EFFECTS OF FLAVANOID KAEMPFEROL ON THE EXPRESSION OF TUMOUR NECROSIS FACTOR ALPHA INDUCED NUCLEAR FACTOR- KAPPA B1 AND NUCLEAR FACTOR- KAPPA B2 ACTIVATION IN HUMAN HEPATOCARCINOMA CELL LINE

CHEN KAH KET

Bachelor of Science (Hons) Biomedical Science

Faculty of Science
University Tunku Abdul Rahman

MAY 2011

EFFECTS OF FLAVANOID KAEMPFEROL ON THE EXPRESSION OF TUMOUR NECROSIS FACTOR ALPHA INDUCED NUCLEAR FACTOR- KAPPA B1 AND NUCLEAR FACTOR- KAPPA B2 ACTIVATION IN HUMAN HEPATOCARCINOMA CELL LINE

By

CHEN KAH KET

A project report submitted to the Department of Biomedical Science
Faculty of Science
University Tunku Abdul Rahman
In partial fulfilment of the requirement for the degree of
Bachelor of Science (Hons) Biomedical Science

MAY 2011

ABSTRACT

EFFECTS OF FLAVANOID KAEMPFEROL ON THE EXPRESSION OF TUMOUR NECROSIS FACTOR ALPHA INDUCED NUCLEAR FACTOR- KAPPA B1 AND NUCLEAR FACTOR- KAPPA B2 ACTIVATION IN HUMAN HEPATOCARCINOMA CELL LINE

CHEN KAH KET

Nuclear factor kappa B (NF-κB) is a transcription factor that plays important roles in many physiological changes especially inflammation. It can be activated by tumour necrosis factor alpha (TNF-α), a pro-inflammatory cytokine. While kaempferol is found in plants and is observed to have anti-inflammatory response. Thus, the objective of this study is to determine the effect of kaempferol on the mRNA and protein expression of NF-κB1 and NF-κB2 in TNF-α treated human hepatocarcinoma cell line (HepG2). To achieve this, HepG2 cells were pre-treated with 20 ng/ml of TNF-α for 24 hrs and subsequently treated with different concentration of kaempferol ranging from 1 to 20 μM for another 24 hrs. Total cellular RNA was extracted and the extracted RNA samples were tested for their integrity, concentration and purity through formaldehyde agarose gel electrophoresis and spectrophotometry. The gene expression of NF-κB1 and NF-κB2 were subsequently quantified using Real Time RT-PCR and the mRNA expression was normalised to β-actin, a housekeeping gene. Nuclear and cytoplasmic protein were extracted and Western Blotting using antibody against total and phosphorylated NF-κB1 and

NF-κB2 proteins were carried out to investigate the protein expression and activation levels. From the results obtained, NF-κB2 but not NF-κB1 phosphorylated levels were increased in cells treated with 20 ng/ml TNF-α alone. Subsequent treatment with kaempferol in low dosage of 1-10 μM reduced the TNF-α induced activation of NF-κB2, but the effect was not significant in NF-κB1 activation. However, treatment with 20 μM of kaempferol alone or in the presence of TNF-α significantly induced NF-κB1 and NF-κB2 phosphorylation to almost 2-fold of untreated cells. These results suggest that the dosage of kaempferol used is crucial in determining the activation or deactivation of NF-κB members. Low dosage of kaempferol would abolish the activation of NF-κB2 by TNF-α, and thus suppressing inflammation; but higher dosage of kaempferol would induce activation of both NF-κB members.

ACKNOWLEDGEMENT

First of all, I would like to thanks my family for their emotional support and encouragement during my laboratory work. I would also like to appreciate Department of Biomedical Science, University Tunku Abdul Rahman, by giving me a chance to learn and practice on my project.

I also wish to express my sincere gratitude to my supervisor, Dr. Chew Choy Hoong for her guidance, advice and encouragement throughout final year project life. I would also like to thank Dr. Chew Choy Hoong's master student, Ng Di Lin, Lim Wyi Sian and Kor Sue Bee for their kindness guidance and advice throughout this project. I am much obliged to all the lab officers for helping me and forgive my mistake.

Last but not least, I would like to show my deepest appreciation to my dear lab mates, Daniel Wong Yew Yen, Michelle Lao Kah Meng, Wong Shin Yee, Chow Xin Hui, Yu Yen Ee, Lim Sh Pot, Lim Sze Yee and Tan Wan Cheng for making my project life happy and enjoyable.

DECLARATION

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

_____

CHEN KAH KET

APPROVAL SHEET

This project report with title "**EFFECTS OF FLAVANOID KAEMPFEROL ON THE EXPRESSION OF TUMOUR NECROSIS FACTOR ALPHA INDUCED NUCLEAR FACTOR-KAPPA B1 AND NUCLEAR FACTOR- KAPPA B2 ACTIVATION IN HUMAN HEPATOCARCINOMA CELL LINE**" was prepared by CHEN KAH KET and submitted as partial fulfilment of the requirement for the degree of Bachelor of Science (Hons) in Biomedical Science at University Tunku Abdul Rahman.

Approved by:

_____ Date:_____

(Dr. CHEW CHOY HOONG)
Assistant Professor
Department of Biomedical Science
Faculty of Science
University Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITY TUNKU ABDUL RAHMAN

Date:_____

PERMISSION SHEET

It is hereby certified that **CHEN KAH KET** (ID No: **08ADB04191**) has completed this final year project entitled **"EFFECTS OF FLAVANOID KAEMPFEROL ON THE EXPRESSION OF TUMOUR NECROSIS FACTOR ALPHA INDUCED NUCLEAR FACTOR-KAPPA B1 AND NUCLEAR FACTOR- KAPPA B2 ACTIVATION IN HUMAN HEPATOCARCINOMA CELL LINE"** supervised by Dr. Chew Choy Hoong from Department of Biomedical Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscript of these research findings for publishing in any form, if I did not prepare it within six (6) months time from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation	Full term
µg	Microgram
µl	Microlitre
A	Absorbance
ANK	Ankyrin repeat domain
APS	Ammonium persulphate
BAFF	B cell activating factor
BCP	1-Bromo-3-Chloropropane
BSA	Bovine serum albumin
Cisplatin	cis- diamminedichloroplatinium(II)
CMV	Cytomegalovirus
C_T	Threshold cycle
DMSO	Diethy sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylene diamine-tetra-acetic acid
et al.	“et alia” (Italian word referring to ‘and others’)
EtBr	Ethidium bromide
FBS	Fetal bovine serum
G	Gram
GAPDH	Glycealdehyde-3-phosphate dehydrogenase
GLUT4	Glucose transporter 4
GRR	Glycine- rich region

HIV-1	Human immunodeficiency virus type one
hr	Hour
IκB	Inhibitory kappa B
IKK	IκB kinase complex
IL	Interleukin
LD_{50}	Median lethal dose
LTβ	Lymphotoxin-β
M	Molar
MEM	Minimum essential medium
min	Minute
ml	Mililitre
MOPS	3-[N-Mopholino]propanesulphonic acid
mRNA	Messenger ribonucleic acid
NEMO	NF- κB essential modulator
NF-κB	Nuclear Factor kappa B
ng	Nanogram
NIK	NF- κB inducing kinase
NLS	Nuclear localisation sequences
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEST	Proline, glutamic acid, serine and threonine
qPCR	Quantitative Polymerase Chain Reaction
RANKL	Receptor activator of NF- κB ligand
Rel	V-rel reticuloendotheliosis viral oncogene homolog

RFU	Relative fluorescence unit
RHD	Real homology domain
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
RT- PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
sec	Second
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLR	Toll- like receptor
T_m	Melting temperature
TNF-α	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TRAIL	Tumour necrosis factor related apoptosis inducing agent
UV	Ultraviolet
xg	Times gravity/ Relative centrifugal force
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

NF-κB family is a transcription factor that is involved in the activation of genes that are involved in various biological processes such as inflammatory and immune responses, anti-apoptosis, cells proliferation, differentiation and maturation (Carmody & Chen, 2007; Gilmore, 2006; Tergaonkar, 2006). Since the discovery of NF-κB family, it has become the focus for therapeutic intervention, especially their upstream signalling pathway that leads to their activation. There are five members in NF-κB family: RelA, RelB, c-Rel, NF-κB1 and NF-κB2. NF-κB1 and NF-κB2 normally exist in precursor form p105 and p100 in cell cytoplasm. Upon activation, p105 and p100 will be cleaved to form p50 and p52, the active form of NF-κB1 and NF-κB2. One of the activator of NF-κB is TNF-α, which is a pro-inflammatory molecule that activates NF-κB during inflammation and has both tumour promoting and anti- tumour functions (Balkwill, 2009). Thus the studies of TNF-α and other modulator induced activation of NF-κB signalling pathway is getting important for the development of drugs for anti-inflammation and anti-cancer (Balkwill, 2009; Gilmore, 2006).

Kaempferol is one of the natural compund extracted from plants which had been found to have antioxidant and anti-inflammation functions. Research discovered that kaempferol is able to control cancer development by inhibiting NF-κB activation and sensitising cancer cells to cancer therapeutic drugs

cisplatin (Chen, Chow, Huang, Lin & Chang, 2004; Luo, Daddysman, Rankin, Jiang, & Chen, 2010).

NF-κB signalling pathway is one of the major pathways that cause inflammation, while TNF-α was shown to be up-regulated during chronic inflammation. Consequently, TNF-α becomes the positive autocrine feedback signal that further enhance activation of NF-κB. Hence, TNF-α induced NF-κB signalling pathway is proposed to be one of the mechanisms that could cause the advancement of chronic inflammation to cancer formation (Carter, Misyak, Hontecillas & Riera, 2009).

However, majority of the studies carried out were designed by stimulating the cells with single type of modulator. The relationship between kaempferol and TNF-α induced NF-κB activation remains unknown. Therefore this study could give useful information for the development of new therapy for cancer and inflammatory diseases. Thus, this project has the following objectives:

1. To determine the effect of kaempferol on TNF-α induce NF-κB1 and NF-κB2 mRNA expression using quantitative PCR
2. To determine the effect of kaempferol on TNF-α induced NF-κB1 and NF-κB2 protein expression using Western Blot
3. To determine the effect of kaempferol on TNF-α induced NF-κB1 and NF-κB2 activation, and
4. To compare the mRNA and protein activation expression in kaempferol pre-treated TNF-α induced HepG2 cells.

CHAPTER 2

LITERATURE REVIEW

2.1 NF-κB family

Nuclear factor kappa B (NF-κB) family are ubiquitously expressed nuclear factor that was discovered more than 20 years ago. NF-κB is involved in many cellular processes like anti-apoptosis, inflammation, innate and adaptive immune responses. The deregulation of NF-κB signalling pathway may lead to cancer formation and inflammatory diseases. Nowadays, more than 200 physiological stimuli have been identified to activate NF-κB signalling pathway these include viral and bacterial antigen, mitogen, growth factor, cytokines, physical and biochemical stress inducers. NF-κB is also found to be important for HIV-1 and CMV viral promoters' transcription (Carmody & Chen, 2007; Gilmore, 2006; Tergaonkar, 2006).

NF-κB family consists of five members: RelA (p65), RelB, c-Rel, NF-κB1 (p50, precursor was p105) and NF-κB2 (p52, precursor was p100). All the members have Rel homology domain (RHD) at N-terminus which facilitates their DNA binding, dimerisation and nuclear translocation processes. However, the difference among RelA, RelB and c-Rel, NF-κB1 and NF-κB2 is NF-κB1 and NF-κB2 lack of trans-activation domain at C-terminus. In most of the mammalian cells, NF-κB forms homo- or heterodimer from these five monomers in the cytoplasm and the generation of all monomers is transcriptionally regulated. NF-κB1 and NF-κB2 are also regulated by their

precursor p105 and p100, respectively. In resting cells NF-κB dimers are inactived by binding with Inhibitory-κB protein (IκB). IκB family includes IκBα, IκBβ, IκBγ, IκBε, IκBζ, BCL3, p100 and p105, which contains ankryin repeats (ANK), a repeated sequence of about 30 amino acids long which helps IκB binding to NF-κB. IκB family functions to inhibit NF-κB in cytoplasm and terminate NF-κB transcriptional activity by translocating it from nuclear to cytoplasm (Figure 2.1) (Bubici, Papa, Dean & Franzoso, 2006; Carmody & Chen, 2007; Gilmore, 2006; Tergaonkar, 2006).

Studies showed that expression of NF-κB dimeric complexes are cell-type and stimulus-specific. Some physiological important dimers like RelA:p50, c-Rel:p50 and RelB:p52 are expressed in all cell types. Dimers that consist of c-Rel are expressed higher in B and T lymphocytes than in other cell types. RelA and NF-κB1 are ubiquitously expressed in all cell types. RelB and NF-κB2 are found to be expressed higher in lymphoid tissue (Shih, Tsui, Caldwell & Hoffmann, 2011).

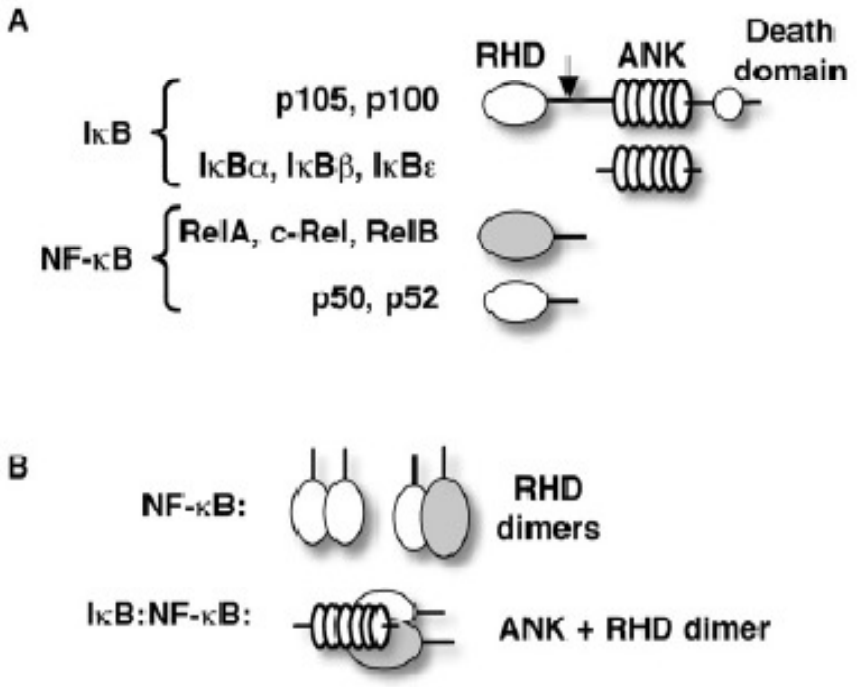


Figure 2.1 NF-*κ*B and I*κ*B family

(A) NF- *κ*B family consists of RelA, RelB, c- Rel, p50 and p52. All of them share similar Rel homology domain (RHD). The precursor of p50 and p52 are p105 and p100, respectively. They belong to I*κ*B family as they have a structure called ankyrin repeat domain (ANK). Both p105 and p100 also have a death domain at the C- terminus. The arrow indicates the site for proteolysis. I*κ*Bα, I*κ*Bβ and I*κ*Bε are the other types of I*κ*B.

(B) NF-*κ*B in its dimeric form, which can be either homodimer or heterodimer. Without being induced by modulators, NF-*κ*B binds to I*κ*B to prevent its activation. (Picture adapted from Savinova, Hoffmann & Ghosh, 2009)

2.2 NF-κB1 and NF-κB2

NF-κB1 protein p105 is the precursor of p50, while NF-κB2 protein p100 is the precursor of p52. Both of them are inhibitors for NF-κB dimers. They also provide "buffering" binding capacity for excess NF-κB because they have IκB activities, which allow them to regulate the activation of NF-κB by repress the preformed NF-κB dimers (Savinova, Hoffmann & Ghosh, 2009).

Human NF-κB1 gene is located in chromosome 4 at the location of 4q24. It has 24 exons spanning 156 kb. The activity of NF-κB1 can be positively regulated by NF-κB itself and the Ets family transcription factor. NF-κB1 consists of 968 amino acids with molecular weight of 105 kDa. NF-κB1 consists of several domains which include a Rel homology domain, a nuclear localisation sequences (NLS), a glycine- rich region (GRR), 7 ankyrin repeats, a proline, glutamic acid, serime, and threonine (PEST) domain and a death domain (Figure 2.2). RHD is responsible for DNA binding, dimerisation of NF-κB and also serves as the binding site for IκB. GRR is located between 375- 400 amino acids. Studies have shown that GRR is a critical element in directing the formation of p50 from p105, where the cleavage site of NF-κB1 is between amino acids 433- 434 (Baldwin, 1996; Chen, Castranova, Shi & Demers, 1999; Ghosh, May & Kopp, 1998; Hunter & Kandarian, 2004; Lin, DeMartino & Greene, 1998; Liptay et al., 1992).

The important roles of NF-κB1 have been shown in several mouse models. Impair development of T cells has been observed in NF-κB1 knockout mouse. The NF-κB1 knockout mouse also exhibits low T cells count in thymus,

spleen and bone marrow. These suggest that NF-κB1 activation is required for T cells proliferation, differentiation and survival (Srikantharajah et al., 2009). Studies also showed that mouse with NEMO or IKKβ deficiency would die from severe TNF-α induced liver damage due to failure in the activation of NF-κB dependent anti-apoptotic genes in hepatocytes (Li, Van Antwerp, Mercurio, Lee & Verma, 1999; Rudolph et al., 2000). Besides, in p105 knockout mice show inflammatory phenotype and have increased susceptibility to opportunistic infections (Savinova, Hoffmann & Ghosh, 2009).

Human NF-κB2 gene is located at chromosome 10 in the location of 10q24. It has 24 exons spanning 8 kb. The activation of NF-κB2 is regulated by two promoters, P1 and P2, in which a number of consensus binding sites for transcription factors, including SP1, AP1 and putative NF-κB were identified. NF-κB2 consists of 900 amino acids with molecular weight of 100 kDa. NF-κB1 protein is formed by numerous domains which consist of a RHD, 2 NLS, a GRR, 7 ankyrin repeats, a PEST domain and a death domain (Figure 2.3). NF-κB2 is cleaved at the site between 454- 455 amino acid through ubiquitin proteosome dependent degradation to generate p52. Recent studies found out that activation of NF-κB2 required phosphorylation by IKKα/NIK complex at C-terminal of NF-κB2 (Baldwin, 1996; Chen et al., 1999; Ghosh et al., 1998; Lin et al., 1998; Liptay et al., 1992; Sentfleben, 2001).

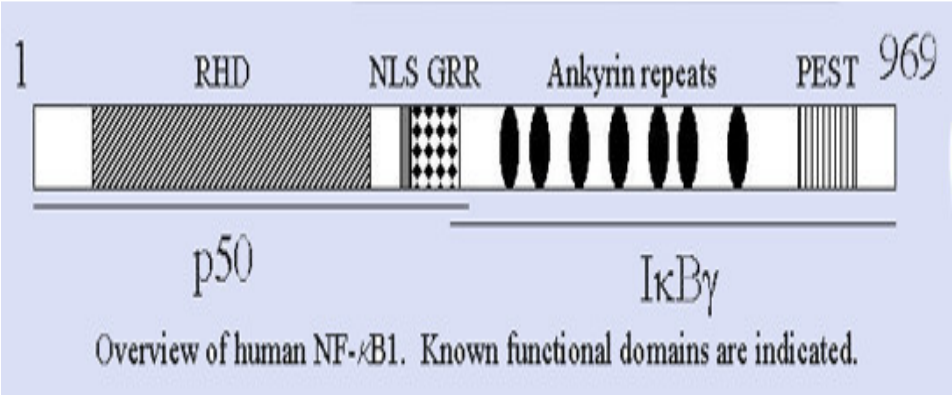


Figure 2.2 Schematic representation of structure of NF-*κ*B1

NF-*κ*B1 protein is formed by several domains. At N-terminal region of NF-*κ*B1, there is a Rel homology domain (RHD) which about 300 amino acids length. Nuclear localisation sequences (NLS) is located after RHD, which is followed by a glycine- rich region (GRR). At the C-terminal region of NF-*κ*B1 contains 7 copies of ankyrin repeats which is belong to I*κ*B family. The C-terminal region of NF-*κ*B1 also contains a proline, glutamic acid, serine and threonine (PEST) domain and a death domain (Adapted from Chen, 2002a).

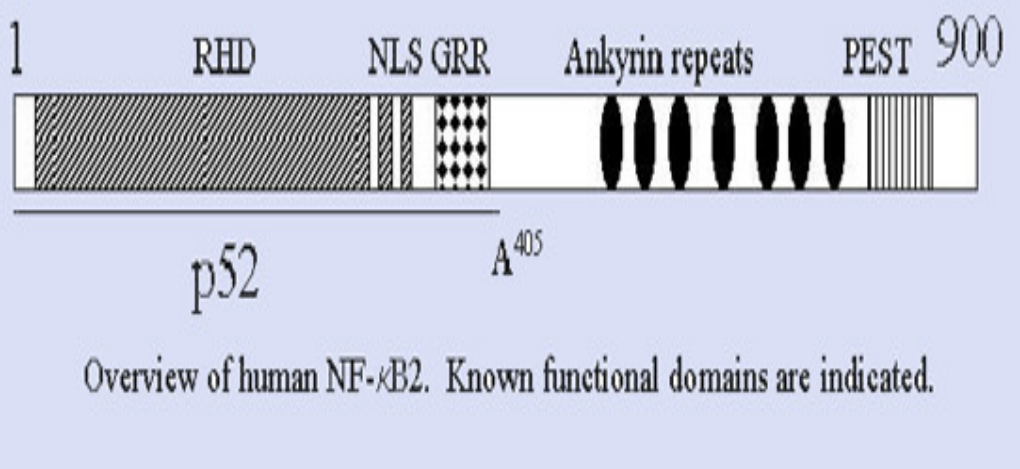


Figure 2.3 Schematic representation of structure of NF-κB2

NF-κB2 protein consists of several domains. Rel homology domain (RHD) is located at N- terminal of NF-κB2. NF-κB2 has 2 nuclear localisation sequences (NLS) which is located after RHD and it is followed by a glycine-rich region (GRR). At the C-terminal region of NF-κB2 contains 7 copies of ankyrin repeats which is belong to IκB family. The C- terminal region of NF-κB2 also contains a proline, glutamic acid, serine and threonine (PEST) domain and a death domain (Adapted from Chen, 2002b).

Several NF-κB2 knockout mouse models were shown to have abnormal B cells differentiation and function (Anver et al., 1998; Barton et al., 1998). Keller and his colleague (2010) also showed that NF-κB2 knockout mouse developed lymphoma due to deregulation of c-Myc expression and reduced activity of NF-κB mediated immune responses, cell survival and transformation. In other cases, animals that have defect in IκB like domain of NF-κB2 was discovered to have deregulatd expression of p52, which in turn, led to the increase in lymphocytes production and gastric mucosal hyperplasia (Bravo, Carrasco, Claudio, Ishikawa & Ryseck, 1997). Other than this, the absence of p100 would cause perinatal lethality in mice, this suggests that NF-κB2 plays a role in fetal development (Savinova et al., 2009). All these models point out that the regulation of NF-κB1 and NF-κB2 expression is biologically important.

2.3 NF-κB signalling pathway

NF-κB activation leads to transcription of several genes that is involved in pro-survival of cells, inflammation and immunity (Carmody & Chen, 2007; Gilmore, 2006; Tergaonkar, 2006). The upstream signalling pathway of NF-κB involve activation of various regulatory receptor like Toll- like receptor (TLR), tumour necrosis factor receptor family (TNFR), Interleukin-1 receptor, B cell and T cell receptor by their ligand. This would induce a cascade of events that lead to the phosphorylation of IκB protein by enzyme IκB kinase complex (IKK). IKK consists of three subunits, IKKα (IKK1), IKKβ (IKK2) and IKKγ (NF-κB essential modulator or NEMO) associate with other proteins like HSP90, CDC37, ELKS and NAP1. IKK1 and IKK2 are catalytic sites for

IKK complex, while NEMO regulates IKK complex function and maintains the structure of IKK complex (Bubici et al., 2006; Carmody & Chen, 2007; Gilmore, 2006; Tergaonkar, 2006).

There are two major signalling pathways for NF-κB: classical (canonical) and alternative (non-canonical) pathway. Classical pathway activation is IKKβ and IKKγ dependent and it mainly leads to activation of p50/RelA and p50/c- Rel dimer. Upon phosphorylation of IκB by IKK complex, it becomes a target for polyubiquitination mediated by E2 and E3 ligases, later follow by 26S proteasomal degradation. This process results in release of NF-κB from IκB and translocation of NF-κB from cytoplasm to nucleus. Classical pathway is responsible for various types of inflammatory responses and the survival of professional immune cells as it enhances the expression of genes involved in inflammation and immune response (Bubici et al., 2006; Carmody & Chen, 2007; Gilmore, 2006; Tergaonkar, 2006; Senftleben, 2008).

IKKα homodimer and NF-κB inducing kinase (NIK) are important elements that regulate the activation of the alternative pathway. The activation of alternative pathway results in phosphorylation of p100, which in turn will be processed into p52 via degradation of its IκB terminus. RelB is the most common subunit that associates with p52 to form dimer and is then translocated into nucleus to activate genes which are involved in B cell maturation and lymphoid organ formation (Bubici et al., 2006; Carmody & Chen, 2007; Gilmore, 2006; Tergaonkar, 2006; Senftleben, 2008).

Classical and alternative pathway are not dogmatically separated because certain ligand like receptor activator of NF-κB ligand (RANKL) and lymphotoxin-β (LTβ) can activate both pathways (Bubici, et al., 2006; Carmody & Chen, 2007; Gilmore, 2006; Tergaonkar, 2006; Senftleben, 2008). Besides, Luedde and co-workers observed that deficiency of IKKβ in hepatocytes has no effect on classical pathway activation by TNF-α. On the other hand, they also found that TNF-α was unable to induce activation NF-κB in NEMO-deficient hepatocytes. Therefore, they suggest that NEMO/IKKα complex is sufficient for NF-κB activation in the absence of IKKβ (Figure 2.4) (Pasparakis, Luedde & Schmidt, 2006).

Negative regulation of NF-κB activation is still not fully understood. Downregulation of NF-κB involves multiple mechanisms including the positive feedback loop of *de novo* IκB protein synthesis that is induced by NF-κB. IκB protein will bind to NF-κB in nucleus and translocate it out of nucleus, thereby terminating the activity of NF-κB. Other mechanism such as post-translational modification is also known to contribute in NF-κB downregulation. Studies found that phosphorylation and acetylation of RelA containing complex is positively correlated with the duration of NF-κB response. Deacetylated RelA is found to have higher binding affinity to IκB. Thus, the association of RelA with histone deacetylase corepressor protein is said to promote the shutdown of NF-κB responses due to deacetylation of RelA and histones (Ashburner, Westerheide & Baldwin, 2001; Chen, Fischle, Verdin & Greene, 2001; Ghosh & Karin, 2002).

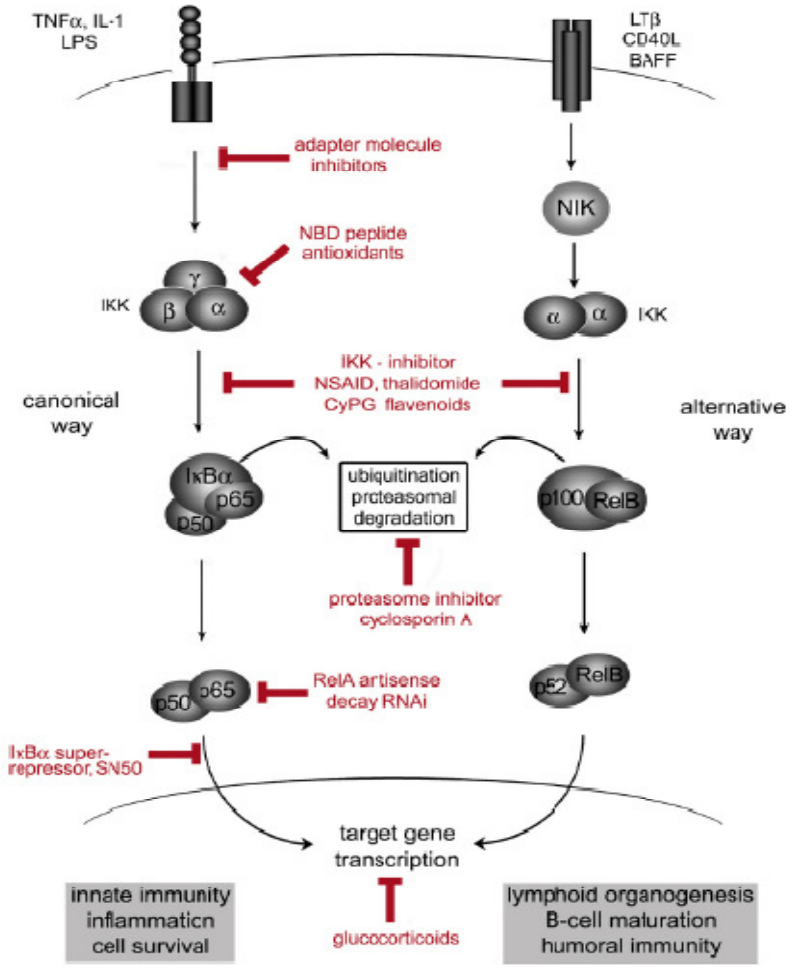


Figure 2.4 NF-*κ*B signalling pathway

Schematic representation of canonical and alternative signalling pathway of NF-*κ*B. Canonical pathway is activated by pro-inflammatory cytokine and microbial product, therefore inducing nuclear translocation of p50/p65 heterodimer. The alternative pathway is activated by TNF superfamily receptors upon binding of ligand such as LTβ, CD40L and B cell activating factor (BAFF). This initial cascade of event leads to the translocation of p52/RelB heterodimer into nucleus to activate gene expression (Adapted from Senftleben, 2008).

2.4 NF-κB and inflammation

NF-κB is known to be the primary regulator of inflammation because it is involved in the activation of immune cells (Ghosh, Hayden & West 2006; Karin & Greten, 2005; Firestein & Tak, 2001). Upon stimulation with pro-inflammatory cytokine such as TNF-α and IL-1β, NF-κB is activated and triggers NF-κB dependent transcription of pro-inflammatory and anti-apoptotic genes. NF-κB signalling pathway also acts as positive feedback to enhance the degree of inflammatory responses. Thus, prolong activation of NF-κB has been associated with chronic inflammatory diseases (Karin & Greten, 2005). These genes which include TNF-α, C-reactive protein (CRP), inducible nitric oxide synthase (iNOS) and cyclooxygenase- 2 (COX- 2), are induced in inflammatory cells and cause inflammatory responses through various of mechanisms such as production of pro-inflammatory mediators such as prostaglandin E2 (Gaynor & Yamamoto, 2001; Ischiropoulos, 1998; Mediavilla et al., 2007).

However, many studies have indicated that improper inhibition of NF-κB signalling pathway will cause adverse effect. Study conducted by Nenci et al. demonstrated that inhibition of NF-κB classical pathway via deletion of NEMO has caused reduction in the production of antimicrobial small bowel peptide. As a consequence, bacterial infection is enhanced and this would trigger higher inflammatory responses, which in turn enhance TNF-α production. Excess TNF-α act as positive regulator to further enhance inflammation and cause apoptosis of cells. Eventually the epithelial barrier

will be broken down and this would allow more infection to occur. This would lead to chronic inflammation (Nenci et al., 2007).

Many inflammatory diseases are linked with activation of NF-κB which include arthritis, inflammatory bowel disease (IBD), atherosclerosis, asthma, systemic lupus erythematosus (SLE) and gastritis (Senftleben, 2008). Thus knowing the relationship between NF-κB and inflammation is important for disease treatment. As the studies of NF-κB signalling pathway become more detailed, more strategies for inhibiting NF-κB activation. Recent studies focusing on upstream inhibition of NF-κB activation and also the natural product like flavonoids have been shown to inhibit the activation of NF-κB (Senftleben, 2008).

2.5 NF-κB and carcinogenesis

NF-κB signalling pathway is closely related with tumourigenesis because it is constitutively activated in most human cancer cells (Sarkar, Lee, Wang & Kong, 2008). The participation of NF-κB in carcinogenesis is supported by the interaction of NF-κB with pathways which can activate carcinogenic genes such as *ras* oncogene (Liptay, Ludwig, Schmid, Adler, Wagner & Weber, 2003). NF-κB signalling pathway is able to promoting proliferation and anti-apoptotic of cell, thus it has been suggested that unregulated NF-κB activity brings the balance between proliferation and apoptosis toward uncontrolled cell growth (Gelinas et al., 2003).

NF-κB was found to be involved in cancer cell metastasis. Studies discovered that inhibition of NF-κB activity by mutant IκBα was able to suppress the metastasis of pancreatic cancer cells to liver cells completely (Fujioka et al., 2003). MMP-9 and uPA are critical proteases that are involved in tumour invasion. Activation of NF-κB can up-regulate their expression. These results suggest that NF-κB activation is one of the factors which could cause cancer cells metastasis (Wang, Abbruzzese, Evan & Chiao, 1999). The successful inhibition of cancer cell proliferation and induction of apoptosis using super inhibitor of NF-κB suggest that NF-κB is a potential target for treatment of cancer (Liptay et al., 2003).

The major issues in cancer therapy are the development of resistance by the cancer cells. Studies have found that many chemotherapeutic agents can activate NF-κB in cancer cells (Chuang et al., 2002; Li, Ahmed, Ali, Philip, Kucuk & Sarkar, 2005; Yeh et al., 2002). Stimulants such as IL-1 are able to induce NF-κB activation and cause chemoresistance in human cancer (Arlt et al., 2002; Muerkoster et al., 2005). Therefore, inhibition of NF-κB activity becomes a useful strategy in cancer treatment.

2.6 Tumour necrosis factor alpha (TNF-α)

TNF-α is a cytokine that is responsible for inflammatory responses, immunity, antiviral responses and a variety of diseases. It is produced by activated macrophages epithelial cells and other cells in response to bacterial infection, inflammatory product and other invasive stimuli. The normal range of TNF-α in human body is varied in different individual. Since TNF-α has several

beneficial functions, it is constitutively produced at a low levels. Firstly, TNF-α is cytotoxic to certain cancer cells, thus it able to induce the death of cancer cells. Besides, low levels of TNF-α also help in maintaining homeostasis by regulating the body's circadian rhythm. Low levels of TNF-α also promotes regeneration of old or injured tissue by stimulate the growth of fibroblast. (Tracey & Cerami, 1994)

TNF-α could also cause many other pathological effects which were harmful to body. TNF-α is also found to be a growth factor for certain cancers. Acute exposure to high levels of TNF-α will cause shock, tissues injuries and other acute inflammatory responses, which will eventually lead to multiple organ failure. The response is due to TNF-α in the circulation which triggers a cascade of classical hormonal and cytokines responses, where the effects can persist for hours and cause lethal organ failure. Chronic exposure to TNF-α would lead to the development of chachexia or tolerance. Chachexia is characterised by anorexia, weight loss, dehydration, depletion of whole body lipid and protein. TNF-α is indirectly up-regulate the catabolism of proteins and lipids in peripheral organs by inducing the release of corticotrophin-releasing factor by hypothalamus. Thus causes insulin resistance in peripheral tissues, depleting body proteins and lipids and also weight lost (Tracey & Cerami, 1994).

As TNF-α has multiple roles in several diseases, the regulation of TNF-α has become the target for treating the diseases as it can be pro-apoptotic or anti-apoptotic. For example, tumour necrosis factor related apoptosis inducing

agent (TRAIL- base), which is a member of TNF family, has been used as chemotherapy agent for cancer, such as sarcoma and melanoma. However, studies showed that TNF-α is able to promote the growth of skin cancer cells and ovarian cancer cells. Thus, the study on the usage of TNF-α is important in regulation and treatment of cancer (Balkwill, 2009).

2.7 Kaempferol

Kaempferol is a natural compound which was discovered in *Camellia sinensis* (common name tea tree). It can be extracted from various plants, fruits, vegetables and herbal medicines in the form of kaempferol glycosides. Green tea is known to have relatively high amount of kaempferol as compared with other plants. Kaempferol belongs to polyphenolic flavonoid family which has been found to have anti-oxidant and anti-inflammation effect. Recent studies discovered that kaempferol has health promoting effect as it able to reduce arteriosclerosis, cardiovascular disease and cancer (Lau, 2008; Luo et al., 2010).

Studies showed that kaempferol and its derivatives have antioxidant and antimicrobial properties. Kaempferol will react with the reactive oxygen species (ROS) that is generated during metabolism, thus it prevent the damage of the ROS to the cell. It also able to inhibit the growth of *Escherichia coli*, *Staphylococcus aureus* and other bacteria (Ramamoorthy & Bono, 2007; Teffo, Aderogba & Eloff, 2009).

Kaempferol is also found to inhibit various enzyme systems that are critically involved in initiation of inflammation and immune responses, such as serine and threonine kinase, phospholipases and lipooxygenases. Study showed that kaempferol has anti-allergic effect because it is able to block the induction of adhesion molecules such as intracellular adhesion molecule-1 upon stimulation by cytokine thereby suppress the inflammatory response (Gerritsen et al., 1995).

Nowdays, kaempferol is widely used in treatment for cancer, cardiovascular disease, neuron disorder and high cholesterol. Kaempferol could enhance the effect of anti-cancer agents such as TRAIL-base and *cis*-diamminedichloroplatinium (II) (cisplatin) and thus reducing the development of drug resistance in cancer cells by sensitising cancer cells to these drugs. Lower doses of the drugs can then be used to achieve same effect. Together with its anti-cancer potential and cytotoxic effect in many type of cancers, kaempferol had become useful component in anti-cancer cocktail therapy (Gao et al., 2009; Lau, 2008; Luo et al., 2010). All these beneficial properties of kaempferol suggests that cosumption of kaempferol in our daily diet will improve our health.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Table 3.1 Materials used in the project and their suppliers

Materials	Supplier (s)
HepG2 cell line	American Type Culture Collection, USA
Fetal Bovine Serum	i-DNA, Malaysia
Tri-Reagent® LS	Molecular Research Center, USA
Tissue culture flask (25cm^2)	Techno Plastic Products (TPP),
Cell scrappers	Switzerland
1ml Cryo-vials	Nunc., USA
Cytokine: Tumour Necrosis Factor Alpha	Chemicon International, USA
Agarose	Cambrex, USA
Glycerol	QRëc™, Singapore
Methanol	Labscan, Poland
Tween® 20	ACRŌS, USA
Guanidine Hydrochloride	Fluka Chemica, USA
TEMED	Bio Basic Inc., USA
Isopropanol, 2- mercaptoethanol	Merck, USA
Ethanol, Butan-1-ol	HmbG® Chemicals, Germany
Sodium acetate, Sodium chloride, Acetic acid, Bromophenol blue	Systerm, USA

Table 3.1, continued

RNase free water, SYBR® green dye, QuantiFast Multiplex RT-PCR master mix, Prestained protein size markers, broad range	QIAGEN, USA
Ethidium bromide, MOPS (sodium salt), Formaldehyde, Phosphate Buffer Saline (PBS), Ammonium persulphate (APS), Acrylamide:bisacrylamide (37.5:1)	Amresco, USA
Bovine Serum Albumin, Hydrochloric acid, Sodium dodecyl sulphate (SDS), Coomassie blue G-250, Glycine	Fisher Scientific, USA
1-Bromo-3-Chloropropane (BCP), Tissue Culture Grade DMSO, Trypsin, EDTA Tris, Glycine, Kaempferol	Sigma Aldrich, USA
Acetone	Bendosen, Norway
Anti-rabbit IgG (HRP-linked antibody), NF-κB1 p105 Antibody, NF-κB1 p105/p50 Antibody, Phospho- NF-κB2 p100 (Ser866/870) Antibody, NF-κB2 p100/p52 (18D10) Rabbit mAb (Human Specific), NF-κB1 forward and reverse primer, NF-κB2 forward and reverse primer, β-actin forward and reverse primer	Cell Signalling Technology, USA
β-actin antibody	Ab Frontier, Korea
Immobilon™ Western Chemiluminescent HRP Substrate (detection reagents), Polyvinylidene fluoride (PVDF) membrane	Milipore, USA
Minimum essential medium (MEM)	Gibco, USA
NE- PER® Nuclear and Cytoplasmic Extraction Reagents	Thermo Scientific, USA
Bio-Rad DC protein assay kit	Bio-Rad, USA

3.2 Cell Culture Media

3.2.1 Minimum essential medium (MEM)

MEM was prepared using the formula shown in Table 3.2. The medium was filter-sterilised before use.

Table 3.2 Composition of MEM (per litre)

Solution	Composition
MEM powder, containing Earle's balanced salts (cell culture reagent) with 2.0 mM L- glutamine	9.5 g
Sodium bicarbonate	2.2 g
Sodium pyruvate, 1 mM	10 ml
Penicillin, 100 U/ml	10 ml
Streptomycin, 100 µg/ml	10 ml
Non essential amino acid, 0.1 mM	10 ml
Sterile deionised water	Top up to 1 L

3.2.2 Phosphate buffered saline (PBS)

Phosphate buffered saline powder was dissolved in 1 litre of deionised water. The solution was autoclaved at 121^{o}C, 975 kPa for 15 min.

3.3 Stock solutions

Stock solutions used in the project were prepared according to composition shown in Table 3.3, 3.4 and 3.5.

Table 3.3 Stock solutions used in formaldehye agarose gel electrophoresis

Solution	Composition
10X MOPS	400 mM MOPS (sodium salts), 100 mM sodium acetate, and 10 mM EDTA pH 8.0, pH7.0
RNA loading dye	95% (w/v) formamide, 0.025% (w/v) SDS, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, and 0.5 mM EDTA

Table 3.4 Stock solutions used in SDS-PAGE and transferring

Solution	Composition
10X SDS running buffer	250 mM Tris, 1.92 M glycine, 1% (w/v) SDS
SDS-PAGE upper gel buffer	0.5 M Tris, 10% (w/v) SDS, pH 6.8
SDS-PAGE lower gel buffer	1.5 M Tris, 10% (w/v) SDS, pH 8.8
SDS-PAGE loading buffer	0.12 M Tris, 25% (v/v) glycerol, 2% (w/v) SDS, 0.004% (w/v) bromophenol blue, 5% (v/v) 2-mercaptoethanol*, pH 6.8
Towbin transfer buffer	25 mM Tris, 192 mM glycine, 20% (v/v) methanol

*added before using

Table 3.5 Stock solutions used in Western Blot

Solution	Composition
Tris buffered saline (TBS)	10 mM Tris, 200 mM sodium chloride, 0.1% (v/v) Tween®20, pH 7.4
Blocking solution	1% (w/v) BSA, top up with TBS

Table 3.5, continued

Stripping solution	0.4 M glycine, 0.2% (w/v) SDS, 2% (v/v) Tween®20, pH 2.2

3.4 Glassware and plasticware preparation

All glassware and plasticware that need to be sterile were autoclaved at 121°C, 975 kPa for 20 min. Stock solutions that used in DNA and RNA works were also autoclaved at 121°C, 975 kPa for 20 min.

CHAPTER 4

RESULTS

4.1 Culture of HepG2 cells

HepG2 cells are cell lines derived from human hepatoblastoma. It has epithelial-like morphology which has one nucleus and 48 to 54 chromosomes. NF-κB1 and NF-κB2 is being expressed constitutively in HepG2 cells and thus it has been widely used to study the mRNA and protein expression of NF-κB1 and NF-κB2 after treatment with various modulators that induce them (Dou, Liu, Wang & Zhang, 2006, Saliou, Rihn, Cillard, Takashi & Packer, 1998, Seow, Liang, Leow & Chung, 2001, Wilkening, Stahl & Bader, 2003). HepG2 cells were cultured in complete medium that consists of Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS) and antibiotic penicillin and streptomycin (Figure 4.1).

4.2 Isolation of total cellular RNA

Treatment of HepG2 cells with TNF-α and kaempferol was done as depicted in Section 3.5.4. Culture flasks of 25 cm^2 were used to culture HepG2 cells. When the cells reached confluency of 70 to 80%, the cells were treated with 20 ng/ml of TNF-α, followed by different concentrations of kaempferol (1 µM, 5 µM, 10 µM and 20 µM). After that, total cellular RNA was extracted from cells using Tri-Reagent® LS (Molecular Research Centre) according to manual given by manufacturer as described at Section 3.6.1. The isolated total cellular RNAs were aliquoted to 5 sets and 1 set was used to perform formaldehyde

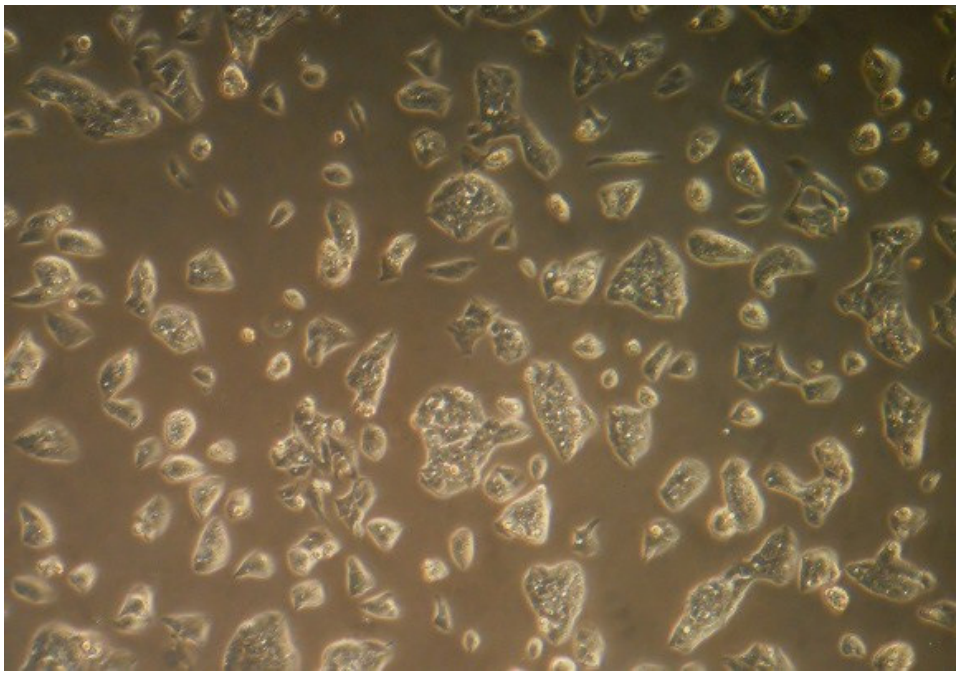


Figure 4.1 HepG2 cells cultured in 25cm^2 tissue culture flask

HepG2 cells are derived from human hepatocellular carcinoma and it has epithelial-like morphology (Magnification: 10X).

agarose gel electrophoresis in order to determine the integrity of total RNA (Section 3.6.3). The results of formaldehyde agarose gel electrophoresis are as shown in Figure 4.2. Sharp and intense bands could be observed which indicated the 28S and 18S ribosomal RNA (rRNA). The 28S rRNA band intensity was twice of the 18S rRNA band which showed that the total cellular RNA was intact and no degradation was observed. Later, the concentration and purity of total cellular RNA were accessed using spectrophotometer (Thermo Scientific). The absorbance of each sample was measured at 260nm and 280nm to obtain the ratio of A_{260}/A_{280}, which was used to indicate the purity of total cellular RNA (Section 3.6.2) (Table 4.1).

4.3 Real Time RT-PCR

Real Time RT-PCR is a rapid, specific and sensitive method to quantify mRNA expression, thus it was applied in this project to quantify the effect of kaempferol on TNF-α induced NF-*κ*B1 and NF-*κ*B2 mRNA expression. The process was carried out on iCycler IQ5 Real Time PCR Detection System (Bio-Rad) using QIAGEN® OneStep RT- PCR Kit according to the manual given by the manufacturer (Section 3.7). The protocol for Real Time RT-PCR and the primers selected were described in Section 3.7.2 and Table 3.6, respectively. Triplicates were used for each RNA samples to determine the significance and accuracy of the results for target gene and housekeeping gene expression (refer to Appendix A).

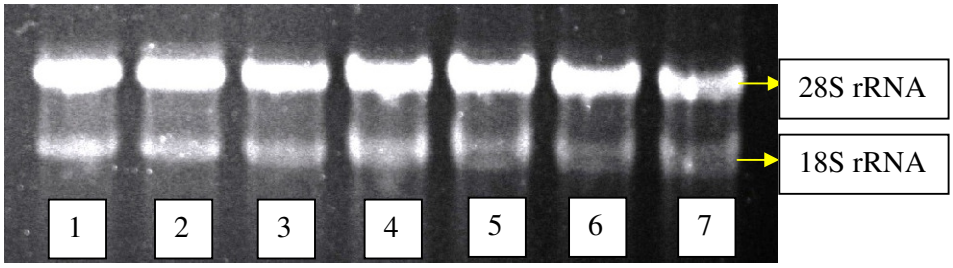


Figure 4.2 1% (w/v) formaldehyde agarose gel electrophoresis of total cellular RNA

Lane 1- Untreated
Lane 2- 20 ng/ml TNF-α
Lane 3- 20 ng/ml TNF-α +1 µM Kaempferol
Lane 4- 20 ng/ml TNF-α +5 µM Kaempferol
Lane 5- 20 ng/ml TNF-α +10 µM Kaempferol
Lane 6- 20 ng/ml TNF-α +20 µM Kaempferol
Lane 7- 20 µM Kaempferol

Lanes 1 to 7 show the RNA extracted from HepG2 cells without any treatment, treated with 20 ng/ml of TNF-α only, 20 ng/ml TNF-α and 1 µM of kaempferol, 20 ng/ml TNF-α and 5 µM of kaempferol, 20 ng/ml TNF-α and 10 µM of kaempferol, 20 ng/ml TNF-α and 20 µM of kaempferol and 20 µM of kaempferol, respectively. The intensity of 28S rRNA and 18S rRNA bands were in the ratio of 2:1, which indicated that the total cellular RNA isolated was intact.

Table 4.1 The concentration and A_{260}/A_{280} ratio of total cellular RNA extracted from 20ng/ml TNF-α and different concentration of kaempferol treated HepG2 cells

The total cellular RNA isolated from HepG2 cells that treated with different treatment using TRI reagent LS and the concentration and purity of RNA was determined using spectrophotometric method. The ratio A_{260}/A_{280} with a range of 1.8-2.0 indicates the RNA extracted is pure.

Treatment	A_{260}	A_{280}	Ratio of A_{260}/A_{280}	Concentration of RNA (µg/ml)
Without treatment	1.827	1.219	1.490	731
20ng/ml TNF-α	1.127	0.582	2.118	451
20ng/ml TNF-α + 1µM kaempferol	0.756	0.414	1.826	302
20ng/ml TNF-α + 5µM kaempferol	1.094	0.569	1.901	443
20ng/ml TNF-α + 10µM kaempferol	0.478	0.357	1.324	198
20ng/ml TNF-α + 20µM kaempferol	0.858	0.455	1.902	340
20µM kaempferol	0.087	0.034	1.828	46.8

The amplification charts of β-actin, NF-κB1 and NF-κB2 are shown in Figure 4.3. The X-axis is represent the cycle number, while the Y-axis is represent the fluorescent signal detected in logarithmic scale which is the fold increase over background fluorescence during amplification. Threshold level in the graph was set approximately higher than the background and the cycle number that immediately achieved the threshold is known as CT value, which is as indicated in the graph (Appendix B).

After PCR amplification, the iCycler IQ5 Real Time PCR Detection System was at melting curve analysis. Melting curve analysis is a method to test for the specificity of the primer used. During melting curve analysis, the temperature was raised by 0.5^{o}C every 10sec from 60 to 95^{o}C and the change in fluorescence intensity was detected by the machine. The fluorescence intensity changed due to the dissociation of double stranded DNA. When it reached melting point of the double stranded DNA, the fluorescence signal would sharply decrease. Figure 4.4 shows the graphical representation of a melting curve analysis in Real Time RT-PCR. The Y-axis of the graph represents the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT), while the X-axis represents the temperature. The peak of the curve is the melting temperature of amplicon. From Figure 4.4, the melting temperature for β-actin, NF-κB1 and NF-κB2 were 87.97^{o}C, 88.29^{o}C and 85.96^{o}C respectively. Each of the graphs showed one sharp peak, which indicated that the Real Time RT-PCR successfully amplified the amplicon specifically.

PCR Amplification/ Cycle Chart for β-actin, NF-*κ*B1 and NF-*κ*B2

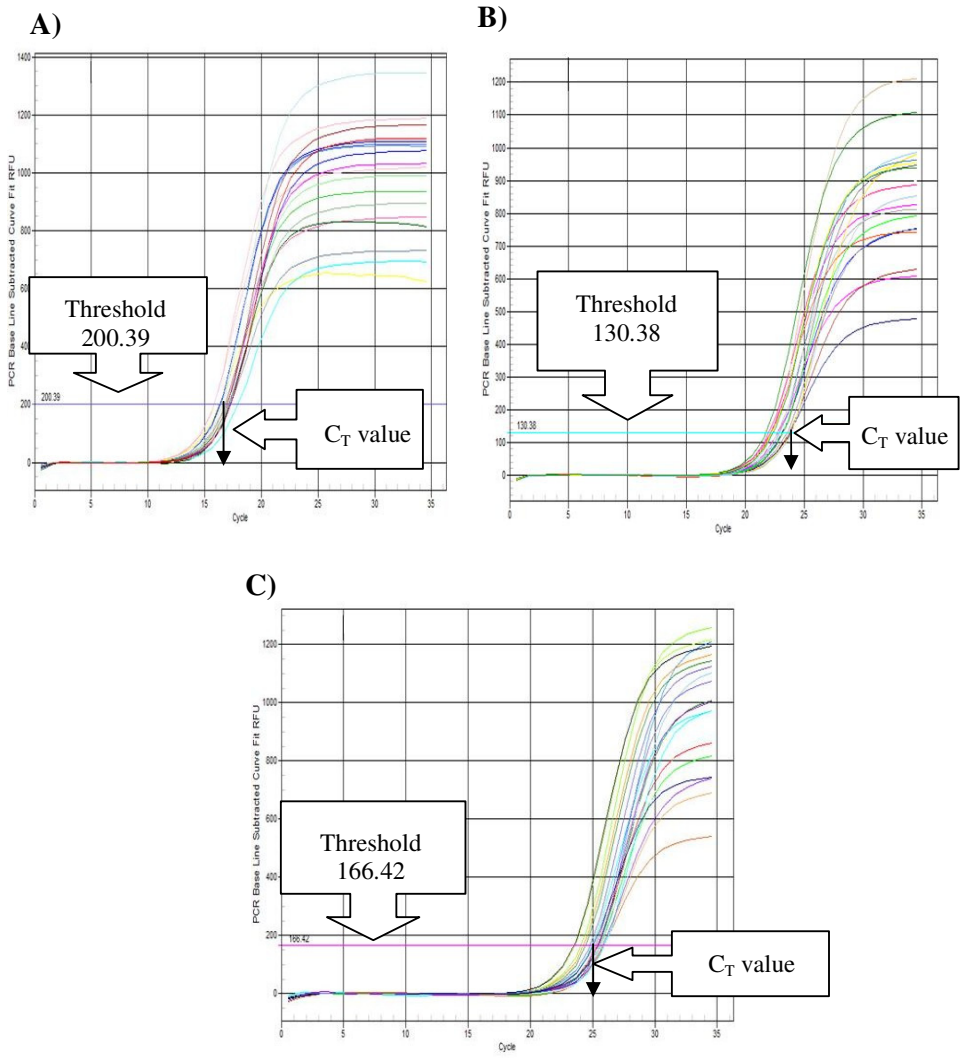


Figure 4.3 Graphical representation of Real Time RT-PCR amplification of A) β-actin, B) NF-*κ*B1 and C) NF-*κ*B2 in logarithmic scale

The PCR amplification graph is a sigmoid curve in which X-axis represents the cycle number and the Y-axis represents arbitrary fluorescence units (which correspond to the fold increase over background fluorescence). Threshold level is set right above the background and CT value is representing the number of cycles required to achieve the threshold level.

Melt Curve Peak Chart for β-actin, NF-*κ*B1 and NF-*κ*B2 (-dF/dt vs T)

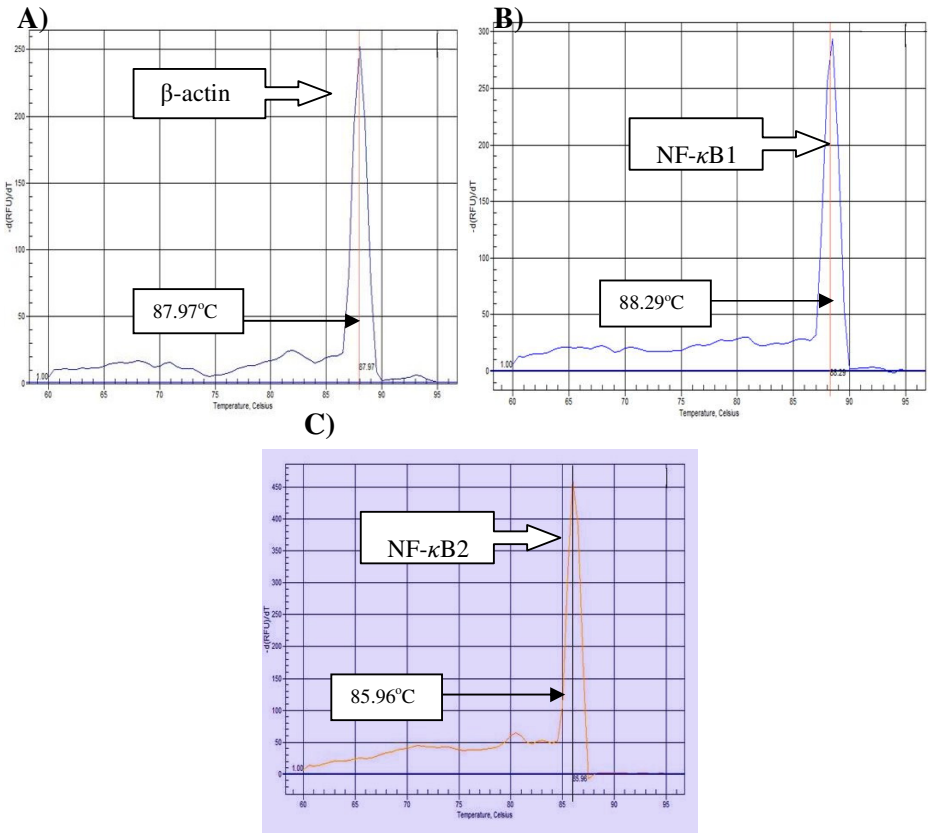


Figure 4.4 Graphical representation of melting curve analysis to determine specific amplification of A) β-actin, B) NF-*κ*B1 and C) NF-*κ*B2 during Real Time RT- PCR

After PCR amplification has finished, the iCycler IQ5 Real Time PCR Detection System was programmed to run a melting curve analysis, whereby the temperature is increased by a fraction of degree. At melting point of double stranded DNA, the two DNA strands will separate and cause rapid decrease of fluorescence. The change in fluorescence is measured by the system and the software plots the graph for the melting curve analysis. The Y-axis of the graph is rate of change of the relative fluorescence unit (RFU) with time (T) (-d(RFU)/dT), while the X-axis represents the temperature. The peak of the curve is the melting temperature (T_m).

The relative fold expression of β-actin, NF-κB1 and NF-κB2 in untreated HepG2 cells are shown in Figure 4.5A. According to the chart, the housekeeping gene, β-actin was higher expressed as compared to the target genes, NF-κB1 and NF-κB2. NF-κB1 mRNA expression was 0.600-fold of the expression of β-actin, while NF-κB2 mRNA expression was 0.341-fold of the expression of β-actin. In other words, β-actin mRNA expression was 1.67-fold higher than NF-κB1 and 2.93-fold higher than NF-κB2 in untreated HepG2 cells.

CHAPTER 5

DISCUSSION

5.1 Overview

There were four objectives in this project. The first objective was to determine the effect of kaempferol on TNF-α induce NF-κB1 and NF-κB2 mRNA expression using quantitative PCR. Secondly, to determine the effect of kaempferol on TNF-α induced NF-κB1 and NF-κB2 protein expression using Western Blot. Thirdly, to determine the effect of kaempferol on TNF-α induced NF-κB1 and NF-κB2 activation. Lastly was to compare the mRNA and protein activation expression in kaempferol-treated TNF-α induced HepG2 cells.

5.2 HepG2 cell as model system

HepG2 cells are originated from human hepatocellular liver carcinoma cell line. HepG2 cells have epithelial-like morphology and its morphological and biochemical characteristics are similar to normal human hepatocytes. Therefore, HepG2 cells are widely used in studies of drugs metabolism in liver cells, hepatocytes functions, liver inflammation and liver cancer (Saliou et al., 1998; Wilkening et al., 2003). HepG2 cells were chosen as model system because studies showed that NF-κB mRNA expression and protein activation could be induced by TNF-α in HepG2 cells (Lluis, Buricchi, Chiarugi, Morales & Fernandez-Checa, 2007; Saliou et al., 1998). The development of hepatocarcinoma involves several stages: inflammation, dysplasia, carcinoma

and metastasis, and thus HepG2 cells are one ideal model for studying the liver carcinogenesis and its therapy (Block, Mehta, Fimmel & Jordan, 2001; Kumar, Abbas, Fausto & Mitchell, 2007).

5.3 Total cellular RNA isolation

The purity and integrity of RNA extracted is variable because RNA is relatively unstable and susceptible to degradation by improper handling of sample after the isolation from cells. Therefore, the RNA extraction work has to be carried out on ice and as rapid as possible. Precautions have been taken on all the equipment and solution used to protect the RNA from RNase that can be found on places such as hands and saliva (Bachert, Cauwenberge, Claeys, Perez-Novo, Speleman & Vandesompole, 2005; Isaac, 1994). The extracted total RNA is also not suitable for long term storage as RNA is fragile and susceptible to various type of degradation. The presence of magnesium ion will catalyse auto-degradation of RNA (Benes, Bustin, Nolan & Pfaffl, 2005; Isaac, 1994). Proper storage of RNA should be free from genomic DNA and protein, thus protease or enzyme inhibitor and DNase treatment can be perform for extended storage (Bustin & Nolan, 2004a; Pfaffl, 2005). Tri-Reagent® LS used in total cellular RNA isolation consists of phenol and guanidine thiocynate in mono-phase solution. These two compounds help to inhibit RNase activity during the extraction processes (Chomczynski & Sacchi, 1987).

5.4 RNA integrity

The integrity of total cellular RNA extracted was determined using formaldehyde agarose gel electrophoresis. Formaldehyde is a denaturant that denature the secondary structure of RNA at room temperature. This would separate the RNA based on their size without being influenced by their shape (Isaac, 1994). Figure 4.2 shows that there were two sharp bands observed for each lane. This two sharp bands were the most abundant RNA, 28S and 18S rRNA. The intensity of 28S rRNA bands should be approximately twice of the 18S rRNA and there was no smearing observed in the gel image which meant that the RNA integrity was high The concentration of total cellular RNA extracted was listed in Table 4.1, whereby the absorbance of 1 at 260 nm was equal to 40 μg/ml of RNA. The 280 nm was used to measure the presence of other contaminants like protein, phenol and DNA. (Sambrook & Russel, 2001).

5.5 Real Time RT-PCR

5.5.1 Overview

HepG2 cells were treated with TNF-α and different concentrations of kaempferol and incubated for 24 hrs as mentioned in Section 3.5.4. Total cellular RNA was isolated and used for Real Time RT-PCR using β-actin, NF-κB1 and NF-κB2 primer (Section 3.7). During the experiment, the primers specificities were tested and dose response effect of kaempferol on TNF-α induced NF-κB1 and NF-κB2 mRNA expression was analysed by normalising NF-κB1 and NF-κB2 expression to β-actin expression.

5.5.2 Basic principle of Real Time RT-PCR

Real Time RT-PCR is one of the most sensitive technique for detection and quantification of targets mRNA. It has high accuracy, sensitivity, wide dynamic range, reproducibility with speed and potential for high throughput (Bustin, 2000). The system operates by detecting and quantitating the fluorophore signals (Bloch, Connell & Lee, 1993; Deetz, Flood, Giusti, Livak & Marmaro, 1995). Non-sequence specific fluorescent intercalating agent, SYBR® green dye, was used in this study. SYBR® green is a fluorogenic minor groove binding dye that produces strong flourescent signal upon binding to double stranded DNA, otherwise it only emits little fluorescence in solution (Morrison, Weis & Wittwer, 1998). The intensity of fluorescence signal is proportional to the double stranded amplicons produced. Hence, high initial copy number of sample will get a significant increase of fluorescence signal sooner. The accumulation of amplicons occurred above the baseline value where the signal significantly increased (Dorak, 2006) (Appendix E).

5.5.3 Advantage of Real Time RT-PCR

The wide usage of Real Time RT-PCR in the detection of mRNA expression nowadays is due to its advantages. Post-PCR processing like DNA gel electrophoresis is eliminated in Real Time RT-PCR and this helps to enhance throughput and reduce the chances of contamination in the sample. Real Time RT-PCR also provides a wider dynamic range of up to 10^7-fold as compared to conventional PCR which is 1000-fold only (Benes et al., 2005; Dorak, 2006). Wide dynamic range means that a wide range of ratio of target and reference can be assayed with equal sensitivity and specificity, thus wide

dynamic range will produce more accurate results. Unlike conventional PCR which is measured at end-point of plateau phase, Real Time RT-PCR collects the data along the exponential amplification of products which makes the quantification more reliable. Real Time RT-PCR also enable use to observe the amplification of products quantitatively through the increase of signal along the processes. In contrast, the conventional PCR would require a DNA gel electrophoresis to be performed in order to observe the success amplification. However, the number of amplicons produced could hardly be quantified by the naked eyes. For these reasons, Real Time RT-PCR has become a more reliable and widely used molecular tool for expression studies in laboratories.

5.5.4 PCR amplification/ cycle chart

Figure 4.3 showed the amplification chart for β-actin, NF-κB1 and NF-κB2. At the initial cycles, the signal produced by SYBR® green reporter was too weak and could not be differentiated from background. As the amount of amplicons increase, the SYBR® green reporter generates fluorescence signal exponentially until the signal saturated and reach plateau phase (Figure 4.3). The saturation of signal is caused by the depletion of elements that are critical for signal generation such as primers, the reporter and the dNTPs (Kubista, Stahlberg & Bar, 2001).

The threshold cycle, C_T, is the number of cycles when the system starts to receive an exponential increase of fluorescence signal, which is related to the exponential growth of amplicons during the log-linear phase. C_T value is an

important parameter for Real Time RT-PCR quantification as it is inversely proportional to the initial amount of target nucleic acid used in the reaction. However, C_T value can also be influenced by factors such as reagents, instruments and primers, therefore C_T value cannot be compared directly. The threshold is placed above all the baseline activity and within the exponential phase (Bustin and Nolan, 2004b; Dorak, 2006; Kubista et al., 2006). The threshold value was determined by the software iCycler IQ5 Real Time PCR Detection System (Bio-Rad) which was 200.39 for β-actin gene, 130.38 for NF-κB1 gene and 166.42 for NF-κB2 gene (Figure 4.3).

5.5.5 Melt curve analysis

Melt curve analysis was performed after PCR amplification to determine and confirm the specificity of the primers used to amplify the target gene. It was based on the detection of flourescence signal generated by reporter, SYBR® green. SYBR® green was a non-sequence specific dye, thus the measurement of amplicons production could include the presence of both specific or non-specific amplification or primer-dimer complex (Morrison et al., 1998). Primer-dimer complex formation would interfere the formation of specific products as they compete for the reagents and might cause error or false results.

The temperature in melt curve analysis was programmed to increase gradually and the fluorescence signal was detected. Along with increasing of temperature, the fluorescence signal would decrease gradually because the increase of temperature would cause an increased in thermal motion that

enable the internal rotation of dye chelated in the double stranded DNA (Kubista, Nygren & Svanvik, 1998). When the temperature reaches the melting temperature (T_M) of double stranded DNA, the double stranded DNA would denature and the dye would be released. This would cause a drop in fluorescence level (Rasmussen, Ririe & Wittwer, 1997). For a specific primer, only one type of products would be produced and they would have same melting temperature unless there was contamination, mispriming or formation of primer-dimer complex. The primer-dimer complex would give a peak that was low melting temperature because they are usually short DNA, while the specific products would have melting temperature above 80^{o}C (Kubista et al., 2006).

The melting temperature of β-actin, NF-κB1 and NF-κB2 were 87.97^{o}C, 88.29^{o}C and 85.96^{o}C, respectively (Figure 4.4). The charts only showed one sharp peak; hence the primers are specific in amplifying target gene.

5.7 Future studies

This project focuses on the study of effect of kaempferol on TNF-α induced activation of NF-κB1 and NF-κB2 in HepG2 cells. However, it was only carried out on the basis of mRNA and cytoplasmic protein expression. Further study of NF-κB1 and NF-κB2 can be carried out on effect of kaempferol on TNF-α induced activation of NF-κB1 and NF-κB2 activation in nuclear protein. Furthermore, the inhibitory properties of kaempferol on the NF-κB1 and NF-κB2 activation can be a platform for a more detailed studies on their application in control inflammatory disease and cancer development. Since

different cell lines will response to the treatment differently, a complete study of kaempferol dose response effect on NF-κB1 and NF-κB2 activation and cytotoxicity in different cancer cell lines can be implemented to discover the therapeutic potential of kaempferol on cancer in different organs. Besides this, other cytokines such as IL-1, IL-6, IL-8 and interferon alpha that are involved in cancer cell growth can be used to replace TNF-α to study the effect of kaempferol on the expression of NF-κB stimulated by them (Elias, Hasskamp & Sharma, 2010). Other flavonoid family like epigallocatechin-3-gallate, epicatechin, luteolin, naringenin and quercetin can be an alternative choice to replace kaempferol to determine the effect of different flavonoids on the TNF-α induced activation of NF-κB1 and NF-κB2 in HepG2 cells. Lastly, the reference genes used for normalisation can be increased in order to obtain a more valid and reliable result.

CHAPTER 6

CONCLUSION

This project was conducted to investigate and determine the effect of kaempferol on TNF-α induced activation of NF-*κ*B1 and NF-*κ*B2 in HepG2 cells using Real Time RT-PCR and Western Blot. Real Time RT-PCR was selected for this study because of its ability to produce high sensitivity, wide quantification range, high throughput and rapid results. While Western Blot was used to analyse protein expression as it provides highly specific and both qualitative and quantitative results.

The results showed that NF-*κ*B2 mRNA expression and activation were induced when treated with 20 ng/ml of TNF-α alone, but the induction was insignificant in NF-*κ*B1. NF-*κ*B1 but not NF-*κ*B2 mRNA expression was further up-regulated in a dose dependent manner in response to increasing concentration of kaempferol in TNF-α pre-treated HepG2 cells. The activation of NF-*κ*B2 was suppressed when treated with low dose of kaempferol ranging from 1 to 10 μM which was contrast to the mRNA expression, while the NF-*κ*B1 activation was not significantly reduced by kaempferol at low dosage. However, both mRNA expression and activation of NF-*κ*B1 and NF-*κ*B2 were induced when treated with 20 μM of kaempferol which contradicted the results obtained with low dosage treatment. Therefore, kaempferol would not inhibit the activation of NF-*κ*B1 but it was said to possess anti-inflammatory effect through inhibition of NF-*κ*B2 at low dosage. Kaempferol would

enhance inflammatory responses at high dosage, although the mechanism remains unclear. Further studies are required to find out the exact mechanism that bring about the sharply elevated mRNA expression and protein activation in HepG2 cell that stimulated by 20 μM kaempferol.

CHAPTER 7

REFERENCES

Anver, M., Carlson, L., Epstein, S., Franzo, G., Grinberg, A., Leonardi, A., et al. (1998). Mice deficient in nuclear factor (NF)-κB/ p52 present with defects in humoral responses, germinal centre reactions, and splenic microarchitecture. *J. Exp. Med*, *187*, 147-159.

Arlt, A., Vorndamm, J., Muerkoster, S., Yu, H., Schmidt, W. E., Folsch, U. R., et al. (2002). Autocrine production of interleukin 1beta confers constitutive nuclear factor kappaB activity and chemoresistance in pancreatic carcinoma cell lines. *Cancer Res*, *62*, 910-916.

Ashburner, B. P., Westerheide, S. D. & Baldwin, Jr. A. S. (2001). The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. *Mol Cell Biol*, 21, 7065-7077.

Bachert, C., Cauwenberge, P. V., Claeys, C., Perez-Novo, C. A., Speleman, F. & Vandesompele, J. (2005). Impact of RNA quality on reference gene expression stability. *Biotechniques*, *29*, 52-56.

Baldwin, Jr. A. S. (1996). The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annual Review of Immunology*, *14*, 649-683.

Balkwill, F. (2009). Tumour necrosis factor and cancer. *Nature Review Cancer*, *9*, 361-371.

Barton, D. S., Bravo, R., Caamano, J. H., Durham, S. K., Raventos-Suarez, C., Rizzo, C., et al. (1998). Nuclear Factor (NF)-κB (p100/ p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. *J. Exp. Med*, *187*, 185-196.

Benes, V., Bustin, S. A., Nolan, T. & Pfaffl, M. W. (2005). Quantitative real time RT-PCR- a perspective. *J Mol Endocrinol*, *34*, 597-601.

Bloch, W., Connell, C. R. & Lee, L. G. (1993). Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic acids Res*, *21*, 3761-3766.

Block, T. M., Mehta, A. S., Fimmel, J. S. & Jordan, R. (2003). Molecular viral oncology of hepatocellular carcinoma. *Oncogene*, 22, 5093-5107.

Bravo, R., Carrasco, D., Claudio, E., Ishikawa, H. & Ryseck,R. P. (1997). Gastric hyperplasia and increased proliferative responses of lymphocytes in mice lacking the COOH-terminal ankyrin domain of NF-*κ*B2. *J. Exp. Med*, *186*, 999-1014.

Bubici, C., Papa, S., Dean, K. & Franzoso, G. (2006). Mutual cross- talk between reactive oxygen species and nuclear factor- kappa B: molecular basis and biological significance. *Nature Oncogene*, 25, 6731-6748.

Bustin, S. A. (2000). Absolute quantification of mRNA using real time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol*, *25*, 169-193.

Bustin, S. A. & Nolan, T. (2004a). Template handling, preparation, and quantification. *The Real Time RT-PCR Encyclopedia A-Z of Quantitative PCR*, *2*, 87-120.

Bustin, S. A. & Nolan, T. (2004b). Pitfalls of quantitative reverse transcription polymerase chain reaction. *J Biomol Tech*, *15*, 155-166.

Carmody, R. J. & Chen, Y. H. (2007). Nuclear Factor-κB: activation and regulation during Toll-like receptor signaling. *Cellular and Immunology*, *4*, 31-41.

Carter, A. B., Misyak, S. A., Hontecillas, R. & Riera, J. B. (2009). DietaryModulation of Inflammation-Induced Colorectal Cancer through PPAR*γ*. *PPAR Research*, *1*, 1-9.

Chen, F. (2002a). NF-*κ*B1 nuclear factor of kappa light polypeptide gene enhancer in B-cells 1. *Atlas Genet Cytogenet Oncol Haematol*, Retrieved April 17, 2011, from http://AtlasGeneticsOncology.org/Genes/NFKB1ID323.html

Chen, F. (2002b). NF-*κ*B2 nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100). *Atlas Genet Cytogenet Oncol Haematol*, Retrieved April 17, 2011, from http://AtlasGeneticsOncology.org/Genes/NFKB2ID362.html

Chen, F., Castranova, V., Shi, X. & Demers, L. M. (1999). New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. *Clinical Chemistry*, *45*(1), 7-17.

Chen, L., Fischle, W., Verdin, E. & Greene, W. C. (2001). Duration of nuclear NF-kappaB action regulated by reversible acetylation. *Science*, *293*, 1653-1657.

Chen, C. C., Chow, M. P., Huang, W. C., Lin, Y. C., & Chang, Y. J. (2004). Flavonoids inhibit tumour necrosis factor-α-induced up-regulation of Intercellular Adhesion Molecule-1 (ICAM-1) in respiratory epithelial cells through Activator Protein-1 and Nuclear Factor-κB: structure-activity relationships. *Molecular Pharmacology*, *66*, 683-693.

Chen, C. Y., Peng, W. H., Tsai, K. D. & Hsu, S. L. (2007). Luteolin suppresses inflammation-associated gene expression by blocking NF-*κ*B and AP-1 activation pathway in mouse alveolar macrophages. *Life Sciences*, *81*, 1602-1614.

Chomczynski, P. & Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. *Anal. Biochem*, *162*, 156.

Chuang, S. E., Yeh, P. Y., Lu, Y. S., Lai, G. M., Liao, C. M., Gao, M., et al. (2002). Basal levels and patterns of anticancer drug-induced activation of nuclear factor-kappaB (NF-kappaB), and its attenuation by tamoxifen, dexamethasone, and curcumin in carcinoma cells. *Biochem Pharmacol*, *63*, 1709-1716.

Coward, W. R., Okayama, Y., Sagara, H., Wilson, S. J., Holgate, S. T. and Church, M. K. (2002). NF-kappa B and TNF-alpha: a positive autocrine loop in human lung mast cells. *J. Immunol. 169*, 5287-5293.

Crawford, D. R., Higgins, P. J. & Suzuki, T. (2000). Control selection for RNA quantitation. *Biotechniques*, *29*, 332-337.

Croubels, S., De Boever, S., Vangestel, C. & De Backer, P. (2007). Identification and validation of housekeeping genes as internal control for gene expression in an intravenous LPS inflammation model in chickens. *Dept of Pharmacol*, *133*, 9820.

Deetz, K. Flood, S. J., Giusti, W., Livak, K. J. & Marmaro, J. (1995). Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleci acid hybridization. *PCR Methods Appl*, *4*, 357-362.

Dheda, K., Huggett, J. & Zumla, A. (2005). Real Time RT-PCR normalization; strategies and considerations. *Genes Immum*, *6*, 279-284.

Dorak, M. T. (2006). Real Time PCR. Retrieved April 19, 2011, from http://www.dorak.info/genetics/realtime.html.

Dou, J., Liu, P. B., Wang, J. & Zhang, X. J. (2006). Preliminary analysis of gene expression profiles in HepG2 cell line induced by different genotype core proteins of HCV. *Cellular and Molecular Immunology*, *3*, 227-233.

Elias, E. G., Hasskamp, J. H. & Sharma, B. K. (2010). Cytokine and growth factor expressed by human cutaneous melanoma. *Cancer*, *2*, 794-807.

Firestein, G. S. & Tak, P. P. (2001). NF-kappaB: a key role in inflammatory diseases. J Clin Invest, 107, 7-11.

Fujioka, S., Sclabas, G. M., Schmidt, C., Frederick, W. A., Dong, Q. G., Abbruzzese, J. L., et al. (2003). Function of nuclear factor kappaB in pancreatic cancer metastasis. *Clin Cancer Res*, *9*, 346-354.

Gao, S. S., Choi, B. M., Chen, X. Y., Zhu, R. Z., Kim, Y., So, H. S., et al. (2009). Kaempferol suppresses Cisplatin-induced apoptosis via inductions of Heme Oxygenase-1 and Glutamate-Cysteine Ligase catalytic subunit in HEI-OC1 cells. *Pharmaceutical research*, *27*, 235-245.

Gaynor, R. B. & Yamamoto, Y. (2001). Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest*, *107*, 135-142.

Gerlinas, C., Kucharczak, J., Simmons, M.J. and Fan, Y. (2003). To be, or not to be: NF-κB is the answer- Role of Rel/NF-κB in the regulation of apoptosis. *Oncogene*, *22*, 8961-8982.

Gerritsen, M. E., Carley, W. W., Ranges, G. E., Shen, C. P., Phan, S. A., Ligon, F. G., et al. (1995). Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. *American Journal of Pathology*, *147*, 278-292.

Ghosh, S., Hayden, M. S. & West, A. P. (2006). NF-kappaB and the immune response. *Oncogene*, *25*, 6758-6780.

Ghosh, S. & Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. *Cell*, *109*, S81-S96.

Ghosh, S., May, M. J. & Kopp, E. B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annual Review of Immunology*, *16*, 225-260.

Gilmore, T. D. (2006). Introduction to NF- κB: players, pathways, perspectives. *Nature Oncogene*, 25, 6680-6684.

Goidin, D., Mamessier, A. & Staquet, M. J. (2001). Ribosomal 18S RNA prevails over glyceraldehye-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. *Anal. Biochem*, *295*, 17.

Goldman, J. & Lin, F. (1994). Appropriate controls for reverse transcription polymerase chain reaction (RT-PCR). *Br J Haematol*, *87*, 218.

Haesman, P. A & Taylor, J. J. (1994). Control genes for reverse transcription polymerase chain reaction (RT-PCR). *Br J Haematol*, *86*, 444-445.

Hohmann, H. P., Piischl, B., Remy, R. & van Loon, A. P. G. M. (1990). Tumour necrosis factor-α andβ bind to the same two types of tumour necrosis factor receptors and maximally activate the transcription factor NF-κB at low receptor occupancy and within minutes after receptor binding. *J Biol Chem*, *265*, 15183-15188.

Huang, W. W., Chiu, Y. J., Fan, M. J., Lu, H. F., Yeh, H. F., Li, K. H., et al. (2010). Kaempferol induced apoptosis via endoplasmic reticulum stress and mitochondria dependent pathway in human osteosarcoma U-2 OS cells. *Molecular Food Nutrition Research*, *54*, 1-11.

Hunter, R. B. & Kandarian, S. C. (2004). Disruption of either the NF-κB1 or Bcl3 gene inhibits skeletal muscle atrophy. *The Journal of Clinical Investigation*, *114* (10), 1504-1511.

Isaac, P. G. (1994). Protocol for Nucleic Acid Analysis by Nonradioactive Probes (Volume 28). Totowa, NJ: Human Press Inc.

Ischiropoulos, H. (1998). Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys*, *356*, 1-11.

Karin, M. & Greten, F. R. (2005). NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol*, *5*, 749-759.

Keller, U., Huber, J., Nilsson, J. A., Fallahi, M., Hall, M. A., Peschel, C., et al. (2010). Myc suppression of NF-κB2 accelerates lymphomagenesis. *BMC Cancer*, *10*, 348-357.

Kim, J. S. & Jobin, C. (2005). The flavonoid luteolin prevents lipopolysaccharide-induced NF-κB signalling and gene expression by blocking IκB kinase activity in intestinal epithelial cells and bone-marrow derived dendritic cells. *Immunology*, *115*, 375-387.

Kor, S. B. (2008). *Quantitative Real Time RT-PCR expression analysis of NF-kappaB2 from tumour necrosis factor-alpha treated human hepatocarcinoma HepG2 cell line*. Unpublished undergraduate's thesis. Universiti Tunku Abdul Rahman, Malaysia.

Kubista, M. Andradem J. M., Bengtsson, M., Forootan, A., Jonak, J., Lind, K. et al. (2006). The real time polymerase chain reaction. *Mol Aspects Med*, *27*, 95-125.

Kubista, M., Nygren, J. & Svanvik, N. (1998). The interaction between the fluorescent dye thiazole orange and DNA. *Biopolymers*, *46*, 39-51.

Kubista, M., Stahlberg, A. & Bar, T. (2001). Light-up probe based real time Q-PCR. *Genomics and Proteomics Technologies*, *4264*, 53-58.

Kumar, V., Abbas, A. K., Fausto, N. & Mitchell, R. (2007). The Liver, Gallbladder and Biliary Tract In *Robin's Basic Pathology* 8th Edition (p632-666). USA: Saunders Elsevier.

Kwon, S. H., Nam, J. I., Kim, S. H., Kim, J. H., Yoon, J. H., & Kim, K. S. (2009). Kaempferol and Quercetin, essential ingredients in Ginkgo biloba extract, inhibit Interleukin-1b-induced MUC5AC gene expression in human airway epithelial cells. *Phytotherapy Research*, *23*, 1708-1712.

Lau, T. (2008). A healthy way to live: The occurrence, bioactivity, biosynthesis and synthesis of kaempferol. *Chemistry*, *150*, 1-3.

Lee, T. K., Denny, E. M., Sanghvi, J. C., Gaston, J. E., Maynard, N. D., Hughey, J. J., et al. (2009). A noisy paracrine signal determines the cellular NF-kappaB response to lipopolysaccharide. *Sci. Signal. 2*, ra65.

Li, Q., Van Antwerp, D., Mercurio, F., Lee, K. F. & Verma, I. M. (1999). Severe liver degeneration in mice lacking the I*κ*B kinase 2 gene. *Science*, *284*, 321-325.

Li, Y., Ahmed, F., Ali, S., Philip, P. A., Kucuk, O. & Sarkar, F. H. (2005). Inactivation of nuclear factor kappaB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. *Cancer Res*, *65*, 6934-6942.

Lin, L., DeMartino, G. N. & Greene, W. C. (1998). Cotranslational biogenesis of NF-kappaB p50 by the 26S proteasome. *Cell*, *92*(6), 819-828.

Lipniacki, T., Puszynski, K., Paszek, P., Brasier, A. R. & Kimmel, M. (2007). Single TNFα trimers mediating NF-κB activation: stochastic robustness of NF-*κ*B signalling. *BMC Bioinformatics*, *8*, 376-395.

Liptay, S., Schmid, R. M., Perkins, N. D., Meltzer, P., Altherr, M. R., McPherson, J. D., et al. (1992). Related subunits of NF-kappa B map to two distinct loci associated with translocations in leukemia, NFKB1 and NFKB2. *Genomics*, *13*(2), 287-292.

Liptay, S., Weber, C. K., Ludwig, L., Wagner, M., Adler, G. & Schmid, R. M. (2003). Mitogenic and antiapoptotic role of constitutive NF-kappaB/Rel activity in pancreatic cancer. *Int J Cancer*, *105*, 735-746.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, *25*, 402-408.

Lluis, J. M., Buricchi, F., Chiarugi, P., Morales, A. & Fernandez-Checa, J. C. (2007). Dual role of mitochondrial reactive oxygen species in hypoxia signaling: activation of Nuclear Factor-κB via c-SRC and oxidant-dependent cell death. *Cancer Research*, *67*, 7368-7377.

Luo, H. T., Daddysman, M. K., Rankin, G. O., Jiang, B. H., & Chen, Y. C. (2010). Kaempferol enhances Cisplatin's effect on ovarian cancer cells through promoting apoptosis caused by down regulation of cMyc. *Cancer Cell International*, 1-9.

Mediavilla, V. G., Crespo, I., Collado, P. S., Esteller, A., Campos, S. S., Tuñón, M. J., et al. (2007). The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells. *European Journal of Pharmacology*, 557, 221-229.

Morrison, T. B., Weis, J. J. & Wittwer, C. T. (1998). Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques*, *24*, 954-958, 960, 962.

Muerkoster, S., Arlt, A., Sipos, B., Witt, M., Grossmann, M., Kloppel, G., et al. (2005). Increased expression of the E3-ubiquitin ligase receptor subunit betaTRCP1 relates to constitutive nuclear factor-kappaB activation and chemoresistance in pancreatic carcinoma cells. *Cancer Res*, *65*, 1316-1324.

Nam, K. W., Oh, G. T., Seo, E. K., Kim, K. H., Koo, U., Lee, S. J., et al. (2009). Nuclear factor *kappa*B-mediated down-regulation of adhesion molecules: Possible mechanism for inhibitory activity of bigelovin against inflammatory monocytes adhesion to endothelial cells. *Journal of Ethnopharmacology*, *123*, 250-256.

Nenci, A., Becker, C., Wullaert, A., Gareus, R., van Loo, G., Danese, S., et al. (2007). Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature*, 446, 557-561.

Nguyen, T. T. T., Tran, E., Ong, C. K., Lee, S. K., Do, P. T., Huynh, T. T. et al. (2003). Kaempferol-induced growth inhibition and apoptosis in A549 lung cancer cells is mediated by activation of MEK-MAPK. *Journal of Cellular Physiology*, *197*, 110-121.

Pasparakis, M., Luedde, T. & Supprian, M. S. (2006). Dissection of the NF-kappaB signalling cascade in transgenic and knockout mice. *Cell Death Differ*, *13*, 861-872.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in Real Time RT-PCR. *Nucleic acids Res*, *29*, 2002-2007.

Pfaffl, M. W. (2005). Nucleic acids: mRNA identification and quantification. *Nucleic acids, Encyclopedia of Analytical Science*, *2*, 417-426.

Prasad, C. N. V., Mohan S. S., Banerji, A., & Gopalakrishnapillai, A. (2009). Kaempferitrin inhibits GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communication*, *380*, 39-43.

Plümpe, J., Malek, N. P., Bock, C. T., Rakemann, T., Manns, M. P. & Trautwein, C. (2000). NF-κB determines between apoptosis and proliferation in hepatocytes during liver regeneration. *American Journal of Physiology*, *278*, G173-G183.

Ramamoorthy, P. K. & Bono, A. (2007). Antioxidant activity, total phenolic flavonoid content of Morinda citrifolia fruit extracts from various extraction processes. *Journal of Engineering Science and Technology*, *2*, 70-80.

Rasmussen, R. P., Ririe, K. M. & Wittwer, C. T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem*, *245*, 154-160.

Rudolph, D., Yeh, W. C., Wakeham, A., Rudolph, B., Nallainathan, D., Potter, J., et al. (2000). Severe liver degeneration and lack of NF-κB activation in NEMO/IKKγ-deficient mice. *Genes Dev*, 14, 854-862.

Said, H. M., Polat, B., Hagemann, C., Anacker, J., Flentje, M. & Vordermark, D. (2009). Absence of GAPDH regulation in tumor-cells of different origin under hypoxic conditions *in-vitro*. *BMC Research Notes*, *2*, 1756-1764.

Saliou, C., Rihn, B., Cillard, J., Takashi, O. & Packer, L. (1998). Selective inhibition of NF- κB by flavanoid hepatoprotector silymarin in HepG2-evidence for different activating pathways. *Federation of European Biochemical Societies Letter*, *400*, 8-12.

Saliou, C., Valacchi, G. and Rimbach, G. (2001). Assessing bioflavonoids as regulations of NF-κB activity and inflammatory gene expression in mammalian cells. *Method in Enzymology*, 335, 380-387.

Sambrook, J. & Russel, D. W. (2001). *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory Press.

Sarkar, F. H., Li, Y. W., Wang, Z. W. & Kong, D. J. (2008). NF-κB signalling pathway and its therapeutic implications in human diseases. *International Reviews of Immunology*, *27*, 293-319.

Savinova, O. V., Hoffmann, A., Ghosh, G. (2009). The NF-κB1 and NF-κB2 proteins p105 and p100 function as the core of high- molecular- weight heterogeneous complexes. *Journal of Molecular Cell*, *34*, 591-692.

Senftleben, U. (2008). Anti-inflammatory interventions of NF-kB signaling: Potential applications and risks. *Biochemical Pharmacology*, *75*, 1567-1579.

Senftleben, U., Cao, Y., Xiao, G., Greten, F. R., Bonizzi, G., Chen, Y., et al. (2001). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science*, *293*(5534), 1495-1499.

Seow, T. K., Liang, R. C. M. Y., Leow, C. K. & Chung M. C. M. (2001). Hepatocellular cainoma: From bedside to Proteomic. *Proteomics*, *1*, 1249-1263.

Shih, F. S., Tsui, R., Caldwell, A. & Hoffmann, A. (2011). A single NF-κB system for both canonical and non-canonical signaling. *Nature Cell Research*, *21*, 86-102.

Sriskantharajah, S., Belich, M. P., Papoutsopoulou, S., Janzen, J., Tybulewicz, V., Seddon, B., et al. (2009). Proteolysis of NF-κB1 p105 is essential for T cell antigen receptor–induced proliferation. *Nature Immunology*, *10* (1), 38-47.

Teffo, L. S., Aderogba, M. A. & Eloff, J. N. (2009). Antibacterial and antioxidant activities of four kaempferol methyl ethers isolated from *Dodonaea viscose* Jacq. var. *angustifolia* leaf extracts. *South African Journal of Botany*, *10*, 1016-1010.

Tergaonkar, V. (2006). NFB pathway: A good signaling paradigm and therapeutic target. *International Journal of Biochemistry& Cell biology*, *38*, 1647-1653.

Tracey, K. J. & Cerami, A. (1994). Tumor necrosis factor: A pleiotropic cytokine and therapeutic target. *Annu. Rev. Med.*, *45*,491-503.

Turner, D. A., Paszek, P., Woodcock, D. J., Nelson, D. E., Horton, C. A., Wang, Y. J., et al. (2010). Physiological levels of TNF-α stimulation induce stochastic dynamics of NF-κB responses in single living cells. *Journal of Cell Science*, *123*, 2834-2843.

Wang, W., Abbruzzese, J. L., Evans, D. B. & Chiao, P. J. (1999). Overexpression of urokinase-type plasminogen activator in pancreatic adenocarcinoma is regulated by constitutively activated RelA. *Oncogene*, *18*, 4554-4563.

Wilkening, S., Stahl, F. & Bader, A. (2003). Comparison of human primary hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. *The American Society for Pharmacology and Experimental Therapeutics*, *31*, 1035-1042.

Yeh, P. Y., Chuang, S. E., Yeh, K. H., Song, Y. C., Ea, C. K. & Cheng, A. L. (2002). Increase of the resistance of human cervical carcinoma cells to cisplatin by inhibition of the MEK to ERK signalling pathway partly via enhancement of anticancer drug-induced NF kappa B activation. *Biochem Pharmacol*, *63*, 1423-1430.

CHAPTER 8.0 APPENDICES

Appendix A

96 wells plate diagram to set each wells in Real Time RT-PCR

	1	2	3	4	5	6	7	8	9	10	11	12
A		Unt-1	Unt-2	Unt-3		Unt-1	Unt-2	Unt-3		Unt-1	Unt-2	Unt-3
B		+-1 β-actin	+-2 β-actin	+-3 β-actin		+-1 NF-κB1	+-2 NF-κB1	+-3 NF-κB1		+-1 NF-κB2	+-2 NF-κB2	+-3 NF-κB2
C		+, 1µM Kaemp-1 β-actin	+, 1µM Kaemp-2 β-actin	+, 1µM Kaemp-3 β-actin		+, 1µM Kaemp-1 NF-κB1	+, 1µM Kaemp-2 NF-κB1	+, 1µM Kaemp-3 NF-κB1		+, 1µM Kaemp-1 NF-κB2	+, 1µM Kaemp-2 NF-κB2	+, 1µM Kaemp-3 NF-κB2
D		+, 5µM Kaemp-1 β-actin	+, 5µM Kaemp-2 β-actin	+, 5µM Kaemp-3 β-actin		+, 5µM Kaemp-1 NF-κB1	+, 5µM Kaemp-2 NF-κB1	+, 5µM Kaemp-3 NF-κB1		+, 5µM Kaemp-1 NF-κB2	+, 5µM Kaemp-2 NF-κB2	+, 5µM Kaemp-3 NF-κB2
E		+, 10µM Kaemp-1 β-actin	+, 10µM Kaemp-2 β-actin	+, 10µM Kaemp-3 β-actin		+, 10µM Kaemp-1 NF-κB1	+, 10µM Kaemp-2 NF-κB1	+, 10µM Kaemp-3 NF-κB1		+, 10µM Kaemp-1 NF-κB2	+, 10µM Kaemp-2 NF-κB2	+, 10µM Kaemp-3 NF-κB2
F		+, 20µM Kaemp-1 β-actin	+, 20µM Kaemp-2 β-actin	+, 20µM Kaemp-3 β-actin		+, 20µM Kaemp-1 NF-κB1	+, 20µM Kaemp-2 NF-κB1	+, 20µM Kaemp-3 NF-κB1		+, 20µM Kaemp-1 NF-κB2	+, 20µM Kaemp-2 NF-κB2	+, 20µM Kaemp-3 NF-κB2
G		20µM Kaemp-1 β-actin	20µM Kaemp-2 β-actin	20µM Kaemp-3 β-actin		20µM Kaemp-1 NF-κB1	20µM Kaemp-2 NF-κB1	20µM Kaemp-3 NF-κB1		20µM Kaemp-1 NF-κB2	20µM Kaemp-2 NF-κB2	20µM Kaemp-3 NF-κB2
H		-ve control-1 β-actin	-ve control-2 β-actin			-ve control-1 NF-κB1	-ve control-2 NF-κB1			-ve control-1 NF-κB2	-ve control-2 NF-κB2	\

20ng/ml of TNF-α represented by "+" sign
Kaempferol in short form "Kaemp"

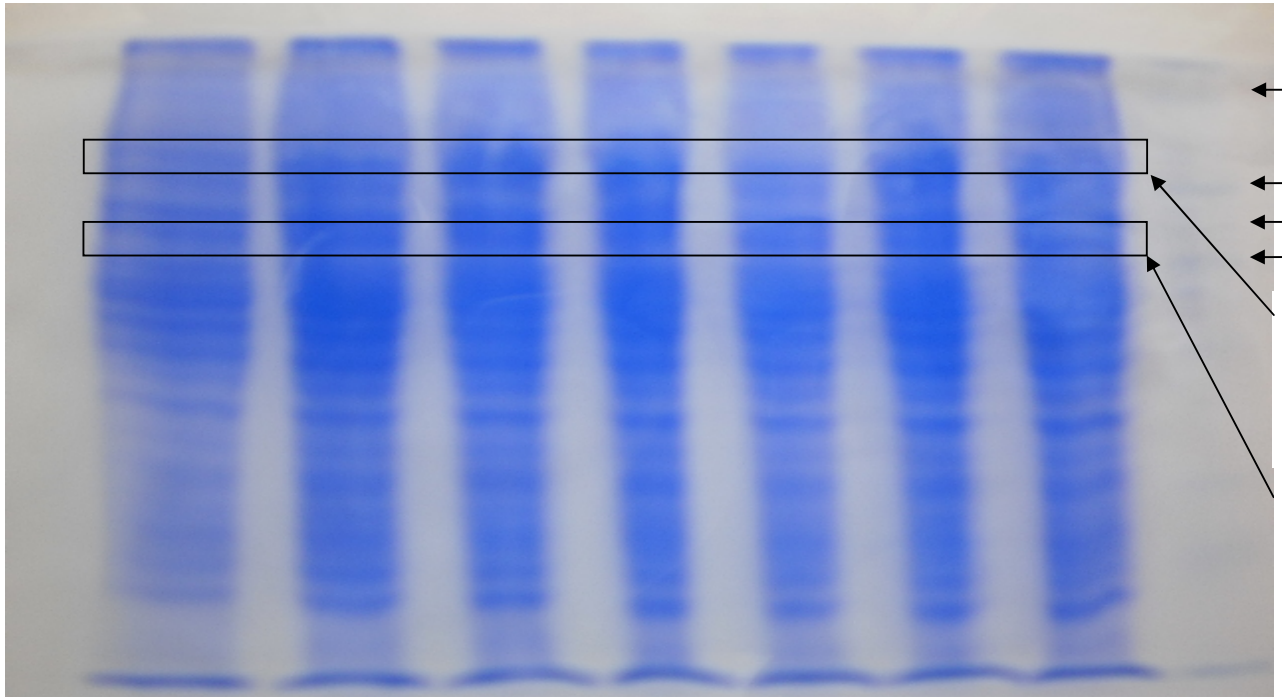
Appendix B

Thershold Crossing Spreadsheet data for PCR amplification chart

Gene	20ng/ml TNF-α	Kaempferol concentration (μM)	Mean CT value	Control
β-actin	-	-	16.20	√
β-actin	+	-	15.99	-
β-actin	+	1	16.18	-
β-actin	+	5	16.80	-
β-actin	+	10	16.84	-
β-actin	+	20	16.92	-
β-actin	-	20	17.41	-
NF-κB1	-	-	25.50	√
NF-κB1	+	-	24.81	-
NF-κB1	+	1	25.27	-
NF-κB1	+	5	25.70	-
NF-κB1	+	10	25.40	-
NF-κB1	+	20	23.64	-
NF-κB1	-	20	24.82	-
NF-κB2	-	-	23.21	√
NF-κB2	+	-	22.09	-
NF-κB2	+	1	22.75	-
NF-κB2	+	5	23.31	-
NF-κB2	+	10	23.39	-
NF-κB2	+	20	22.42	-
NF-κB2	-	20	22.32	-

Efficiency for β-actin, NF-κB1 and NF-κB2 gene are 100%

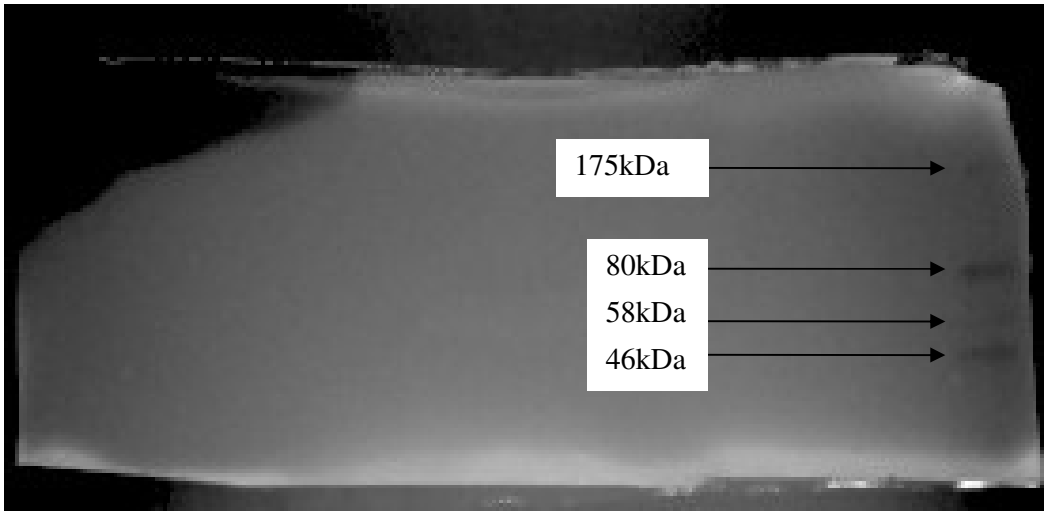
Appendix C



20ng/ml TNF-α	-	+	+	+	+	+	+	Protein
Kamepferol (μM)	-	-	1	5	10	20	20	Ladder

Polyacrylamide gel stained with Coomassie Brilliant Blue- the bands indicate the presence of proteins

Appendix D

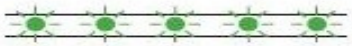


Protein Ladder on the membrane was viewed using AlphaView® FluorChem System (Alpha Innotech) and compare to the target protein.

Appendix E

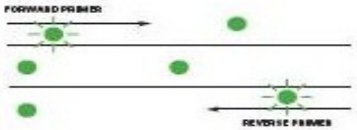
SYBR® Dye Assay

1. **Reaction setup:** The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.

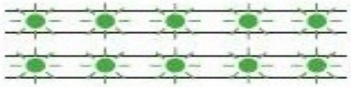


2. **Denaturation:** When the DNA is denatured, the SYBR® Green I Dye is released and the fluorescence is drastically reduced.

3. **Polymerization:** During extension, primers anneal and PCR product is generated.



4. **Polymerization completed:** When polymerization is complete, SYBR® Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.



Appendix F

Universal Plaffl formula used to calculate the relative expression ratio for Real Time RT-PCR samples

$$\text{Relative Expression Ratio} = \frac{(E_{target})^{\Delta CT \text{ target (control-treated)}}}{(E_{target})^{\Delta CT \text{ target (control-treated)}}}$$