



UNIVERSITI PUTRA MALAYSIA

**IDENTIFICATION OF PROTEIN KINASE INHIBITORY ACTIVITIES
FROM STREPTOMYCES STRAIN H7372 FOR POTENTIAL USE AS
ANTI-CANCER AGENT**

MASRIANA HASSAN

FPSK(M) 2007 18

02 SEP 2009

**IDENTIFICATION OF PROTEIN KINASE INHIBITORY ACTIVITIES FROM
STREPTOMYCES STRAIN H7372 FOR POTENTIAL USE AS ANTI-CANCER
AGENT**

By

MASRIANA HASSAN

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

February 2007



Specially dedicated to,

***My husband, Mohd. Solvee, son, Muhammad Sofwan Wazeen, daughter Sofea
Adriana and parents, Hassan and Sapura***

For their love, understanding, encouragement, patience and moral support.

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

**IDENTIFICATION OF PROTEIN KINASE INHIBITORY ACTIVITIES FROM
STREPTOMYCES STRAIN H7372 FOR POTENTIAL USE AS ANTI-CANCER
AGENT**

By

MASRIANA HASSAN

February 2007

Chairman: Professor Seow Heng Fong, PhD

Faculty: Medicine and Health Sciences

Aberrations in the phosphatidylinositol 3-kinase (PI3K)/Akt pathway have been found in a wide spectrum of human cancers. Activation of Akt and inactivation of the downstream substrates such as GSK-3 β , BAD and Forkhead family (FKHR) proteins are relevant to promote cell survival, proliferation and growth. Another related pathway linked with PI3K/Akt is the Ras/Raf/MEK/ERK, which is known to promote cancer as a result of ras-transformation. The discovery of new drugs targeted at specific molecules of these pathways is a 'hot' field in cancer research. Blocking the constitutively active PI3K/Akt pathway provides a new strategy for cancer therapy. Thus, inhibitors of this signaling pathway would be potential anti-cancer agents. The *Streptomyces* strain H7372 isolated from mangrove soils in Sabah was found to inhibit the Ras/Raf-1 protein interaction in the yeast two-hybrid screening system. The present study was undertaken to determine the cytotoxic effect of H7372 fractionated extract on a breast cancer cell line, MCF-7 and a non-tumorigenic epithelial cell line, MCF-10A and quantitatively measure kinase inhibition, apoptosis induction and cell cycle disruption. The crude

extracts of H7372 were fractionated into eight fractions using reverse phase HPLC. Fraction 5 was found to be the most cytotoxic in an MTT assay. The crude extract and fraction 5 of H7372 were found to exert growth inhibition of MCF-7 at IC_{50} of 15 μ g/ml and 1.4 μ g/ml, respectively. Western blot analyses showed that activated PI3K and Akt (Thr308) but not Akt (Ser473) by stimulation of IGF-I were inhibited by the crude extract and fraction 5 after 72 hours. Interestingly, phosphorylation of Raf-1 (Ser259) and ERK1 were also inhibited by fraction 5, indicated that there is a cross-talk between PI3K/Akt and MAPK pathways. By using the flow cytometry technique, we found that fraction 5 inhibited the proliferation of MCF-7 cell line by causing them to arrest in the G_1 phase of the cell cycle. The induction of growth arrest by fraction 5 was associated with accumulation of cells in G_1 and decreasing cells in S and G_2/M phases. The results were supported by inhibition of cyclin D1 in MCF-7 cells. The apoptosis study showed that fraction 5 but not crude extract was increased the percentage of cells in early apoptotic stage at all concentrations. Furthermore, treatment of MCF-7 cells with fraction 5 resulted in reduction in phosphorylation of GSK-3 β (Ser9), phospho-BAD (Ser112) and phospho-FKHR (Ser256). These results could contribute the apoptosis in MCF-7 cell line. Thus, we discovered that fraction 5 of H7372, a naturally occurring microbe, contains an inhibitor of cell proliferation, cell cycle progression and is able to induce apoptosis via the PI3K/Akt and MAPK pathways.



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

**PENGENALPASTIAN AKTIVITI PERENCAT PROTEIN KINASE DARIPADA
STREPTOMYCES STRAIN H7372 BAGI KEGUNAAN YANG BERPOTENSI
SEBAGAI AGEN ANTI-KANSER**

Oleh

MASRIANA HASSAN

Februari 2007

Pengerusi: Profesor Seow Heng Fong, PhD

Fakulti: Perubatan dan Sains Kesihatan

Gangguan dalam lintasan phosphatidylinositol 3-kinase (PI3K)/Akt (Protein Kinase B) telah ditemui secara meluas di dalam kanser manusia. Pengaktifan Akt dan penyahaktifan substrat bawahan seperti protein GSK-3 β , BAD and Forkhead family (FKHR) adalah berkaitan bagi pemanjangan hayat, penambahan dan pertumbuhan sel. Lintasan lain yang berkaitan dengan PI3K/Akt adalah Ras/Raf/MEK/ERK di mana ia telah dikenalpasti bagi menyebabkan kanser yang disebabkan oleh transformasi raf. Penemuan ubat-ubatan baru bagi sasaran molekul yang spesifik untuk lintasan ini adalah bidang yang hangat dalam kajian kanser. Penghalangan lintasan PI3K/Akt yang aktif berterusan memberi strategi baru untuk terapi kanser. Oleh itu, perencat lintasan isyarat ini akan menjadi agen antikanser yang berpotensi. *Streptomyces* strain H7372 yang diasingkan daripada tanah paya bakau di Sabah telah merencat interaksi protein Ras/Raf-1 di dalam sistem penyaringan 'yeast two-hybrid'. Kajian ini telah dilakukan untuk menentukan kesan sitotoksik oleh ekstrak H7372 yang telah difraksi terhadap sel kanser payudara, MCF-7 dan sel epithelium bukan tumor, MCF-10A, serta mengukur secara

kuantiti perencatan kinase, induksi apoptosis dan pemusnahan kitaran sel. Ekstrak mentah H7372 telah difraksikan kepada lapan fraksi menggunakan fasa berbalik HPLC. Fraksi ke-5 telah dikenalpasti sebagai fraksi yang paling sitotoksik di dalam asei MTT. Ekstrak mentah telah dikenalpasti dapat merencat pertumbuhan pada $IC_{50} 15 \mu\text{g/ml}$ manakala, fraksi ke-5 pada $1.4 \mu\text{g/ml}$. Analisis dari kaedah 'Western blot' telah menunjukkan bahawa pengaktifan PI3K dan Akt (Thr308) tetapi bukan Akt (Ser473) dengan rangsangan IGF-I telah direncat oleh ekstrak mentah dan fraksi ke-5 selepas 72 jam. Fosforilasi Raf-1 (ser259) dan ERK 1/2 juga telah direncat oleh fraksi ke-5 dan ianya menunjukkan bahawa terdapatnya hubungan antara lintasan PI3K/Akt dan lintasan MAPK. Dengan menggunakan teknik 'flow cytometry', kami mendapati bahawa fraksi ke-5 telah merencat penambahan sel MCF-7 dan dengan ini menyebabkan sel tersebut ditahan pada fasa G_1 di dalam kitaran sel. Induksi bagi penahanan pertumbuhan oleh fraksi ke-5 telah menyebabkan pengumpulan sel di dalam fasa G_1 dan mengurangkan jumlah sel di dalam fasa S dan G_2/M . Keputusan-keputusan ini telah disokong oleh perencatan cyclin D1 dalam sel MCF-7. Kajian apoptosis telah menunjukkan bahawa fraksi ke-5 tetapi bukan ekstrak mentah telah meningkatkan peratusan sel pada tahap awal apoptosis bagi setiap kepekatan. Tambahan pula, rawatan sel MCF-7 oleh fraksi ke-5 telah menyebabkan penurunan di dalam fosforilasi GSK-3 β (Ser9), BAD (Ser112) dan FKHR (Ser256). Keputusan-keputusan ini boleh menyumbang kepada apoptosis bagi sel MCF-7. Oleh itu, kajian ini telah mengenalpasti bahawa fraksi ke-5 daripada mikroorganisma semulajadi mengandungi perencat bagi penambahan sel, progresi kitaran sel dan juga berupaya untuk merangsang apoptosis melalui lintasan PI3K/Akt dan MAPK.



ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Prof. Dr. Seow Heng Fong for her invaluable guidance, encouragement and endless support throughout this study. Her careful review and constructive criticism have been crucially important for this thesis.

Special thanks go to my co-supervisors, Prof. Ho Coy Choke, Dr. Maha Abdullah and Dr. Anthony Ho Siong Hock for their advices and assistance throughout the entire progress of this study.

My sincere thanks go to Dr. Khor Tin Oo, Cheah Hween-Yee, Foo Sek Hin, Puah Seok Hwa, Lim Pei Ching, Leong Pooi Pooi, Loh Hui Woon and Yip Wai Kien, thank you for being caring and readily assisting me when I need it most.

I am indebted to my labmates, See Hui Shien, Leslie Than, Jee Jap Meng, Vincent, Siti Aishah and Mr. Anthonysamy for their collaboration and sharing everyday joys and miseries in the making of science.

Last but not least, I would like to express my heartfelt gratitude to my husband, Mohd. Solvee, thank you for his love and patient, encouragement and endless support throughout this study. Especially thanks to my beloved parents for their understanding and support during the entire study in UPM.

This thesis 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 are as follows:

Seow Heng Fong, PhD

Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Ho Coy Choke, PhD

Professor
School of Science and Technology
Universiti Malaysia Sabah
(Member)

Maha Abdullah, PhD

Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Anthony Ho Siong Hock, PhD

Lecturer
Faculty of Engineering and Science
Universiti Tunku Abdul Rahman
(Member)

AINI IDERIS, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 10 MAY 2007

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	v
ACKNOWLEDGEMENTS	vii
APPROVAL	viii
DECLARATION	x
LIST OF TABLES	xv
LIST OF FIGURES	xvi
LIST OF ABBREVIATIONS	xxiii
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	4
2.1 Cell signaling and Carcinogenesis	4
2.2 Growth Factors as activators of protein kinases	6
2.2.1 Insulin-like Growth Factor	6
2.3 PI3K/Akt Signaling Pathway	8
2.3.1 Phosphatidylinositol 3-kinase (PI3K)	8
2.3.2 Akt (Protein Kinase B)	11
2.3.3 Akt targets in cell survival- the apoptotic machinery	14
2.3.3.1 Bcl-2 family	15
2.3.3.2 Transcription factor Forkhead (FKHR)	16
2.4 Glycogen Synthase Kinase-3 β	17
2.5 Mitogen Activated Protein Kinase (MAPK) Signaling Pathway	19
2.5.1 ERKs signaling pathway	19
2.5.2 Cross-talk between PI3K/Akt and MAPK signaling pathways	21
2.6 Signal transduction and chemotherapeutic agents	22
2.6.1 MEK inhibitor	23
2.6.2 Inhibitors of PI3K/Akt signaling pathway	24
2.7 Microbial secondary metabolites	26
2.7.1 Actinomycetes	27
2.7.2 <i>Streptomyces sp.</i> as producer of protein kinase inhibitors	28
2.7.2.1 Staurosporine	28
2.7.2.2 Geldanamycin	29
2.7.2.3 Actinomycetes, strain H7372	31
2.8 Apoptosis	33
2.9 Necrosis	38

2.10	Cell Cycle	39
2.10.1	Cell cycle and carcinogenesis	40
2.10.2	Cell cycle regulation by signaling pathway	41
3	MATERIALS AND METHODS	43
3.1	Materials	43
3.1.1	Cell lines	43
3.1.2	Actinomycetes extract	43
3.2	Culture of actinomycetes extract	43
3.2.1	Growth media: Oatmeal Agar	43
3.2.2	Storage and maintainance of actinomycetes strains	44
3.2.3	Aerobic liquid culture of actinomycetes	44
3.2.4	Extraction of secondary metabolites	45
3.2.5	Freeze drying of extract	45
3.3	High Performance Liquid Chromatography (HPLC)	45
3.3.1	Sample pretreatment	47
3.4	Cell culture	47
3.4.1	Maintenance and subculture of the MCF-7 cell line	47
3.4.2	Maintenance and subculture of the MCF-10A cell line	48
3.5	Trypan blue exclusion test	49
3.6	MTT assay	50
3.7	Preparation of extract	50
3.7.1	Cells treatment with H7372 or fractions and stimulation of insulin or IGF-1	51
3.8	Analysis of inhibition of protein kinases by Western Blot	51
3.8.1	Cell extract preparation	51
3.8.2	Protein concentration determination	51
3.8.2.1	Bradford assay (Bradford, 1976)	51
3.8.3	Western Blot	52
3.9	Flow cytometry analysis	55
3.9.1	Flow cytometry analysis using RNase A/Propidium Iodide	55
3.9.2	Flow cytometry analysis using Annexin V-FITC and Propidium Iodide	56
3.10	Statistical analysis	56
4	RESULTS	57
4.1	Extraction of H7372	57
4.1.1	Purification of H7372 by HPLC	57
4.2	Cytotoxic effects of actinomycetes extract, H7372	61
4.2.1	Cytotoxic effect of H7372 or H7763 extract on MCF-7 and MCF-10A	61
4.2.2	Screening of cytotoxic effect of fractions H7372 on MCF-7 and MCF-10A cell lines	63
4.3	PI3K/Akt signaling pathway	66

4.3.1	Effects of crude extracts of H7372 on PI3K/Akt signaling pathway in MCF-7	66
4.3.2	Effects of H7372 fraction 5 on PI3K/Akt pathway on MCF-7	69
4.3.3	Effects of H7372 fraction 5 on PI3K/Akt pathway on MCF-10A	71
4.3.4	Effects of the downstream target of Akt signaling pathway, GSK-3 β , BAD and FKHR in MCF-7 cell line	72
4.4	Effects of H7372 fraction 5 on MAP Kinase signaling pathway	75
4.5	Flow cytometry analysis using RNase/Propidium Iodide	77
4.5.1	Effects of crude extract of H7372 on cell cycle progression in MCF-7	77
4.5.2	Effects of H7372 fraction 5 on cell cycle progression in MCF-7	84
4.5.3	Effects of H7372 fraction 5 on cell cycle progression in MCF-10A	90
4.6	Effects of H7372 fraction 5 on cyclin D1 in MCF-7	96
4.7	Flow cytometry analysis using Annexin V-FITC/Propidium Iodide	99
4.7.1	Effects of crude extract of H7372 on apoptosis in MCF-7	101
4.7.2	Effects of H7373 fraction 5 on apoptosis in MCF-7	104
4.7.3	Effects of H7372 fraction 5 on apoptosis in MCF-10A	107
5	DISCUSSION	109
5.5	Purification of H7372 extract	109
5.6	Effects of H7372 on viability of MCF-7 cell line	110
5.7	Inhibitory activity of signaling pathway by actinomycetes strain H7372	111
5.3.1	Inhibition of PI3K/Akt and MAPK signaling pathways by H7372	112
5.8	Cross-talk between Raf and Akt pathways	114
5.9	Cell cycle progression of H7372-treated MCF-7 and MCF-10A cell lines	115
5.5.1	Role of PI3K/Akt and MAPK signaling pathways in the cell cycle progression of MCF-7 cell line by inhibition of cyclin D1	116
5.10	Effects of H7372 on apoptosis	120
6	CONCLUSIONS AND FUTURE RECOMMENDATIONS	125
6.5	Conclusions	125
6.6	Future recommendations	127

REFERENCES	128
APPENDICES	144
BIODATA OF THE AUTHOR	158



LIST OF TABLES

Table		Page
2.1	Akt-regulated cell processes (Vara <i>et al.</i> , 2004).	13
2.2	Akt implication in different processes characteristic of cancer (Vara <i>et al.</i> , 2004).	14
3.1	Primary antibodies used in this study.	54
4.1	IC ₅₀ of MCF-7 and MCF-10A treated with H7372.	63
4.2	IC ₅₀ of MCF-7 treated with fractionated H7372 extracts.	64
4.3	IC ₅₀ of MCF-10A treated with fractionated H7372 extracts.	64



LIST OF FIGURES

Figure		Page
2.1	Main signaling cascades of the IGF-IR. Binding of IGF-I or IGF-II to IGF-IR causes autocatalytic phosphorylation of the IGF-IR tyrosine kinase domain, which also phosphorylates additional IGF-IR tyrosine residues important for the recruitment of adapter molecules like Shc and IRS. These in turn activate kinase cascade, primarily the MAP kinase pathway and the PI3K/Akt pathway that ultimately lead to signal transduction to the nucleus and mitochondrion (Adapted from Grimberg, 2003).	7
2.2	Regulation and targets of PKB/Akt. Ligation of receptor tyrosine kinases (RTKs) with their ligands leads to recruitment of members of the PI3K family of lipid kinases to the plasma membrane where they encounter the substrate lipid phosphatidylinositol 4,5 biphosphate (PIP₂), leading to formation phosphatidylinositol of 3,4,5 triphosphate (PIP₃). This molecule recruits PH domain containing proteins to the lipid bilayer including Akt and PDK1, where the former becomes activated. Substrates of Akt include transcription factors such as FKHR which regulates expression of Fas ligand and other molecules that promote apoptosis and metabolism. Akt phosphorylation of Bad, FKHR, p27Kip1 and GSK-3 inactivates these proteins, helping to suppress apoptosis and promote glucose utilization. (Adapted from Scheid and Woodgett, 2003).	12
2.3	Flow chart of the three MAPK modules (ERKs, JNKs and p38) (Cowan and Storey, 2003).	19
2.4	A model for the Akt-Raf interaction in MCF-7 cells. Phosphorylation of Raf by Akt leads to cross-talk and inhibition of the Ras-Raf-MEK-ERK cascade and induction of proliferation in the presence of high IGF-I concentration (thick arrows). LY294002 relieves this block and allows Raf to induce growth arrest. At low IGF-I concentration (thin arrows) no Akt-Raf cross-talk takes place. PMA directly affect Raf-MEK-ERK (Adapted from Moelling <i>et al.</i>, 2002).	22
2.5	Chemical structure of staurosporine (Picture adapted from http://www.lclabs.com).	29
2.6	Chemical structure of geldanamycin (Picture adapted from http://www.fermentek.co.il).	31
2.7	Pro- and anti- apoptotic pathways. The death receptor ligands, such	37

as TNF and TRAIL, initiate pro-apoptotic pathways such as sphingomyelinase activation, leading to ceramide and cytochrome c release from mitochondria, in addition, caspase-8 and NFκB can be concurrently activated, leading to simultaneous stimulation of both apoptotic and anti-apoptotic signals. The relative constitutive expression of anti-apoptotic pathways such as PI3K, PKC and the MAPKs influences the efficacy of pro-apoptotic signals (Adapted from Wu, 1996).

- 2.8 Mechanism of Ras and Akt signaling pathway regulate cell cycle in G1/S phase transition. The protein kinase ERK and GSK-3β, the transcription factors Myc and FOXO and the CDK inhibitors p21 and p27, function as key nodes for signal integration (Massagué, 2004). 42
- 3.1 Haemocytometer grids on the counting chamber of a haemocytometer. Squares A to D represented the 1-mm square that used for cell counting. The concentration of cell in 1 ml was obtained by using the above equation. Picture adapted from <http://www.pharma.ethz.ch/> 49
- 4.1 HPLC chromatogram of crude extract, H7372 using Waters analytical HPLC column with a gradient mode and a flow rate of 1 mL/min. A peak in the green box was observed in the fraction 5. 58
- 4.2 HPLC chromatogram of H7372 using semi preparative Licospher C18 HPLC column with a gradient mode and a flow rate of 7.9 mL/min. A peak in the green box was observed in the fraction 5. 59
- 4.3 HPLC chromatogram of H7372 fraction 5 using Waters analytical HPLC column with a gradient mode and a flow rate of 1 mL/min. Fraction was collected in the retention time between 17 to 21 minute and reinject into the HPLC. 59
- 4.4 Effect of H7372 or H7763 on the growth of MCF-7 cells. Cells were treated with various concentrations of H7372 for 24, 48 and 72 hours, and H7763 for 72 hours. The results represent the mean ± SD of three independent experiments. 61
- 4.5 Effect of H7372 or H7763 on the growth of MCF-10A cells. Cells were treated with various concentrations of H7372 or H7763 for 72 hours. The results represent the mean ± SD of three independent experiments. 62
- 4.6 Effect of fractions on the growth of MCF-7 cells. Cells were treated with various concentrations of fractions for 72 hours. The results represent the mean ± SD of three independent experiments. 65

4.7	Effect of fractions on the growth of MCF-10A cells. Cells were treated with various concentrations of fractions for 72 hours. The results represent the mean \pm SD of three independent experiments.	65
4.8	Effects of crude extract, H7372 on PI3K/Akt signaling pathway in MCF-7 cells. The cells were stimulated with IGF-I with and without various concentrations of H7372 crude extract for (A) 24 hours, (B) 48 hours, and (C) 72 hours and stimulated with IGF-I.	68
4.9	Comparison line chart on the normalized level of expression of PI3K as compared to baseline β -actin; and phospho-Akt (Ser473 and Thr308) was compared to baseline total Akt. Graphical data represent the MCF-7 cell line was treated and untreated with H7372 crude extract for (A) 24 hours, (B) 48 hours and (C) 72 hours.	69
4.10	Effects of fraction 5 on PI3K, phospho-Akt (Thr308) and phospho-Akt (Ser473). MCF-7 cell line was treated and untreated with various concentrations of fraction 5 for 72 hours and stimulated with IGF-I.	70
4.11	Comparison line chart on the normalized level of expression of PI3K as compared to baseline β -actin; and phospho-Akt (Ser473 and Thr308) was compared to baseline total Akt. Graphical data represent the MCF-7 cell line was treated and untreated with various concentrations of fraction 5 for 72 hours.	70
4.12	Effects of H7372 fraction 5 on PI3K, phospho-Akt (Thr308) and phospho-Akt (Ser473). MCF-10A cell line was treated and untreated with various concentrations of fraction 5 for 72 hours and stimulated with IGF-I.	71
4.13	Comparison line chart on the normalized level of expression of PI3K as compared to baseline β -actin; and phospho-Akt (Ser473 and Thr308) was compared to baseline total Akt. Graphical data represent the MCF-10A cell line was treated and untreated with various concentrations of fraction 5 for 72 hours.	72
4.14	Effect of fraction 5 on phospho-GSK-3 β (Ser9), phospho-BAD (Ser136) and phospho-FKHR (Ser256). MCF-7 cells were treated and untreated with fraction 5 in various concentrations for 72 hours.	73
4.15	Comparison line chart on the normalized level of expression of phospho-GSK-3 β (Ser9), as compared to baseline of total GSK-3 β ; and phospho-BAD (Ser136) and phospho-FKHR (Ser256) as compared to baseline of β -actin. Graphical data represent the MCF-7 cell line was treated and untreated with fraction 5 at various concentrations for 72 hours.	74

- 4.16 Effects of H7372 fraction 5 on phospho-ERK1/2 and phospho-Raf (Ser259). MCF-7 cell line was treated and untreated with various concentrations of fraction 5 for 72 hours and stimulated with IGF-I. 75
- 4.17 Comparison line chart on the normalized level of expression of phospho-ERK1/2 and phospho-Raf (Ser259) as compared to baseline total ERK and Raf respectively. Graphical data represent the MCF-7 cell line was treated and untreated with various concentrations of fraction 5 for 72 hours. 76
- 4.18 Effects of crude extract, H7372 on the cell cycle profile. Histogram shows the phases in the cell cycle: G₁ (M1); S (M2); G₂/M (M3) and Sub-G₁ (M4). The MCF-7 cells were cultured in medium alone as a control or cultured in the presence of H7372 for various concentrations for (A) 24 hours, (B) 48 hours and (C) 72 hours. I; Control, II; 7.5 µg/mL H7372, III; 15 µg/mL H7372, IV; 30 µg/mL H7372. 79
- 4.19 Percentage of MCF-7 cells in G₁ phase treated with the crude extracts of H7372 based on DNA content in two independent experiments. The percentage of cells in G₁ phase which were treated with the H7372 extract slightly increased at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly greater than control, p < 0.05. 80
- 4.20 Percentage of MCF-7 cells in S phase treated with the crude extracts of H7372 based on DNA content in two independent experiments. The percentage of cells in S phase which were treated with the H7372 extract decreased at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly lower than control, p < 0.05. 81
- 4.21 Percentage of MCF-7 cells in G₂/M phase treated with the crude extracts of H7372 based on DNA content in two independent experiments. The percentage of cells in G₂/M phase which were treated with the H7372 extract slightly decreased at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. 82
- 4.22 Percentage of MCF-7 cells in Sub-G₁ phase treated with the crude extracts of H7372 based on DNA content in two independent experiments. The percentage of cells in Sub-G₁ phase which were treated with the H7372 extract have no significant different at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. 83

- 4.23 Effects of fraction 5, H7372 on the cell cycle profile. Histogram shows the phases in the cell cycle: G₁ (M1); S (M2); G₂/M (M3) and Sub-G₁ (M4). The MCF-7 cells were cultured in medium alone as a control or cultured in the presence of fraction 5 for various concentrations for (A) 24 hours, (B) 48 hours and (C) 72 hours. I; Control, II; 0.7 µg/mL Fraction 5, III; 1.4 µg/mL Fraction 5, IV; 2.8 µg/mL Fraction 5. 85
- 4.24 Percentage of MCF-7 cells in G₁ phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in G₁ phase which were treated with the fraction 5 shows significant increase at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly greater than control, p < 0.05. 86
- 4.25 Percentage of MCF-7 cells in S phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in S phase which were treated with the fraction 5 shows significant decrease at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly lower than control, p < 0.05. 87
- 4.26 Percentage of MCF-7 cells in G₂/M phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in G₂/M phase which were treated with the fraction 5 shows significant decrease at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly lower than control, p < 0.05. 88
- 4.27 Percentage of MCF-7 cells in Sub-G₁ phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in Sub-G₁ phase which were treated with the fraction 5 shows significant increase after 72h incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly greater than control, p < 0.05. 89
- 4.28 Effects of fraction 5 on the cell cycle profile. Histogram shows the phases in the cell cycle: G₁ (M1); S (M2); G₂/M (M3) and Sub-G₁ (M4). The MCF-10A cells were cultured in medium alone as a control or cultured in the presence of fraction 5 in various concentrations for (A) 24 hours, (B) 48 hours and (C) 72 hours. I; Control, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5. 91
- 4.29 Percentage of MCF-10A cells in G₁ phase treated with fraction 5 92

based on DNA content in two independent experiments. The percentage of cells in G₁ phase which were treated with the fraction 5 shows no significant increase at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar.

- 4.30 Percentage of MCF-10A cells in S phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in S phase which were treated with the fraction 5 shows significant decrease after 24h incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly lower than control, p < 0.05. 93
- 4.31 Percentage of MCF-10A cells in G₂/M phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in G₂/M phase which were treated with the fraction 5 shows significant decrease at 2.8µg/mL after 48h incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly lower than control, p < 0.05. 94
- 4.32 Percentage of MCF-10A cells in Sub-G₁ phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in Sub-G₁ phase which were treated with the fraction 5 have no significant different at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. 95
- 4.33 Effects of fraction 5 on cyclin D1. MCF-7 cell line was with and without fraction 5 at various concentrations for 72 hours and stimulated with IGF-I. 96
- 4.34 Comparison line chart on the normalized level of expression of cyclin D1 as compared to baseline β-actin. The expression level of cyclin D1 was decreased by fraction 5 in a dose-dependent manner. Graphical data represent the MCF-7 cell line was treated and untreated with fraction 5 in various concentrations for 72 hours. 97
- 4.35 MCF-7 treated with H7372 for 24 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL H7372, II; 7.5 µg/mL H7372, III; 15 µg/mL H7372, IV; 30 µg/mL H7372. 101
- 4.36 MCF-7 treated with H7372 for 48 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL H7372, II; 7.5 µg/mL H7372, III; 15 µg/mL H7372, IV; 30 µg/mL H7372. 101
- 4.37 MCF-7 treated with H7372 for 72 hours. The cells were stained with 101

Annexin V and PI. I; 0 µg/mL H7372, II; 7.5 µg/mL H7372, III; 15 µg/mL H7372, IV; 30 µg/mL H7372.

- 4.38 Percentage of (A) early apoptotic cells, (B) late apoptotic cells, and (C) necrotic cells in two independent experiments. MCF-7 cells were treated with crude extracts of H7372 at indicated time and concentrations. The results shown were mean \pm 1 SD bar. *, indicate significantly greater than control, $p < 0.05$. 102
- 4.39 MCF-7 treated with fraction 5 for 24 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5. 104
- 4.40 MCF-7 treated with fraction 5 for 48 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5. 104
- 4.41 MCF-7 treated with fraction 5 for 72 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5. 104
- 4.42 Percentage of (A) early apoptotic cells, (B) late apoptotic cells, and (C) necrotic cells in two independent experiments. MCF-7 cells were treated with fraction 5 at indicated time and concentrations. The results shown were mean \pm 1 SD bar. *, indicate significantly greater than control, $p < 0.05$. 105
- 4.43 MCF-10A treated with fraction 5 for 24 hours. The cells were stained with Annexin V and PI. I; 0µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5. 107
- 4.44 MCF-10A treated with fraction 5 for 48 hours. The cells were stained with Annexin V and PI. I; 0µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5. 107
- 4.45 MCF-10A treated with fraction 5 for 72 hours. The cells were stained with Annexin V and PI. I; 0µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5. 107
- 4.46 Percentage of (A) early apoptotic cells, (B) late apoptotic cells, and (C) necrotic cells. MCF-10A cells were treated with fraction 5 at indicated time and concentrations. 108
- 5.1 Regulation of the G₁/S phase transition (Adapted from Roy and Thompson, 2006). 118

LIST OF ABBREVIATIONS

~	Approximately
%	Percentage
α	Alpha
β	Beta
γ	Gamma
κ	Kappa
μ	Micro
cm	Centimetre
$^{\circ}\text{C}$	Degree of Celsius
CO_2	Carbon dioxide
g	Gram
kDa	Kilo Dalton
L	Liter
M	Molar
mg	Miligram
min	Minute
mL	Milliliter
mm	Millimetre
ng	Nanogram
rpm	Revolutions per minute
μg	Microgram
μL	Microliter

μm	Micrometer
U	International unit
V	Volt
AMP	Adenosine monophosphate
APS	Ammonium persulphate
ATCC	American Type Cell Collection
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
DNA	Deoxyribonucleic acid
DTT	Dithioretol
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GSK-3 β	Glycogen synthase kinase-3Beta
HCl	Hydrochloride acid
HPLC	High performance liquid chromatography
Hsp90	Heat shock protein 90
IDV	Integrated density value
IGF	Insulin-like growth factor
IGF-IR	Insulin-like growth factor-I receptor
ILK	Integrin-linked kinase
IR	Insulin receptor
IRS	Insulin-response sequence