD GHAZALI, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 10 December 2009



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.

YUSMAZURA ZAKARIA

Date: 10 December 2009



TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	v
ACKNOWLEDGEMENTS	ix
APPROVAL	x
DECLARATION	xi
TABLE OF CONTENTS	xiii
LIST OF TABLES	xvi
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xxii

CHAPTER

1	INTRODUCTION	
	1.1 Overview	1
	1.2 Objective of study	
	1.2.1 General objective	6
	1.2.2 Specific objective	6
2	LITERATURE REVIEW	
	2.1 Cancer	8
	2.1.1 Classification of cancer	8
	2.1.2 Epidemiology of cancer	9
	2.1.3 Treatment of cancer	12
	2.2 Cell Biology	15
	2.3 Cell Death	18
	2.3.1 Apoptosis	18
	2.3.2 Necrosis	23
	2.4 Liver Cancer	24
	2.4.1 Treatment of liver cancer	29
	2.4.2 Cancer Chemoprevention	34
	2.5 Traditional Medicine	35
	2.6 Natural Products in Anti-cancer Treatment	38
	2.7 <i>Eurycoma longifolia</i> Jack	46
	2.7.1 <i>E. longifolia</i> uses described in folk medicine	48
	2.7.2 Phytochemistry	49
	2.7.3 Biological activities	51



3	CYTOTOXICITY AND PHYTOCHEMICAL STUDY	
3.1	Introduction	54
3.1.1	Chemotherapeutic targets	54
3.2	Cytotoxicity Assay	56
3.2.1	MTT assay	57
3.3	Cell Culture and Cytotoxicity Test	58
3.3.1	Materials and methods	58
3.3.2	Sample preparation	60
3.3.3	MTT assay protocol	61
3.4	Phytochemical Investigation of <i>E. longifolia</i>	62
3.4.1	Preparation of plant material	62
3.4.2	Extraction and isolation of eurycomanone	62
3.5	Cytotoxicity Assay of Eurycomanone against HepG2 Cell	65
3.6	Results and Discussion	65
3.6.1	Preliminary screening of <i>E. longifolia</i> towards several cancer cell lines	65
3.6.2	Extraction and isolation of eurycomanone	71
3.6.3	Cytotoxicity activity of eurycomanone towards HepG2 cell	79
4	THE EFFECTS OF EURYCOMANONE IN AN ANIMAL MODEL	
4.1	Introduction	88
4.2	Materials	90
4.2.1	Chemicals	90
4.2.2	Cell lines	90
4.2.3	Animal	90
4.3	Methodology	91
4.3.1	Sample preparation	91
4.3.2	Cell culture and preparation for transplantation	91
4.3.3	Human tumor xenotransplant	92
4.3.4	Body weight and tumor measurement	93
4.3.5	Histology study	93
4.4	Results and Discussion	94
4.4.1	Inhibitory effects of <i>E. longifolia</i> crude extract and eurycomanone towards xenotransplanted nude mice	94
5	THE MECHANISM OF ACTION OF EURYCOMANONE	
5.1	Introduction	115
5.1.1	The Bcl-2 family members	117
5.1.2	The involvement of p53 with Bcl-2 proteins in apoptosis	125

5.2	Materials and Methods	135
5.2.1	Morphology evaluation of apoptosis	135
5.2.2	Determination of cell cycle by DNA analysis	137
5.2.3	Apoptosis detection by Annexin V- FITC staining	138
5.2.4	Detection of proteins involve in Apoptosis (Bcl-2; Bax; p53; Cytochrome C)	140
5.3	Results and Discussion	141
5.3.1	Morphology of apoptosis Observation by phase contrast microscopy	141
5.3.2	Morphology of apoptosis by Hoechst staining	146
5.3.3	Cell cycle analysis	148
5.3.4	Apoptosis detection by flow cytometry	164
5.3.5	The involvement of pro- and anti- Apoptotic proteins in eurycomanone mediated apoptosis	183
6	CONCLUSION	198
	REFERENCES	205
	APPENDICES	243
	BIODATA OF STUDENT	258

LIST OF TABLES

Table	Page
2.1 Terminology of cancer	10
2.2 Cancer incidence by sex in Malaysia	13
2.3 Differences between necrosis and apoptosis	20
2.4 Summary of anticancer agents derived from natural products.	43
3.1 Summary of antiproliferative effect of crude extract and eurycomanone from the roots of <i>Eurycoma longifolia</i> and tamoxifen as a control drug.	68
4.1 Mitotic index of CE and eurycomanone treatment on subcutaneous xenotransplanted tumor, HepG2, in nude mice.	109
5.1 The correlations of tumor markers with the p53 status in HCC.	133

LIST OF FIGURES

Figure		Page
2.1	Ten most frequent cancer in male and female in Malaysia in 2002	14
2.2	Diagram cell cycle phases	16
2.3	Illustration of morphological differences between necrosis and apoptosis.	19
2.4	<i>Eurycoma longifolia</i> Jack.	47
2.5	Structure of eurycomanone	52
3.1	Extraction procedure for <i>E. longifolia</i> material.	63
3.2	Dose-response curve of <i>E. longifolia</i> crude extract against malignant cells HM3KO, Hela, HepG2 and CaOV3 and non-malignant cells, CCD11114sk and Chang's liver.	67
3.3	Dose-response curve of positive control drug tamoxifen against malignant cells HM3KO, Hela, HepG2 and CaOV3 and non-malignant cells, CCD11114sk and Chang's liver.	70
3.4	HPLC result after columns chromatography of butanol extract of <i>E. longifolia</i> .	73
3.5	Quantitative determination of eurycomanone by HPLC by Nova Laboratories Sdn. Bhd.	74
3.6	HPLC result after second columns chromatography of butanol extract of <i>E. longifolia</i> .	75
3.7	The TLC result of fractions F1, F2, F3, F4, F4 and F6 with compared to standard eurycomanone.	77
3.8	HPLC profile for F1 to F6 using C18 column (30mm) with mobile phase 45% ACN.	78
3.9	Dose-response curve of eurycomanone which significantly shown a reduced number of viable HepG2 cells in a dose dependent manner.	80

3.10	Dose-response curve of eurycomanone which significantly shown a reduced number of viable Chang's Liver cells in a dose dependent manner.	81
3.11	Dose response curve of eurycomanone and vinblastine sulfate which significantly shown a reduced number of viable WLR-68 cells in a dose dependant manner.	83
4.1	Figures (A-G) showed the development and tumor progression of nude mice from day 0 to day 30.	97
4.2	The photographs of isolated subcutaneous xenograft tumor of human hepatoma in nude mice.	98
4.3	The graph of mean tumor weight of nude mice treated with CE and eurycomanone.	100
4.4	The effect of eurycomanone and CE against tumor volume of nude mice.	102
4.5	Animal body weight curve.	103
4.6	RTV at day n (0,3,6,9,12,15,18,21,24,27,30) and the efficacy of CE and eurycomanone in tumor suppression.	105
4.7	Percentage of relative tumor growth ratio in CE and eurycomanone towards nude mice bearing tumor, HepG2.	106
4.8	Inhibitory effects of CE and eurycomanone on tumor implantation, HepG2 in nude mice.	107
5.1	Homology of Bcl-2 family proteins.	119
5.2	Role of Bcl-2 family proteins in the life - death decision point within the common pathway of apoptosis.	122
5.3	Morphological characteristics of untreated HepG2 cells visualized with a phase-contrast microscope for 24 h, 48 h and 72 h.	143
5.4	Morphological characteristics of HepG2 cells treated with CE visualized with a phase-contrast microscope for 24 h, 48 h and 72 h.	144
5.5	Morphological characteristics of HepG2 cells treated with eurycomanone visualized with a phase-contrast microscope for 24 h, 48 h and 72 h.	145

5.6	Morphological characteristics of HepG2 cells treated with tamoxifen visualized with a phase-contrast microscope for 24 h, 48 h and 72 h.	147
5.7	Hoecsht 33258 nuclear staining of untreated HepG2 cells for 24 h, 48 h and 72 h.	149
5.8	Hoecsht 33258 nuclear staining of HepG2 cells treated with eurycomanone for 24 h, 48 h and 72 h.	150
5.9	Hoecsht 33258 nuclear staining of HepG2 cells treated with tamoxifen for 24 h, 48 h and 72 h.	151
5.10	DNA content histogram of untreated HepG2 cells for 24 h, 48 h and 72 h.	153
5.11	The histogram show untreated HepG2 cells (negative control).	154
5.12	DNA content histogram of HepG2 cells treated with eurycomanone (5 µg/ml) for 24 h, 48 h and 72 h.	155
5.13	The histogram show HepG2 cells treated with eurycomanone (5 µg/ml).	156
5.14	DNA content histogram of HepG2 cells-treated vinblastine sulfate (2.5 µg/ml) for 24 h, 48 h and 72 h.	159
5.15	The histogram show HepG2 cells treated with vinblastine sulfate (2.5 µg/ml).	160
5.16	DNA content histogram of HepG2 cells treated with genistein (5 µg/ml) for 24 h, 48 h and 72 h.	161
5.17	The histogram show HepG2 cells treated with genistein (5 µg/ml).	162
5.18	Scatter plots of Ann/PI-stained untreated HepG2 cells under four situations in quadrant analysis; living cells (A3), apoptotic cells (A4), late apoptotic or dead cells (A2) and necrotic or dead cells (A1). Exposed for 24 h, 48 h and 72 h.	166
5.19	The histogram show the percentage of viable, apoptotic and necrotic of untreated HepG2 cells (negative control), analyzed by flow cytometry.	167

5.20	Scatter plots of Ann/PI-stained HepG2 cells treated with eurycomanone (5 µg/ml), under four situations in quadrant analysis; living cells (A3), apoptotic cells (A4), late apoptotic or dead cells (A2) and necrotic or dead cells (A1). Exposed for 24 h, 48 h and 72 h.	168
5.21	The histogram show the percentage of viable, apoptotic and necrotic of HepG2 cells treated with eurycomanone (5 µg/ml), analyzed by flow cytometry.	170
5.22	Scatter plots of Ann/PI-stained HepG2 cells treated with vinblastine sulfate (2.5 µg/ml), under four situations in quadrant analysis; living cells (A3), apoptotic cells (A4), late apoptotic or dead cells (A2) and necrotic or dead cells (A1). Exposed for 24 h, 48 h and 72 h.	171
5.23	The histogram show the percentage of viable, apoptotic and necrotic of HepG2 cells treated with vinblastine sulfate (2.5 µg/ml), analyzed by flow cytometry.	173
5.24	Scatter plots of Ann/PI-stained HepG2 cells treated with genistein (5 µg/ml) under four situations in quadrant analysis; living cells (A3), apoptotic cells (A4), late apoptotic or dead cells (A2) and necrotic or dead cells (A1). Exposed for 24 h, 48 h and 72 h.	174
5.25	The histogram show the percentage of viable, apoptotic and necrotic of HepG2 cells treated with genistein (5 µg/ml), analyzed by flow cytometry.	175
5.26	Scatter plots of Ann/PI-stained untreated WLR-68 cells under four situations in quadrant analysis; living cells (A3), apoptotic cells (A4), late apoptotic or dead cells (A2) and necrotic or dead cells (A1). Exposed for 24 h, 48 h and 72 h.	176
5.27	The histogram show the percentage of viable, apoptotic and necrotic of untreated WLR-68 cells (positive control), analyzed by flow cytometry.	177
5.28	Scatter plots of Ann/PI-stained WLR-68 cells treated with eurycomanone (5 µg/ml) under four situations in quadrant analysis; living cells (A3), apoptotic cells (A4), late apoptotic or dead cells (A2) and necrotic or dead cells (A1). Exposed for 24 h, 48 h and 72 h.	178
5.29	The histogram show the percentage of viable, apoptotic and necrotic of WLR-68 cells treated with eurycomanone (5 µg/ml), analyzed by flow cytometry.	180

5.30	Scatter plots of Ann/PI-stained WLR-68 cells treated with vinblastine sulfate (2.5 µg/ml) under four situations in quadrant analysis; living cells (A3), apoptotic cells (A4), late apoptotic or dead cells (A2) and necrotic or dead cells (A1). Exposed for 24 h, 48 h and 72 h.	181
5.31	The histogram show the percentage of viable, apoptotic and necrotic of WLR-68 cells treated with vinblastine sulfate (2.5 µg/ml), analyzed by flow cytometry.	182
5.32	The profile of Bax expression proteins for untreated HepG2 cells, HepG2 cells treated with eurycomanone and vinblastine sulfate at 24 h, 48 h and 72 h.	186
5.33	The profile of Bcl-2 expression protein for untreated HepG2 cells, HepG2 cells treated with eurycomanone and vinblastine sulfate at 24 h, 48 h and 72 h.	187
5.34	The profile of p53 expression proteins for untreated HepG2 cells, HepG2 cells treated with eurycomanone and vinblastine sulfate at 24 h, 48 h and 72 h.	190
5.35	The profile of cytochrome C expression proteins for untreated HepG2 cells, HepG2 cells treated with eurycomanone and vinblastine sulfate at 24 h, 48 h and 72 h.	191
5.36	The histogram of the expression levels of Bcl-2, Bax, p53 and Cytochrome C in untreated HepG2 cells, HepG2 cells treated with eurycomanone and HepG2 cells treated with vinblastine sulfate.	192
6.1	The propose mechanism of action for eurycomanone-mediated apoptosis in human liver cancer cells, HepG2.	204

LIST OF ABBREVIATIONS

γ -GT	gamma-glutamyl transpeptidase
ACN	Acetonitrile
ACUC	Animal Care Unit Committee
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 <i>E. longifolia</i>
CKI	Cyclin-dependent kinase inhibitor
DCP	des- γ -carboxy prothrombin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EGCG	Epigallocatechin gallate
ELISA	Enzyme-Linked Immunosorbent Assay

